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Late-night salivary cortisol cut-offs for diagnosis of Cushing syndrome using second-generation electrochemiluminescence immunoassay kits

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Abstract

Context: Late-night salivary cortisol (LNSC) is a simple and reliable screening test for Cushing syndrome (CS). With improved analytical performance of the current second-generation electrochemiluminescence immunoassay (ECLIA; Elecsys Cortisol-II; Roche Diagnostics), there is a need to revisit the LNSC cut-offs, especially in a South-Asian population.

Objective: To derive LNSC cut-offs for diagnosis of CS using second-generation ECLIA kits.

Design: Diagnostic accuracy study.

Methods: We prospectively recruited 155 controls aged 18–60 years, including, normal-weight (body mass index [BMI] < 25 kg/m^2 and no hypertension or diabetes [n = 53]) and overweight/obese (BMI 25–30 kg/m^2 and hypertension and/or diabetes [n = 52] or BMI ≥ 30 kg/m^2 with/without comorbidities [n = 50]) participants. All participants submitted LNSC samples collected at home; overweight/obese controls additionally underwent dexamethasone suppression test to exclude CS. We also reviewed records of adults with endogenous CS (cases, n = 92) and a valid LNSC result using the same method.

Results: The 95th percentile for LNSC in controls was 6.76 nmol/L. The mean ± SD LNSC levels were 40.47 ± 49.63 nmol/L in cases and 3.37 ± 1.18 nmol/L in controls (p < 0.001). Receiver operating characteristic (ROC) analysis showed excellent diagnostic performance of LNSC for CS, with area under curves (AUCs) of 0.994 (cases vs. all controls) and 0.993 (cases vs. overweight/obese controls), respectively. The best diagnostic performance was achieved at cut-offs ≥6.73 nmol/L (sensitivity: 97.8%, specificity: 94.8%) and ≥7.26 nmol/L (sensitivity: 97.8%, specificity: 95.1%), respectively.

Conclusions: LNSC measured using second-generation ECLIA demonstrated high diagnostic accuracy for CS. Based on this study, we propose a LNSC cutoff ≥6.73 nmol/L to diagnose CS.

KEYWORDS

Cushing syndrome, ECLIA, late-night salivary cortisol (LNSC), salivary cortisol, South Asian

1 | INTRODUCTION

A diagnosis of Cushing syndrome (CS) is suspected in several clinical settings including uncontrolled diabetes, hypertension, obesity, polycystic ovary syndrome, incidental adrenal mass and unexplained osteoporosis.[1] However, the diagnosis is challenging and often delayed due to inadequate awareness about the disease process and complexities of the testing protocol.[2] The Endocrine Society guidelines recommend dexamethasone suppression test (DST), 24-h urinary free cortisol measurement (24-h UFC) and late-night salivary cortisol (LNSC) as the initial screening tests, which complement each other in the diagnostic work-up of CS.[3]

Measurement of cortisol in saliva samples collected at late-night between 2300 and 2400 h (LNSC) constitutes a simple, convenient and reliable test for CS. LNSC provides an effective measure of circulating free cortisol in plasma and is essentially based on the abnormal circadian rhythm of cortisol secretion in CS, that is, the loss of midnight nadir.[2,3] Saliva samples can be collected at home in non-stressed environment, are stable at room temperature for several weeks and can be easily transported to the laboratory without the need for any stringent protocols.[3] Furthermore, LNSC does not involve cumbersome 24-h collection and the results are not affected by changes in cortisol binding globulin levels,[4,5] common problems faced with 24-h UFC and DSTs, respectively. Salivary cortisol can be measured by different methods including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), automated platform immunoassays, and more recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) assay.[6] At present, salivary cortisol is measured in most laboratories using automated platform immunoassays (such as electrochemiluminescent immunoassay or ECLIA) which are easy to perform and provide a rapid turnaround time.

LNSC reference ranges and diagnostic thresholds for CS are highly dependent on the assay method. For instance, Beko et al.[7] reported that while LNSC concentrations measured using automated ECLIA and RIA methods showed strong between-method correlation, best LNSC cut-off was 9.7 nmol/L using ECLIA, but only 8.0 nmol/L with RIA. Recently, second-generation cortisol ECLIA kits with remarkably improved functional sensitivity (3 nmol/L compared with 8 nmol/L with first-generation kits) and specificity have been introduced commercially.[8] The improved functional sensitivity implies that lower analyte concentrations can be measured more reliably; this has a bearing on the overall performance of the assay and the resultant LNSC cut-offs, which need a revisit. To this end, a study by Gagnon et al.[9] found that salivary cortisol measured using second-generation ECLIA ($r = 0.97$) showed higher correlation with LC-MS/MS than first-generation ECLIA ($r = 0.69$) and the second-generation assay demonstrated an overall better analytical performance. There are two studies that evaluated the diagnostic performance of LNSC for CS using these improved ECLIA kits in Caucasian population[10,11]; however, to the best of our knowledge, there are no similar studies in South Asian population. There are known ethnic variations in the activity of HPA axis and the circadian rhythm of cortisol secretion. For instance, Chong et al. and Cohen et al.,[12,13] in two separate studies found that Blacks demonstrate a less robust cortisol response to a standardised psychosocial stress test and an overall flatter cortisol diurnal rhythm compared to Whites. For these reasons, LNSC cut-offs should not only be assay-specific but also derived locally for a population.[14] With this background, the present study aimed to evaluate the diagnostic performance of LNSC measured using second-generation ECLIA kits and derive optimal cut-off for the diagnosis of CS in an Indian population.

2 | MATERIALS AND METHODS

2.1 | Settings and study design

This was a diagnostic accuracy study conducted in the department of Endocrinology and Metabolism at All India Institute of Medical Sciences, New Delhi, a tertiary care hospital in North India. The study protocol was approved by the institutional ethics committee (Ref. No.: IEC-429/06.05.2022, RP-57/2022).

2.2 | Study participants

Study participants included: a) patients without endogenous Cushing syndrome (controls, $n = 155$), and b) patients with endogenous Cushing syndrome (cases, $n = 92$). Control group participants, aged 18–60 years, were recruited prospectively between July and November 2022 from endocrine clinics of our department and further subgrouped into: a) normal-weight controls: body mass index (BMI) < 25 kg/m^2 and no hypertension or type 2 diabetes ($n = 53$), b) overweight/obese controls: BMI 25–30 kg/m^2 plus one or more of the comorbidities, that is, hypertension and type 2 diabetes, that increase the suspicion of endogenous CS ($n = 52$), or, BMI ≥ 30 kg/m^2 with or without any of the above mentioned comorbidities ($n = 50$). For overweight/obese controls, endogenous CS was excluded with a DST. Written informed consent was obtained from all controls. Cases were adults (≥18 years) with endogenous CS managed in our department between May 2016 and June 2022; data for such participants were derived through a retrospective record review. The departmental laboratory switched to second-generation cortisol ECLIA (Elecsys Cortisol-II) from May 2016 and therefore, we chose this as the starting point for recruitment of cases into our study.

For control group, we excluded pregnant and lactating females and also persons with conditions that affect salivary cortisol levels, including: a) history of acute febrile illness or acute physical or emotional stress in the last 2 weeks, b) diagnosed chronic psychiatric condition, c) chronic heavy alcohol use (defined as >14 standard drinks/week in men and >7 standard drinks/week in women), d) gingivitis, e) smoking, f) use of licorice or tobacco in any form, g) night-shift work schedule, h) any use of glucocorticoid lasting >3 weeks in last 3 months or any short-term glucocorticoid use within last 3 weeks, h) any systemic disease such as chronic liver or kidney disease, and i) uncontrolled hypothyroidism or hyperthyroidism. Because a DST was mandatory in overweight/obese controls, the use of drugs that affect dexamethasone metabolism, either CYP3A4 inducers (e.g., rifampicin, phenytoin and carbamazepine) and

CYP3A4 inhibitors (e.g., fluoxetine, itraconazole, diltiazem and ritonavir) or drugs that increase cortisol-binding globulin (e.g., oral contraceptive pills) constituted additional exclusion criteria in this group. For cases, we excluded paediatric patients with endogenous Cushing syndrome and adults without a valid LNSC result (such as due to insufficient volume or "solidified" samples unfit for analysis). For cases with >1 LNSC results (two values, $n = 46$ and three values, $n = 18$), the mean of all valid readings was taken.

2.3 | Procedure of sample collection

All controls were provided written instructions on saliva sample collection. Briefly, participants were advised to collect sample at their home between 2300 and 2400 h by passive drooling method. On the night of sample collection, they were advised to rinse mouth with tap water after dinner, not to eat or exercise at least 3 h before sample collection and avoid brushing of teeth. All participants were provided with serum separator tubes (containing separator gel and clot activator) to collect nearly 2–3 mL of saliva, which they submitted on a subsequent day. Those without a valid LNSC result were re-explained and invited to submit a fresh sample. As per the departmental protocol, all cases also collected saliva samples at late-night by passive drooling technique.

Overweight/obese controls additionally underwent an overnight DST (ONDST) to exclude CS (normal <50 nmol/L). For this purpose, participants were instructed to take two tablets of 0.5 mg dexamethasone (immediately after collecting saliva sample), and submit their blood and saliva samples the next morning between 0800 and 1000 h. A 2-day low-dose DST (0.5 mg dexamethasone q 6 h for 48 h; normal <50 nmol/L) was performed in participants with unsuppressed ONDST cortisol ($n = 2$).

2.4 | Cortisol measurement

Cortisol concentrations in serum and saliva samples were measured by competitive-binding ECLIA using second-generation kits on cobas-e-411 autoanalyser (Elecsys Cortisol-II; Roche Diagnostics). Briefly, the assay involves competition between endogenous cortisol liberated from binding proteins using danazol and a ruthenium-labelled exogenous cortisol derivative for the limited number of binding sites on a biotinylated cortisol-specific monoclonal antibody. Saliva samples do not require any special preparation and can be analysed after centrifugation, similar to serum and plasma samples. The minimum volume of saliva (including dead volume of sample container [150 µL]) needed for a single analysis is 160 µL. The analytical and functional sensitivity of this assay are 1.5 and 3.0 nmol/L, respectively, with a measuring range of 1.5–1750 nmol/L. For this study, salivary cortisol results lesser than the lower detection limit (1.5 nmol/L) were fixed at that limit. According to the manufacturer, the intra- and inter-assay coefficients of variation for serum samples are 1.4%–7.1% and 2.5%–12.7%, respectively, while the corresponding values for saliva samples are 2.5%–6.1% and 3.6%–11.8%, respectively. The cross-reactivity for structurally related steroids added in a concentration of 10 µg/mL are: 11-deoxycorticosterone, 0.64%; 11-deoxycortisol, 4.9%; 17-hydroxyprogesterone, 0.08%; corticosterone, 2.48%; cortisone, 6.58%; dexamethasone, not detectable; fludrocortisone, 0.2%; prednisone, 2.23%; and progesterone, 0.035%. The 95th and 97.5th percentile for LNSC among healthy individuals as determined by the manufacturer are 7.56 and 11.3 nmol/L, respectively.[8]

2.5 | Study definitions

Overweight and obesity was defined as per the World Health Organization (WHO) definition, that is, BMI 25–30 kg/m^2 (overweight) and ≥30 kg/m^2 (obesity). Obesity was further classified as: Class I (BMI 30–35 kg/m^2), Class II (BMI 35–40 kg/m^2), and Class III (BMI ≥ 40 kg/m^2). Diabetes was defined as fasting plasma glucose ≥7 mmol/L and/or HbA1c ≥ 6.5% (48 mmol/mol) or previously diagnosed case on diet and lifestyle modifications and/or anti-hyperglycemic medications. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or use of antihypertensive medications. Endogenous CS was diagnosed using Endocrine Society guidelines[3] and the details about methods of diagnosis and subtyping are available in Appendix S1 and our previous publication.[15]

2.6 | Sample size calculation

Based on the study by Aberle et al.,[11] anticipating a mean LNSC difference of 10.0 nmol/L between the two groups and a combined SD of 15.4 nmol/L and assuming a significance level of 5% and power of 90%, we needed 50 cases and 50 controls. Since we aimed to derive LNSC cut-offs to differentiate cases from controls, we planned to further increase the sample size and ended up recruiting 92 cases and 155 controls.

2.7 | Statistical analysis

Statistical analyses were performed using Stata 15.0 (StataCorp LP). Data are presented as n (%), mean ± SD, mean (95% CI) or median (q25–q75). Pearson χ^2 test was used to compare qualitative variables between different groups. Quantitative variables with normal distribution were compared using one-way analysis of variance (ANOVA) with Bonferronni correction, while variables without normal distribution were compared using Kruskal–Wallis test (three groups), followed by Wilcoxon rank sum test (pairwise comparisons). To estimate diagnostic performance of LNSC for CS, receiver operating characteristic (ROC) curves were drawn and area under curve (AUC; 95% CI) values were derived. Using the ROC analysis, optimal LNSC cut-offs for diagnosis of CS were derived and the corresponding sensitivity (95% CI), specificity (95% CI) and likelihood ratio positive (LR+, 95% CI) were reported. A p value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Study participants

We identified 216 controls, of whom we finally recruited 155, including 53 normal-weight and 102 overweight/obese (overweight, $n = 52$ and obese, $n = 50$) participants (Figure 1). Among obese participants, 35 (70%) had Class I obesity, 11 (22%) had Class II obesity and 4 (8%) had Class III obesity. A total of 78 (76.5%) overweight/obese participants had diabetes, 40 (39.2%) had hypertension, and 32 (31.4%) had both hypertension and diabetes. Among participants with diabetes, 29 (37.2%) had suboptimal glycemic control, defined as HbA1c > 8% (64 mmol/mol). We also identified 92 cases of endogenous CS (adrenocorticotrophic hormone [ACTH]-dependent, $n = 81$ [Cushing disease, $n = 53$], and ACTH-independent, $n = 11$) with a valid LNSC result during the study period. In overweight/obese controls, all but two participants (50.2 nmol/L and 196.4 nmol/L, respectively) suppressed serum cortisol to <50 nmol/L following ONDST. A 2-day LDDST adequately suppressed serum cortisol to 20.7 nmol/L and 12.7 nmol/L, respectively in these participants.

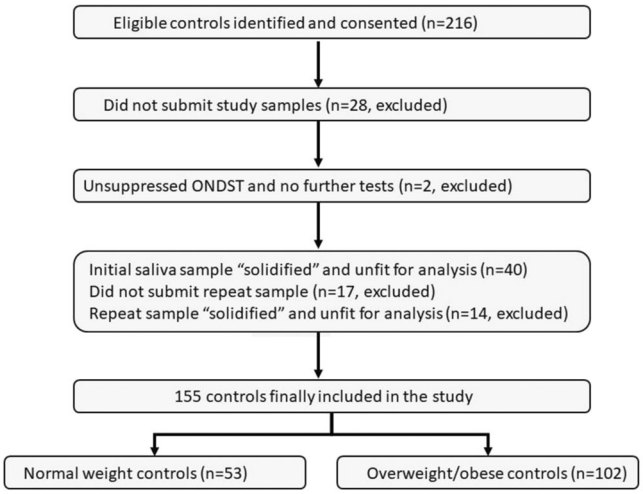

FIGURE 1 Flow diagram showing prospective recruitment of study controls. ONDST, overnight dexamethasone suppression test.

3.1.1 | Baseline characteristics

The baseline characteristics of study participants have been summarised in Table 1. The mean age was 32.7 ± 10.5 (range, 18–69) years in cases, 32.2 ± 6.3 (range, 22–43) years in normal-weight controls ($p = 1.000$ vs. cases) and 43.6 ± 9.5 (range, 18–59) years in overweight/obese controls ($p < 0.001$ vs. cases). Cases were more likely to be hypertensive ($p < 0.001$) and had significantly higher systolic blood pressure ($p < 0.001$), diastolic blood pressure ($p < 0.001$), and serum glutamic-pyruvic transaminase (SGPT; $p < 0.001$) levels. The mean BMI ($p = 0.003$) and prevalence of diabetes ($p = 0.001$) was significantly higher in overweight/obese controls compared to cases (Table 1). The mean 0800 h serum cortisol in cases was 756.2 ± 289.3 nmol/L (<550 nmol/L in 19 [20.7%], 550–1100 nmol/L in 62 [67.3%] and ≥1100 nmol/L in 11 [12.0%]).

TABLE 1 Baseline characteristics of study participants.

Variable	Cases ($n = 92$)	NW controls ($n = 53$)	OW/obese controls ($n = 102$)	p value (overall)	p value (cases vs. NW controls)	p value (cases vs. OW/obese controls)
Age (years)	32.7 ± 10.5	32.2 ± 6.3	43.6 ± 9.5	<0.001	1.000	<0.001
Gender (male)	30 (32.6%)	21 (39.6%)	33 (32.4%)	0.622	0.394	0.970
BMI[$] (kg/m^2)	28.6 ± 6.0	21.7 ± 2.4	30.9 ± 4.8	<0.001	<0.001	0.003
SBP (mmHg)	141.2 ± 16.4	110.0 ± 12.9	124.2 ± 17.4	<0.001	<0.001	<0.001
DBP (mmHg)	89.7 ± 11.1	71.7 ± 9.4	78.2 ± 11.7	<0.001	<0.001	<0.001
HTN	80 (87%)	0	40 (39.2%)	<0.001	-	<0.001
SGPT[@] (IU/L)	40 (23, 64)	21 (17, 27)	27 (21, 50)	<0.001	<0.001	<0.001
HbA1c[^] (%)	6.9 ± 2.1	5.4 ± 0.4	7.5 ± 1.9	<0.001	<0.001	0.057
DM	50 (54.4%)	0	78 (76.5%)	<0.001	-	0.001

Note: Data are expressed as n (%), mean ± SD or median (q25–q75). p value not expressed for comparison of HTN and DM categories between cases and NW controls because, by study design, both conditions were mandatorily absent in NW controls.

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; DM, diabetes; HTN, hypertension; NW, normal weight; OW/obese, overweight/obese; SBP, systolic blood pressure; SGPT, serum glutamic-pyruvic transaminase.

[$] $n = 87$ for column 2.

[@] $n = 101$ for column 4.

[^] $n = 90$, 52 and 101 for columns 2, 3 and 4, respectively.

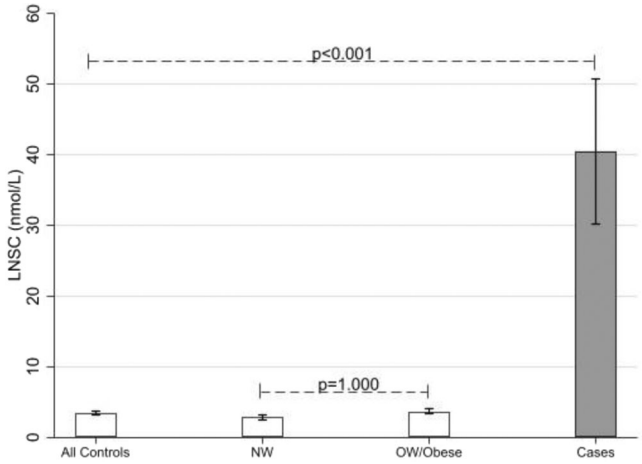

FIGURE 2 Mean (95% CI) of late-night salivary cortisol among cases (n = 92) and controls (n = 155). LNSC, late-night salivary cortisol; NW, normal weight controls; OW/Obese, overweight/obese controls.

3.1.2 | Late-night salivary cortisol (LNSC) data for cases and controls

The 5th and 95th LNSC percentiles for controls were 1.50 nmol/L and 6.76 nmol/L, respectively. The corresponding percentiles for normal-weight controls were 1.50 nmol/L and 6.51 nmol/L, respectively, while for overweight/obese controls, these were 1.50 nmol/L and 7.56 nmol/L, respectively. The mean ± SD LNSC levels were significantly higher in cases compared with controls (40.47 ± 49.63 nmol/L versus 3.37 ± 1.18 nmol/L; $p < 0.001$). LNSC levels were not significantly different between normal-weight and overweight/obese controls [2.81 ± 1.56 nmol/L versus 3.67 ± 1.90 nmol/L; $p = 1.000$] (Figure 2).

3.1.3 | Receiver operating characteristic (ROC) analysis

ROC analysis showed excellent diagnostic performance of LNSC, with AUC (95% CI) of 0.994 (0.987–1.000) for differentiating cases from all controls, and AUCs of 0.996 (0.991–1.000) and 0.993 (0.984–1.000) for differentiating cases from normal-weight and overweight/obese controls, respectively (Figure 3).

In the analysis involving cases and all controls, the best diagnostic performance was achieved at a LNSC cut-off ≥6.73 nmol/L (sensitivity [95% CI]: 97.8% [92.4%–99.4%], specificity [95% CI]: 94.8% [90.2%–97.4%], LR + [95% CI]: 18.9 [14.8–24.2]). There were two false negatives (1 in ACTH-dependent and 1 in ACTH-independent group) and eight false positives (2 in normal-weight and 6 in overweight/obese controls). The same LNSC cut-off best demarcated cases from normal-weight controls (sensitivity [95% CI]: 97.8% [92.4%–99.4%], specificity [95% CI]: 96.2% [87.3%–98.9%], LR + [95% CI]: 25.9 [9.7–69.1]). Finally, a LNSC cut-off ≥7.26 nmol/L best demarcated cases from overweight/obese controls (sensitivity: [95% CI]: 97.8% [92.4%–99.4%], specificity [95% CI]: 95.1% [89.0%–97.9%], LR + [95% CI]: 19.9 [13.5–29.6]) (Table 2).

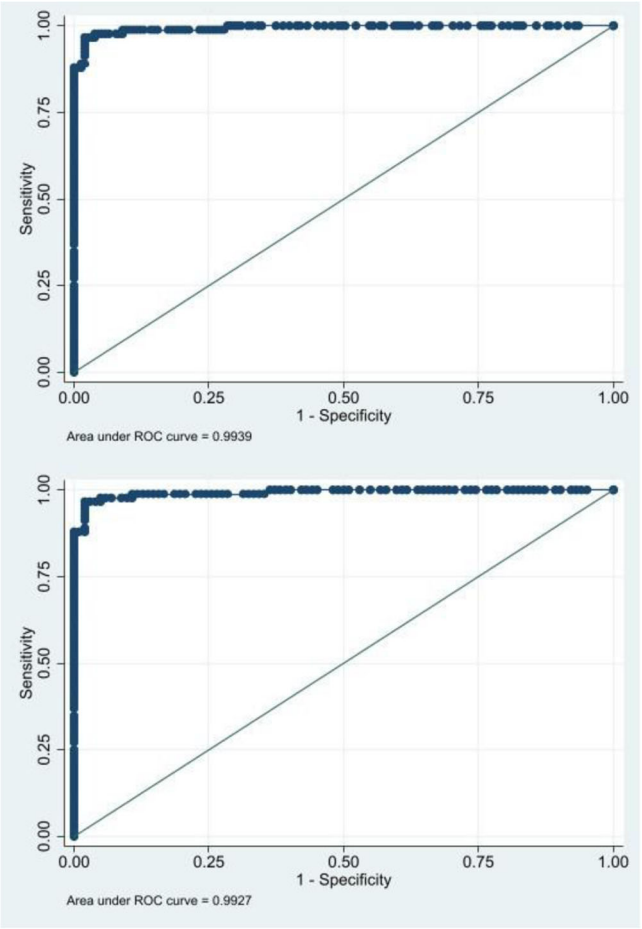

FIGURE 3 Receiver operative characteristic (ROC) curve demonstrating performance of late-night salivary cortisol (LNSC) for discriminating cases from all controls (upper panel) and cases from overweight/obese controls (lower panel). [Color figure can be viewed at wileyonlinelibrary.com]

3.1.4 | Late-night salivary cortisol (LNSC) performance using 95th percentile as the cut-off

Using LNSC 95th percentile (6.76 nmol/L) in controls as the cut-off, the sensitivity (95% CI) and specificity (95% CI) for diagnosis of CS were 97.8% (92.4%–99.4%) and 95.5% (91.0%–97.8%), respectively. In the analysis involving cases and normal-weight controls, the 95th percentile (6.51 nmol/L) cut-off yielded sensitivity (95% CI) and specificity (95% CI) of 97.8% (92.4%–99.4%) and 96.2% (87.3%–98.9%), respectively, while in the analysis involving cases and overweight/obese controls, the corresponding level (7.56 nmol/L) yielded sensitivity (95% CI) and specificity (95% CI) of 96.7% (90.9%–98.9%) and 95.1% (89.0%–97.9%), respectively.

TABLE 2 Diagnostic performance of late-night salivary cortisol and best cut-offs for diagnosis of Cushing syndrome.

Groups compared	LNSC Cut-off	True positive	False positive	True negative	False negative	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95% CI)	AUC (95% CI)
Cases and all controls	≥6.73 nmol/L	90	8	147	2	97.8% (92.4–99.4)	94.8% (90.2–97.4)	18.9 (14.8–24.2)	0.994 (0.987–1.000)
Cases and NW controls	≥6.73 nmol/L	90	2	51	2	97.8% (92.4–99.4)	96.2% (87.3–98.9)	25.9 (9.7–69.1)	0.996 (0.991–1.000)
Cases and OW/obese controls	≥7.26 nmol/L	90	5	97	2	97.8% (92.4–99.4)	95.1% (89.0–97.9)	19.9 (13.5–29.6)	0.993 (0.984–1.000)

Note: To convert cortisol from nmol/L to μg/dL, multiply the value in nmol/L by 0.0363.

Abbreviations: AUC, area under curve; LNSC, late-night salivary cortisol; LR+ , likelihood ratio positive. NW, normal-weight; OW/obese, overweight/obese.

4 | DISCUSSION

In this diagnostic accuracy study, we evaluated performance of LNSC in a South Asian population using the improved second-generation ECLIA kits. We included cases with both ACTH-dependent and independent CS, and normal-weight as well as overweight/obese controls, and demonstrated excellent discriminatory potential for LNSC in the study population. We also derived LNSC cut-offs for diagnosing CS and demonstrated high sensitivity and specificity for differentiation of CS from conditions that raise the suspicion of this disorder in the general population.

The diagnostic performance of LNSC for CS has been previously investigated by several groups and the proposed cut-offs vary from 3.6 to 15.2 nmol/L (Table 3).[7,10,11,14,16–32] The reasons for a wide variability in diagnostic cut-points include differences in the types of controls (apparently healthy vs. obese vs. pseudo-CS vs. suspected CS), sampling method (passive drooling technique vs. salivette device), assay methodology (RIA vs. ELISA vs. automated platform immunoassays vs. LC-MS/MS), statistical method used to derive the LNSC cut-point (upper limit of reference range vs. arbitrary thresholds vs. ROC analysis) and the numbers of cases and controls.[33] At the proposed cut-offs, sensitivity and specificity of LNSC vary between 68%–100% and 84%–100%, respectively, with most studies reporting both parameters in excess of 90%.[33] Previous studies performed using first-generation ECLIA kits proposed LNSC cut-offs ranging from 6.1 to 14.2 nmol/L, with sensitivity and specificity estimates of 69%–100% and 88%–100%, respectively.[7,14,24,27–29] With the emergence of second-generation ECLIA kits (Elecsys Cortisol-II) that employ monoclonal instead of polyclonal antibodies and offer greater specificity as well as improved analytical performance and reliability at lower levels relevant to salivary measurement,[8,9] LNSC diagnostic thresholds need to be reevaluated. Recently, using these newer kits, post-ACTH stimulation cortisol cut-offs for diagnosis of adrenal insufficiency were revised to 397 nmol/L (from a historical value of 497 nmol/L).[34]

We demonstrated that LNSC measured using Elecsys Cortisol-II assay was a reliable and accurate measure for the diagnosis of CS, with an overall AUC of 0.994 and a sensitivity of 98% and a specificity of 95% at cut-off level ≥6.73 nmol/L. Different statistical approaches have been used in the literature to arrive at the LNSC diagnostic cut-points. Some groups have set the cut-off at upper limits of reference range in normal population,[14,16,17] while others have used arbitrary thresholds.[18,20] We employed a ROC analysis to derive the optimal LNSC cut-off with highest sensitivity and specificity, an approach most consistently used in the literature.[19,21–32] Our ROC-derived LNSC cut-off was also very close to the 95th percentile determined in healthy population by the manufacturer, that is, 7.56 nmol/L[8] and the 97.5th percentile determined in healthy community-dwelling Asian Indians by Prasad et al.[35] with the same assay, that is, 6.89 nmol/L. Furthermore, we found that an alternative approach of using 95th percentile of LNSC (6.76 nmol/L) in our controls as the cut-off yielded a similarly high sensitivity (98%) and specificity (96%).

TABLE 3 Studies on late-night salivary cortisol for diagnosis of Cushing syndrome.

First author, year, country, Ref.	Assay	Cut-off method	Cut-off (nmol/L)	Sensitivity (%)	Specificity (%)
Raff, 1998, USA[16]	RIA	UL of RR	3.6	92	100
Castro, 1999, Brazil[17]	RIA	90thcentile of controls	7.7	93	93
Papanicolaou, 2002, USA[18]	RIA	Arbitrary	15.2	93	100
Putignano, 2003, Italy[19]	RIA	ROC	9.7	93	93
Yaneva, 2004, France[20]	RIA	Arbitrary	5.5	100	96
Trilck, 2004, Germany[21]	RIA	ROC	5.2	100	88
Viardot, 2005, Switzerland[22]	RIA	ROC	6.1	100	100
Doi, 2008, Japan[23]	RIA	ROC	5.8	93	100
Yaneva, 2009, Bulgaria[24]	ECLIA-I	ROC	14.2	93	94%
Nunes, 2009, France[25]	RIA	ROC	12.0	100	100
Cardoso, 2009, Argentina[26]	RIA	ROC	3.8	100	98
Carrozza, 2010, Italy[27]	ECLIA-I	ROC	8.3	100	97
Beko, 2010, Hungary[7]	ECLIA-I	ROC	9.7	100	88
Beko, 2010, Hungary[7]	RIA	ROC	8.0	100	71
Jeyaraman, 2010, India[14]	ECLIA-I	97.5th centile of controls	10.87	69	100
Deutschbein, 2012, Germany[28]	ECLIA-I	ROC	6.1	95	91
Belaya, 2012, Russia[29]	ECLIA-I	ROC	9.4	84	92
Bukan, 2015, India[30]	ELISA	ROC	5.04	96	100
Mészáros, 2018, Hungary[10]	ECLIA-II	ROC	7.28	97	92
Mészáros, 2018, Hungary[10]	LC-MS/MS	ROC	5.1	95	94
Aberle, 2018, Germany[11]	ECLIA-II	ROC	12.3	68	85
Lin, 2019, Taiwan[31]	RIA	ROC	4.7	98	100
van Baal, 2021, Germany[32]	CLIA	ROC	10.1	94	84
Our study, 2023, India	ECLIA-II	ROC	6.73	98	95

Note: To convert cortisol from nmol/L to μg/dL, multiply the value in nmol/L by 0.0363.

Abbreviations: CLIA, chemiluminescence immunoassay; ECLIA-I, electrochemiluminescence immunoassay first-generation; ECLIA-II, electrochemiluminescence immunoassay second-generation; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ROC, receiver operating characteristic; RIA, radioimmunoassay; RR, reference range; UL, upper limit.

The LNSC cut-off in the present study was lower than that proposed in a previous study from our hospital (10.87 nmol/L [sensitivity: 69%, specificity: 100%]) using Elecsys Cortisol-I assay,[14] albeit with a better diagnostic performance. Our study findings can be compared with two studies that have previously evaluated the diagnostic performance of LNSC using Elecsys Cortisol-II assay.[10,11] Mészáros et al.[10] found high diagnostic accuracy (AUC: 0.985) and a very similar cut-off value (7.28 nmol/L; sensitivity: 97%, specificity: 92%) in a Hungarian population (21 patients with active CS, 27 patients with CS in remission, 185 patients with suspected CS and 52 healthy participants). On the other hand, Aberle et al.[11] found a lower diagnostic accuracy (AUC: 0.8) and a higher cut-off value (12.3 nmol/L) with lower sensitivity (68%) and specificity (85%) in a German population (34 patients with Cushing disease, 83 obese controls and 40 healthy controls). These differences are accounted by several factors discussed previously and highlight the importance of deriving region and context-specific cut-offs, even when using the same assay. Recently, LC-MS/MS assays have been developed for measuring salivary steroids. Although, LNSC cut-offs for CS are lower with these assays, the diagnostic accuracy is similar to that of Elecsys Cortisol-II assay[10]; this supports the continued utility of less demanding immunoassays in routine laboratory diagnosis of CS. Regardless of the assay method, the sensitivity of LNSC is reported to be lower among patients with adrenal or ACTH-independent CS, attributed to a higher prevalence of mild hypercortisolism in these cases.[36,37] In a recent study, Kannankeril et al.[37] reported that 11 out of 16 patients (sensitivity: 31.3%) with adrenal CS had non-elevated LNSC measurement by an enzyme immunoassay. We found that LNSC result was below the proposed cut-off (or non-elevated) in only 1 out of 11 patients with adrenal CS; a possible

reason for this difference is an inclusion of patients with more severe or overt hypercortisolism in our study.

The specificity of a test for diagnosis of CS heavily depends on the characteristics of reference population and it is considered ideal to have a reference group that is eucortisolemic but closely mimics CS.[38] For instance, the specificity of ONDST for CS is 98.9% when reference population includes only "normal controls", but false positives increase and specificity plummets to 80.5% in a reference population comprising of "obese" and "other controls".[38] We found that mean LNSC levels were not different between normal-weight and overweight/obese controls and LNSC faired as an accurate and reliable test to separate patients with CS not only from normal-weight controls (AUC: 0.996; sensitivity: 98%, specificity: 96%), but also overweight/obese controls (AUC: 0.993; sensitivity: 98%, specificity: 95%). Our data confirm results from other groups that demonstrate a high diagnostic value of LNSC for CS in adults with obesity.

The strengths of our study are its large sample size, inclusion of a spectrum of controls, including those where CS is often suspected and investigated, and the relevance of results to a South Asian population where LNSC cut-offs using Elecsys Cortisol-II assay were previously not available. We acknowledge certain limitations. First, causes of pseudo-CS, other than obesity and uncontrolled diabetes, such as depression, chronic alcoholism and pregnancy were excluded in this study. Thus, we did not specifically address the utility of LNSC in the differential diagnosis of true and pseudo-CS. We included patients with "confirmed CS" but not those referred as "suspected CS" and found to be eucortisolemic on clinical and biochemical evaluation; inclusion of such borderline subjects could have added value to the ROC analysis. Second, overweight/obese controls were older compared to cases; this was not by design, rather due to CS predominantly being a disease of younger population and an expected increase in prevalence of obesity and related comorbidities with age. Since salivary cortisol measurements were not found to be affected by age (in adults <65 years of age) in a recent study by Raff et al.,[39] we do not expect a significant implication of this difference for our study findings. Third, we used serum separator tubes (which are routinely available in our hospital) to collect saliva samples rather than the more commonly used plain polypropylene tubes.[17,35] However, the approach was similar in both cases and controls; whether this nonconventional approach affects concentration of cortisol in saliva samples needs a formal evaluation. Finally, we excluded paediatric subjects and therefore our findings are only applicable for diagnosis of CS in adults.

To conclude, LNSC measured using second-generation cortisol ECLIA demonstrated high diagnostic accuracy for CS in our population. We propose a LNSC cutoff ≥6.73 nmol/L to diagnose CS with 98% sensitivity and 95% specificity.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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REFERENCES

1. Guaraldi F, Salvatori R. Cushing syndrome: maybe not so uncommon of an endocrine disease. *J Am Board Family Med*. 2012;25(2):199-208.
2. Fleseriu M, Auchus R, Bancos I, et al. Consensus on diagnosis and management of Cushing's disease: a guideline update. *Lancet Diab Endocrinol*. 2021;9(12):847-875.
3. Nieman LK, Biller BMK, 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.
4. Ambroziak U, Kondracka A, Bartoszewicz Z, Krasnodębska-Kiljańska M, Bednarczuk T. The morning and late-night salivary cortisol ranges for healthy women may be used in pregnancy. *Clin Endocrinol*. 2015;83(6):774-778.
5. Manetti L, Rossi G, Grasso L, et al. Usefulness of salivary cortisol in the diagnosis of hypercortisolism: comparison with serum and urinary cortisol. *Eur J Endocrinol*. 2013;168(3):315-321.
6. El-Farhan N, Rees DA, Evans C. Measuring cortisol in serum, urine and saliva - are our assays good enough? *Ann Clin Biochem: Int J Lab Med*. 2017;54(3):308-322.
7. Beko G, Varga I, Glaz E, et al. Cutoff values of midnight salivary cortisol for the diagnosis of overt hypercortisolism are highly influenced by methods. *Clin Chim Acta*. 2010;411:364-367.
8. Elecsys Cortisol-II Assay Kit Insert. Available from Accessed 23 February 2023. https://labogids.sintmaria.be/sites/default/files/files/cortisol_ii_2020-03_v6.pdf
9. Gagnon N, Fréchette I, Mallet PL, Dubé J, Houde G, Fink GD. Establishment of reference intervals for the salivary cortisol circadian cycle, by electrochemiluminescence (ECLIA), in healthy adults. *Clin Biochem*. 2018;54:56-60.
10. 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.
11. Aberle J, Schulze ZurWiesch C, Flitsch J, et al. Specificity of late-night salivary cortisol measured by automated electrochemiluminescence immunoassay for Cushing's disease in an obese population. *J Endocrinol Invest*. 2018;41(11):1325-1331.
12. Chong RY, Uhart M, McCaul ME, Johnson E, Wand GS. Whites have a more robust hypothalamic-pituitary-adrenal axis response to a psychological stressor than blacks. *Psychoneuroendocrinology*. 2008;33(2):246-254.
13. Cohen S, Schwartz JE, Epel E, Kirschbaum C, Sidney S, Seeman T. Socioeconomic status, race, and diurnal cortisol decline in the coronary artery risk development in young adults (CARDIA) study. *Psychosom Med*. 2006;68(1):41-50.

14. Jeyaraman K, Ammini AC, Nandita G, Dwivedi SN. Late-night salivary cortisol in normal subjects and in patients with Cushing's syndrome. *Postgrad Med J*. 2010;86(1017):399-404.
15. Attri B, Goyal A, Kalaivani M, et al. Clinical profile and treatment outcomes of patients with ectopic ACTH syndrome compared to Cushing disease: a single-center experience. *Endocrine*. 2023. doi:10.1007/s12020-022-03298-z
16. Raff H. Late-night salivary cortisol as a screening test for Cushing's syndrome. *J Clin Endocrinol Metab*. 1998;83:2681-2686.
17. Castro M, Elias PCL, Quidute ARP, Halah FPB, Moreira AC, Castro M. Out-patient screening for Cushing's syndrome: the sensitivity of the combination of circadian rhythm and overnight dexamethasone suppression salivary cortisol tests. *J Clin Endocrinol Metab*. 1999;84(3):878-882.
18. Papanicolaou DA, Mullen N, Kyrou I, Nieman LK. Nighttime salivary cortisol: a useful test for the diagnosis of Cushing's syndrome. *J Clin Endocrinol Metabo*. 2002;87(10):4515-4521.
19. Putignano P, Toja P, Dubini A, Giraldi FP, Corsello SM, Cavagnini F. Midnight salivary cortisol versus urinary free and midnight serum cortisol as screening tests for Cushing's syndrome. *J Clin Endocrinol Metab*. 2003;88:4153-4157.
20. Yaneva M, Mosnier-Pudar H, Dugué MA, Grabar S, Fulla Y, Bertagna X. Midnight salivary cortisol for the initial diagnosis of Cushing's syndrome of various causes. *J Clin Endocrinol Metab*. 2004;89:3345-3351.
21. Trilck M, Flitsch J, Lüdecke D, Jung R, Petersenn S. Salivary cortisol measurement- a reliable method for the diagnosis of cushing's syndrome. *Exp Clin Endocrinol Diabetes*. 2005;113:225-230.
22. Viardot A, Huber P, Puder JJ, Zulewski H, Keller U, Müller B. Reproducibility of nighttime salivary cortisol and its use in the diagnosis of hypercortisolism compared with urinary free cortisol and overnight dexamethasone suppression test. *J Clin Endocrinol Metab*. 2005;90:5730-5736.
23. Doi M, Sekizawa N, Tani Y, et al. Late-night salivary cortisol as A screening test for the diagnosis of Cushing's syndrome in Japan. *Endocr J*. 2008;55:121-126.
24. Yaneva M, Kirilov G, Zacharieva S. Midnight salivary cortisol, measured by highly sensitive electrochemiluminescence immunoassay, for the diagnosis of Cushing's syndrome. *Open Med*. 2009;4:59-64.
25. 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.
26. Cardoso EML, Arregger AL, Tumilasci OR, Contreras LN. Diagnostic value of salivary cortisol in Cushing's syndrome (CS). *Clin Endocrinol*. 2009;70(4):516-521.
27. Carrozza C, Corsello SM, Paragliola RM, et al. Clinical accuracy of midnight salivary cortisol measured by automated electrochemiluminescence immunoassay method in Cushing's syndrome. *Ann Clin Biochem: Int J Lab Med*. 2010;47(Pt 3):228-232.
28. Deutschbein T, Broecker-Preuss M, Flitsch J, et al. Salivary cortisol as a diagnostic tool for Cushing's syndrome and adrenal insufficiency: improved screening by an automatic immunoassay. *Eur J Endocrinol*. 2012;166(4):613-618.
29. Belaya ZE, Iljin AV, Melnichenko GA, et al. Diagnostic performance of late-night salivary cortisol measured by automated electrochemiluminescence immunoassay in obese and overweight patients referred to exclude Cushing's syndrome. *Endocrine*. 2012;41(3):494-500.
30. Bukan AP, Dere HB, Jadhav SS, et al. The performance and reproducibility of late-night salivary cortisol estimation by enzyme immunoassay for screening Cushing disease. *Endocrine Practice*. 2015;21(2):158-164.
31. Lin D, Tsai P, Lin Y. Midnight salivary cortisol for the diagnosis of Cushing's syndrome in a Chinese population. *Singapore Med J*. 2019;60(7):359-363.
32. van Baal L, Wichert M, Zwanziger D, et al. Distinct late-night salivary cortisol cut-off values for the diagnosis of hypercortisolism. *Horm Metab Res*. 2021;53(10):662-671.
33. 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.
34. Javorsky BR, Raff H, Carroll TB, et al. New cutoffs for the biochemical diagnosis of adrenal insufficiency after ACTH stimulation using specific cortisol assays. *J Endocr Soc*. 2021;5(4):bvab022.
35. Prasad N, Jabbar PK, Jayakumari C, et al. Late-night salivary cortisol in healthy, community-dwelling Asian Indians assessed by second-generation ECLIA. *J Clin Endocrinol Metab*. 2020;105(8):e2807-e2814.
36. 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.
37. Kannankeril J, Carroll T, Findling JW, et al. Prospective evaluation of late-night salivary cortisol and cortisone by EIA and LC-MS/MS in suspected Cushing syndrome. *J Endocr Soc*. 2020;4(10):bvaa107.
38. Aron DC. Cushing's syndrome: why is diagnosis so difficult? *Rev Endocrine Metab Disord*. 2010;11(2):105-116.
39. 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.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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