

Late Night Salivary Cortisol for the Screening of Cushing's Syndrome in Patients with Chronic Renal Failure

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Abstract

Background: The thresholds of Late Night Salivary Cortisol (LNSC) vary widely among studies due to differences in assay methodologies and in control groups.

We aimed to verify the analytical performance of the LNSC by electrochemiluminescence assay (ECLIA) and to establish cut-off values of LNSC for the screening of Cushing's Syndrome (CS) according to renal function.

Methods: Patients with suspected CS underwent screening tests including LNSC and 1 mg Dexamethasone Suppression Test (DST). Subjects with abnormal response underwent a 2 mg DST, ACTH assessment and complementary radiological explorations. For the analytical evaluation, the study was performed with guidance from the Clinical and Laboratory Standards Institute.

Results: Reference range for LNSC was 1.06 - 1.58 ng/mL in healthy subjects. The intra assay and inter assay coefficients of variation were 9.84 % and 10.5 % respectively. The detection and quantification limits for salivary cortisol measurement were 0.05 ng/ml and 0.08 ng/ml respectively. In group of patients, LNSC was correlated with nocturnal serum cortisol ($r = 0.56$, $p = 0.004$), with serum cortisol after 1 mg DST ($r = 0.82$, $p = 0.04$) and after 2 mg DST ($r = 0.89$, $p < 0.001$) but not with serum creatinine. BMI was correlated with serum cortisol after 1 mg DST but not with salivary cortisol. In patients with unaffected renal function, using a 2.41 ng/ml cutoff, LNSC gave a sensitivity of 96.2 % and specificity 86.8% for the screening of CS. Patients with chronic renal failure, using a 2.74 ng/ml cutoff, the sensitivity and specificity of LNSC to identify CS was 83.3% et 90.0% respectively.

Conclusion: Our data confirm the usefulness of LNSC measurement as an initial and simple test for screening for CS, using an automated ECLIA, especially in patients with chronic renal failure.

Keywords: Cushing's Syndrome; Cut Off; Immunoassay; Saliva; Cortisol; Chronic Renal Failure

Introduction

Cushing's syndrome (CS) is considered a rare and disabling disease resulting from prolonged exposure to glucocorticoids [1]. Clinical features of CS are various including central obesity, hypertension, purple striae, hirsutism, menstrual irregularities, glucose intolerance etc [2]. Thus, the diagnosis may be challenging when the presenting features are mild and common in the general population [3], in renal failure, in incidental adrenal masses, or during pregnancy [3].

The Clinical Guideline Committee of The Endocrine Society recommends the use of late night salivary cortisol LNSC for the initial testing for CS [4]. LNSC is much more practical and can be easily performed on ambulatory patients. However, the normal reference ranges and thresholds of LNSC vary widely among studies due to differences in assay methodologies and in control groups [5]. Moreover, there is still lack of standardization and harmonization for kits and techniques used for LNSC testing [6]; the assay should have a good sensitivity to allow detecting low concentrations of LNSC.

Aim of the Study

We aimed in the present study to:

- Verify the analytical performance of the LNSC testing by electrochemiluminescence assay (ECLIA).
- Establish the cutoff values of LNSC for the diagnosis of CS according to renal function, using an automated ECLIA.

Methods

This was a prospective study conducted in Endocrinology Department of Hedi Chaker hospital (Sfax, Tunisia) from September 2011 to July 2017. All patients gave informed written consent and the study was approved by the regional ethics committee.

We included patients who referred for endocrinological evaluation because of erythrosis face, buffalo hump, obesity/rapid weight gain, hypertension with hypokalemia or because of the presence of incidentally discovered adrenal tumors. Height, weight, waist circumference (WC), blood pressure (BP) were measured and body mass index (BMI) was calculated for all enrolled subjects. All biochemical samples were analyzed in the one - site laboratory: glycaemia (Gly), total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C). Serum creatinine concentration on admission was measured by the modified Jaffe's techniques and was used to calculate the glomerular filtration rate (GFR) by using the Modification of Diet in Renal Disease (MDRD) equation [7]. Chronic renal failure was diagnosed according to National Kidney Foundation classification [8]. Subjects underwent screening tests for CS including an assessment of serum cortisol (8 a.m, 4p.m, 11 p.m), LNSC and serum cortisol after 1 mg dexamethasone suppression test (DST). Saliva was collected in test tube at 11 p.m without using collection device, kept at 4°C and conducted to laboratory at the morning. Subjects with abnormal response underwent a 2 mg DST, ACTH assessment and complementary radiological explorations. Unfortunately, 24-hours urine cortisol was unavailable.

Salivary and serum cortisol were both stored at -20°C until assayed. They were measured by an automated ECLIA (Elecsys 2010, Roche-diagnostics, Mannheim, Germany) using a commercial kit (Cortisol Elecsys® Roche and Cobas analysers). 18 ng/mL was the cut off used for CS diagnosis after DST [9]. Based on the final diagnosis, each patient was included into one of the following four groups: group A: patients with CS, group B: patients with subclinical CS, group C: patients with pseudo CS, group D: patients without HPA disturbances. CS was diagnosed if the patient had the characteristic clinical symptoms of CS and confirmed by histopathological examination of the resected tumors [10]. Subclinical CS was defined as the presence of an altered glucocorticoid secretion with absence of the classical signs of overt CS (a biological hypercortisolism) [11]. Pseudo CS was defined as an overactivity of the HPA axis, it can present with a similar clinical phenotype and is associated with chronic alcoholism, psychiatric disorders, severe obesity, poorly controlled diabetes, and extreme physical stress. Serum cortisol after DST were used to differentiate between CS and pseudo CS [9].

For the analytical evaluation, the study was performed with guidance from the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS), protocol EP5-A2.20 according to the International Standards Organization ISO 15189 recommendations [12].

For determination of reference range: we collected saliva at 23 pm from 20 healthy subjects, LNSC was tested by the same method and reference range was calculated based on 95% confidence intervals limits [13].

When evaluating the precision of a method it is necessary to assess the repeatability (within-run) and the between-run precision [14]. Saliva pool ranged between 1.87 and 2.83 ng/mL, were assayed using same lots of reagents at the same moment 20x times (within run precision) and at separate times for 20 days (between run precision). Each reagent lot used a single calibration curve throughout the study. Analytical and functional sensitivities were obtained by measuring the limits of detection and quantification respectively, using the calibrator matrix without antigen (cal 0), 20 times. The limit of detection was defined as Standard deviation (SD) of Cal 0 x 3 and limit of quantification was calculated as SD of Cal0 x 10 [12] then compared to the manufacturers’ data sheet.

Statistical analysis

Statistical analysis was performed with the software Statistical Package for Social Sciences (SPSS) for Windows (version 20.0, SPSS Inc., Chicago, IL). Quantitative variables were expressed as mean ± SD or median [minimum-maximum], depending on the nature of the distribution. Qualitative variables were expressed as frequencies. For the comparison of means, we used Student’s t-test or the Mann-Whitney U test whenever the normality of the distributions was not observed (non-Gaussian distribution) or the size of one of the groups was small (< 10). Categorical variables were analyzed by the c2 test or Fisher’s exact test whenever a population was < 5. Differences between results were considered significant at a value of p < 0.05. For multiple comparisons we used the Bonferroni correction. In order to see the power of the different biomarkers in the prediction of ICU stay, we used linear regression to calculate predicted length of stay by each marker and we compared the predicted stays with the observed one. To determine the different threshold values, we used the ROC (receiver operating characteristic). The best threshold value is the value having the best sensitivity and specificity.

Results

A total of 56 patients with mean age 58.1 ± 15.5 years were finally enrolled (30 men and 26 women). 22 patients from 56 had clinically CS (group A), 7 patients had subclinical CS (group B) and 8 patients had a pseudo CS (group C). Group D (n = 19) was composed of non-functioning adenomas, Conn adenomas, male osteoporosis, metabolic syndrome etc.

16 patients (28.5%) had also chronic renal failure, among them 7 were diagnosed as CS.

Of the patients with CS, one patient had ACTH-producing pituitary adenomas and 21 had adrenal CS due to unilateral (n = 15) or bilateral (n = 6) adrenal tumors. Almost patients with adrenal CS underwent surgical intervention, and histological examination confirmed the adrenocortical tumor. Adrenocortical carcinomas were diagnosed in 5 cases. Subclinical CS was discovered incidentally during abdominal imaging in all examined cases. Clinical features and metabolic parameters of subgroups were summarized in table 1. LNSC was correlated with nocturnal serum cortisol (r = 0.56, p = 0.004, table 2), with serum cortisol after 1 mg DST (r = 0.82, p = 0.04, table 2) and after 2 mg DST (r = 0.89, p < 0.001, table 2) but not with GFR. Serum cortisol level at 8 am was correlated with GFR (Figure 1A).

BMI was correlated with serum cortisol after 1 mg DST but not with salivary cortisol (Table 3).

	Group A, n = 22	Group B, n = 7	Group C, n = 8	Group D, n = 19
Age, years	58.71 ± 15.8	58.8 ± 22.3	62.3 ± 13.6	55.3 ± 15.18
BMI, kg/m ²	31.6 ± 5.7	33.69 ± 7.52	27.9 ± 2.5	31.7 ± 5.2
Serum cortisol at 8h00, ng/mL	374 ± 81.8	174 ± 72.6	96.6 ± 65	165.12 ± 70.2
Serum cortisol at 16h00, ng/mL	127 ± 83.1	110.7 ± 56.6	74.2 ± 11	100.4 ± 44.26
Serum cortisol at 23h00, ng/mL	117.8 ± 87.3	87.9 ± 71.9	40.9 ± 35	121.6 ± 94.1
Salivary cortisol at 23h00, ng/mL	7.62 ± 10.75	3.63 ± 3.91	3.08 ± 1.9	2.8 ± 2.1
Serum cortisol after 1 mg DST, ng/mL	37.2 ± 32.3	27.8 ± 8.1	38.2 ± 31.9	17.2 ± 6.2
Serum cortisol after 2 mg DST, ng/mL	55.7 ± 100.7	60.2 ± 45.6	15.3 ± 6.7*	35.5 ± 59.1

ACTH, pg/mL	44 ± 66.2	18	ND	ND
Central obesity (n)	15	4	1	6
Erythrosis face	11	0	2	2
Fragile skin	7	0	2	2
Buffalo hump	4	2	0	2
Acanthosis Nigricans	1	1	1	1
Purple striae	2	0	0	2
Diabetes	18	3	5	9
Hypertension	19	4	5	12
Dyslipidemia	10	3	4	7
Chronic renal failure	6	1	3	6
Metabolic syndrome	12	3	6	7
Adrenal adenoma	11	5	1	4
Bilateral adrenal hyperplasia	3	3	0	2

Table 1: Demographic and hormonal characteristics of the studied population.

CS: Cushing’s Syndrome, Group A: Clinically CS, B: Subclinical CS, C: Pseudo CS, D: Non CS group,

BMI: Body mass index, DST: Dexamethasone suppression test, ND: Not done.

Salivary cortisol	r	p
Serum cortisol 11 pm	0.564	0.004*
Serum cortisol after 1 mg DST	0.827	0.04*
Serum cortisol after 2 mg DST	0.893	< 0.001*
GFR _{MDRD}	0.077	0.686

Table 2: Correlations between serum creatinine, salivary and serum cortisol in the studied population (n = 56)

DST: Dexamethasone suppression test, GFR: glomerular filtration rate, r: coefficient of correlation, * p < 0.05, significant

BMI	r	p
Serum cortisol after 1 mg DST	0.642	0.03
Salivary cortisol	0.165	0.280
Serum cortisol after 2 mg DST	-0.114	0.639

Table 3: Correlations between BMI, salivary and serum cortisol in the studied population (n = 56).

BMI: Body Mass Index, DST: Dexamethasone suppression test, r: coefficient of correlation, p: p-value.

Patients with GFR < 30 ml/min had higher levels of serum cortisol at 8 a.m and 11p.m compared with those with GFR > 30 mL/min (217.78 ± 49.13 ng/mL vs 153.92 ± 45.08 ng/mL, p = 0.08; and 94.60 ± 24.96 ng/mL vs 90.33 ± 27.2 ng/mL, p = 0.851 respectively, Figure 1B).

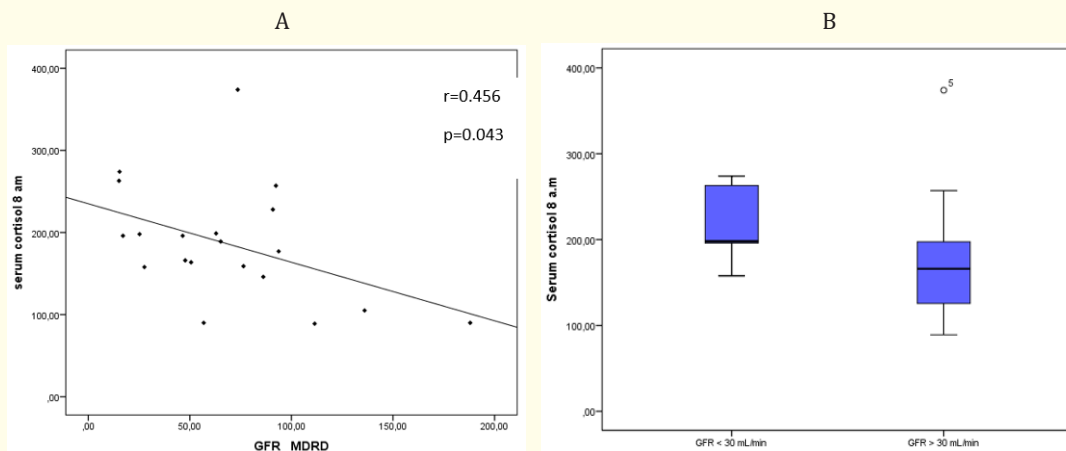


Figure 1: A: Correlation between Serum cortisol 8a.m and Glomerular Filtration Rate estimated by MDRD (GFR); B: box plot showing differences between serum cortisol 8 a.m according to GFR.

Using 2.41 ng/mL threshold derived from ROC analysis, the sensitivity and specificity of LNSC to identify CS was 96.2 % and 81.8% respectively (Figure 2A).

LNSC cutoff value 2.41 ng/ ml demonstrated higher sensitivity and specificity than serum cortisol after 1 mg DST in the screening of CS (AUC were 0.812 and 0.662 respectively, figure 2A).

In patients with chronic renal failure, using a 2.74 ng/ ml cutoff, the sensitivity and specificity of LNSC to identify CS was 83.3% et 90.0% respectively (Figure 2B).

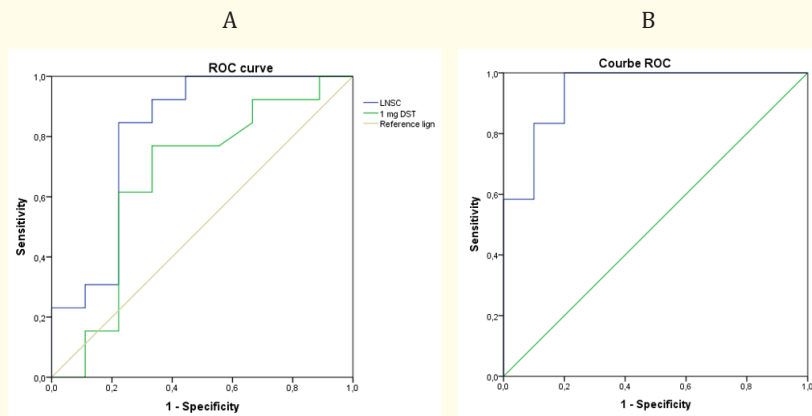


Figure 2: Cut off value of LNSC and serum cortisol after 1 mg DST for the screening of Cushing’s syndrome in patients with conserved renal function (A) and with chronic renal failure (B) using ROC curve.

Variables	AUC	P	Confidence interval 95%	
Serum cortisol 1 mg DST	0,662	0,205	0,409	0,916
LNSC (A)	0,812	0,015	0,604	1,000
LNSC (B)	0,942	< 0.001	0,845	1,000

Specific performance data

The within and between run precision were expressed by intra assay and inter assay coefficients of variation (CV) (9.84% and 10.5% respectively, table 4). The detection and quantification limits for salivary cortisol measurement were 0.6 ng/ml (analytical sensitivity) and 0.18 ng/ml (functional sensitivity) respectively (Table 4). LNSC reference range was 1.06 - 1.58 ng/mL (confidence interval 95%).

20 µl saliva	Analytical/Functional Sensitivity, ng/mL (n = 20)	Within run precision, % (n = 20)	Between Run precision, % (n = 20)
Mean (ng/mL)	0.67	2.25	2.35
SD (ng/mL)	0.06	0.22	0.24
CV (%)	8.96	9.84	10.5
	0.18 /0.6		

Table 4: Analytical performance of LNSC by ECLIA method.
SD: Standard Deviation; CV: Coefficient of Variation.

Discussion

CS describes the symptoms due to prolonged supraphysiological levels of circulating glucocorticoids [15]. It may be caused by an excess of adrenocorticotrophic hormone (ACTH) secretion (80 - 85%), usually by a pituitary corticotroph tumor by an ectopic ACTH secreting adenoma (10 - 15%). Moreover, ACTH-independent CS may result from excessive glucocorticoid secretion by unilateral adrenocortical tumors or by bilateral adrenal hyperplasia [15]. According to recent guidelines, the diagnostic algorithm includes measurement of diurnal variation of plasma cortisol, determination of 24-h urinary free cortisol, measurement of LNSC and determination of plasma cortisol after 1 mg DST [16]. Unfortunately, measurement of 24-h urinary free cortisol was not possible in our study.

Salivary cortisol sampling is easy to collect and less stressful than blood collection. In almost studies, LNSC was measured by using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) methods [17] while few studies used an automated assay to evaluate LNSC in healthy subjects and only one study in nine CS patients [18,19]. Recently, automated ECLIA have come into use for measuring cortisol in saliva. The serum cortisol assay on the cobas 6000 analyzers is a competitive polyclonal antibody immunoassay that employs a magnetic separation step followed by electrochemiluminescence quantitation (Roche Diagnostics) [20].

It has been reported that it offers an acceptable analytical performance even in the very low concentration range and have several advantages, including no pretreatment of samples and a quicker turnaround time [21]. Our results supported these findings; indeed, the detection and quantification limits were tested according to international norms. The limits of detection and quantification were < 0.18 and < 0.6 ng/mL respectively, which is similar to manufacturer’s sheet (< 0.18 ng/mL and < 0.7 ng/mL respectively) [20].

LNSC tested by ECLIA showed good analytical and functional sensitivities, when compared with the liquid chromatography- tandem mass spectrometry method used in a larger number of CS patients [19,22]. However, there is little information on the normal reference range and cut-off for LNSC measured by automated ECLIA [23]. To our best knowledge, few studies focused on the diagnostic value of LNSC in Cushing’s syndrome with chronic renal failure. Our data confirm the results of other groups from overseas [24]. The diagnostic performance of LNSC was similar whether saliva was collected in collection device or not.

To assess a best specificity with reliable high sensitivity, the diagnostic accuracy of LNSC in this study was performed by the ROC analysis. Above 2.41 ng/ml, LNSC gave a sensitivity of 96.2 % and specificity 86.8% for the screening of CS. Various cut off points have been reported from several studies ranging from 2.23 to 5.11 ng/ml [24]. Our cut off value is comparable to those of Yaneva., *et al.* (2 ng/ml) [25] and Viardot., *et al.* (2.2 ng/ml) [26].

Difference between cut offs may be due to preanalytical phase (population studied, different sample collections) and analytic procedures (storage, measurement etc).

Inherent differences between sensitivity and specificity may be due to analytical differences which cause difficulties in determining optimal diagnostic criteria; for example, the two different antibody based techniques, enzyme-linked immunosorbent assay (ELISA) and RIA, gave different results from the same sample [27]. Thus, the cut off values of LNSC must be carefully adjusted using a standardized collection and assays. According to Timo D., *et al.* [28], serum cortisol after 1mg DST is preferable to LNSC when screening for CS in patients with adrenal incidentalomas, because of its higher specificity. It is important to keep in mind that there are also some disadvantages to the serum cortisol after 1 mg DST suppression. For instance, Crapo., *et al.* [29] reported that the rate of false-positive results is considerably higher in the presence of obesity, psychiatric disorders, chronic illness, and certain drugs respectively. In these cases, further confirmatory testing may be required. In pseudo CS, mean serum cortisol after 1 mg DST is almost elevated while mean LNSC was just upper the cut off. Besides, some CS patients retain cortisol suppression to dexamethasone [30]. This unusual sensitivity may be due to individual differences in the absorption and metabolism of dexamethasone. For instance, patients with liver and/or renal failure may suffer from impaired glucocorticoid clearance, whereas drugs as well as alcohol may induce its hepatic metabolism [31]. Because plasma free cortisol is filtered through the glomeruli with partial tubular reabsorption, the amount of free cortisol appearing in the urine is theoretically dependent on the glomerular filtration rate. Allen Chan KC., *et al.* [32] demonstrated that the urinary free cortisol excretion rate is significantly reduced in patients with moderate to severe renal impairment and consequently has diminished sensitivity in the diagnosis of CS in such patients. He proposed to use late-night serum salivary cortisol for the screening and diagnosis CS in patients with significantly impaired renal function [32].

In our study, serum cortisol was correlated with GFR, however LNSC was not, so it can be used in renal failure rather than serum cortisol, for the diagnosis of CS. The cutoff of LNSC in renal failure was higher (2.74 ng/ ml) than in patients with unaffected kidney.

We found also a correlation between BMI and serum cortisol after 1 mg DST leading probably to falsely positive response in obese patients, unlike LNSC. In fact, there was no correlation between LNSC and BMI. Thus, LNSC is a better screening test for CS in overweight and obese patients.

The strength of the present study includes the fact it's the first study in Tunisia and North Africa that establish new cutoffs for LNSC using an automated ECLIA, according to renal function, which is rapid, safe and provides a good analytical performance.

Some limitations of our study had been reported including the unavailability of 24 h urinary cortisol, the remote possibility of cyclic CS [33] and the lack of biochemical follow- up of some subjects with an abnormal screening result.

Conclusion

In conclusion, our data confirm the usefulness of LNSC measurement as an initial and simple test for screening Cushing's syndrome in Tunisia. Larger series are needed to confirm our findings. Some authors propose to extend the use of LNSC as screening test for subclinical Cushing's syndrome in large high risk population including metabolic syndrome, obesity or diabetes but it is still controversial.

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Competing Interests

The authors declare no competing interest.

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