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Distinct Late-Night Salivary Cortisol Cut-Off Values for the Diagnosis of Hypercortisolism

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ABSTRACT

Due to high morbidity and mortality of untreated hypercortisolism, a prompt diagnosis is essential. Measurement of late-night salivary cortisol provides a simple and non-invasive method. However, thresholds and reference ranges differ among studies. The goal of this study was to define a threshold of late-night salivary cortisol for the diagnosis of hypercortisolism based on the used assay. Moreover, the influence of different aetiologies of hypercortisolism and individual comorbidities were investigated. Prospective analyses of 217 patients, including 36 patients with proven hypercortisolism were carried out. A sum of 149 patients with suspicion of hypercortisolism but negative endocrine testing and 32 patients with hypercortisolism in remission served as control group. Late-night salivary cortisol was measured using an automated chemiluminescence immunoassay. Cut-off values were calculated by ROC analysis. The calculated cut-off value for the diagnosis of hypercortisolism was 10.1 nmol/l (sensitivity 94 %; specificity 84 %). Only slightly lower thresholds were obtained in patients with suspected hypercortisolism due to weight gain/obesity (9.1 nmol/l), hypertension or adrenal tumours (both 9.8 nmol/l) or pituitary adenomas (9.5 nmol/l). The late-night salivary cortisol threshold to distinguish between Cushing's disease and Cushing's disease in remission was 9.2 nmol/l. The cut-off value for the diagnosis of ectopic ACTH-production was 109.0 nmol/l (sensitivity 50 %, specificity 92 %). Late-night salivary cortisol is a convenient and reliable parameter for the diagnosis of hypercortisolism. Except for ectopic ACTH-production, thresholds considering different indications for evaluation of hypercortisolism were only slightly different. Therefore, they might only be useful if late-night salivary cortisol results near the established cut-off value are present.

Introduction

Diagnosis of hypercortisolism (HC) is still one of the most challenging tasks in clinical endocrinology. Due to the high morbidity and mortality of untreated HC, a prompt diagnosis is essential [1]. The current Endocrine Society guideline suggests one of the following screening tests for the diagnosis of HC: 24-hours urinary free cortisol (UFC), 1 mg overnight dexamethasone suppression test (DST), or late-night salivary cortisol (LNSaC) [2, 3].

The advantage of LNSaC is its non-invasive measurement, its independency from plasma levels of cortisol-binding globulin and from interference with oral oestrogen medication. Hence LNSaC has been proposed as a feasible diagnostic tool in patients with sus-

pected HC, in particular in the outpatient setting with an optimal time point for collection of salivary cortisol between 11 and 12 PM [4]. Variability in LNSaC cut-offs may arise from comorbidities with recent studies showing higher levels of LNSaC in subjects with higher age, hypertension, and diabetes [5]. Higher levels of LNSaC have also been described when patients smoked before sample collection or if sample contamination with blood after teeth brushing occurred [6]. To avoid interferences, a collection at least half an hour after eating, drinking, or teeth-brushing is recommended [7].

For LNSaC measurement, several methods have been developed and include radioimmunoassays, automated electrochemiluminescence immunoassays, and recently liquid chromatography-tandem mass spectrometry [8].

Although measurement of LNSaC is recommended, reference ranges and cut-off levels depend on the analytic method and the used assay. Moreover, published studies often included healthy controls or control groups that did not meet criteria for suspicion of HC to define thresholds (▶ **Table 1**). Described thresholds differ from 2.2–13.5 nmol/l (0.08–0.49 µg/dl) for the diagnosis of HC [9–11], with reported sensitivities and specificities of up to 100 % [9, 10, 12–17]. Therefore, the goal of this prospective single centre study was to evaluate a cut-off value for LNSaC for the diagnosis of HC using a chemiluminescence immunoassay and to investigate the impact of different comorbidities and aetiologies on this cut-off in a tertiary endocrine referral centre with the so far largest meaningful control group.

Subjects and Methods

Compliance with Ethical Standards

The study was a single centre prospective study at the Department of Endocrinology, Diabetes and Metabolism, University Hospital Essen, Germany, from 2017–2019, tertiary endocrine referral centre in the Ruhr Metropolitan area, Nord Rhine Westphalia, with a catchment area of 5 million inhabitants.

All procedures performed in this study involving human participants were in accordance with the ethical standards of the ethics committee of the University Hospital Essen and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Subjects

Two hundred and seventeen patients (146 female, 71 male) were studied prospectively, including 36 patients with proven HC. One hundred forty-nine patients with suspicion of HC but subsequent negative testing and 32 patients with HC in remission served as controls (▶ **Fig. 1**). Patients under medication influencing cortisol metabolism (e. g., oral contraceptives, somatostatin analogue, mitotane), pregnant women and patients under systemic, or topical steroid therapy were excluded from the study. Written consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used.

Patients with proven HC

Thirty-six patients (25 females, 11 males; age: 48.58 ± 14.34 years) with confirmed HC [13 Cushing's disease, 8 ectopic ACTH-production, 15 adrenal Cushing's syndrome (of these 7 metastasized adrenocortical carcinomas)] were enrolled. These patients were referred to our centre with clinically overt HC. Most clinical signs and symptoms of HC were hypertension ≥ WHO grade II (prevalence: 69 %), weight gain/obesity (prevalence: 65 %), skin complaints (prevalence: 59 %) or proximal myopathy (prevalence: 43 %). The biochemical diagnosis for HC was established by at least two of the following parameters: elevated 24-hour urinary-free cortisol levels [measured by electrochemiluminescence immunoassay (ECLIA, Cobas e411, Roche Diagnostics, Rotkreuz, Switzerland)] in at least two measurements, elevated midnight serum cortisol (measured by ECLIA, Atellica IM, Siemens Healthcare, Erlangen, Germany) and insufficient serum cortisol suppression during a 1 mg dexamethasone suppression test. A final diagnosis of the cause of HC was established based on measurement of ACTH and in case of an ACTH-depending hypercortisolism due to the results of inferior petrosus sinus sampling (IPSS). ACTH-producing pituitary adenomas and ectopic ACTH-production as well as adrenal adenomas were proven histologically.

Patients with suspected HC

One hundred and forty-nine patients (99 females, 50 males; age: 47.95 ± 15.82 years) with suspected HC due to the leading clinical sign [38 with weight gain/abdominal obesity (median weight gain of 18 kg in 12 months, median BMI 33.9 kg/m^2), 21 with hypertension WHO grade > II, 21 with other features for HC, e. g., osteoporosis, hyperhidrosis, or hypokalaemia] or with newly diagnosed non-functioning endocrine tumour (32 pituitary, 37 adrenal tumours) were enrolled. In each of the subgroups, no other suggestive clinical signs (e. g., myopathy, skin lesions, depression) for HC were present. In this context it is worth mentioning that hypertension WHO grade I-II, a long-lasting general obesity without significant weight gain or a well-controlled diabetes were not defined as a suggestive clinical sign for HC. In all these patients HC was excluded in our department due to normal urinary free cortisol in two measurements, cortisol < 1.8 µg/dl after 1 mg dexamethasone and, where available, awake midnight serum cortisol < 5.0 µg/dl. Therefore, an autonomous cortisol secretion in patients with adrenal tumours could also be excluded [18]. Patients with autonomous cortisol secretion were not detected within the study period. In patients with newly diagnosed non-functioning endocrine tumour a hormone excess was excluded by respective biochemical parameters. Patients with adrenal or pituitary mass were recruited sequentially and not selected based on a clinical suspicion of HC.

Patients with HC in remission

Thirty-two patients (22 females, 10 males; age: 51.11 ± 15.02 years) being followed-up at our department for previous HC were enrolled. Median follow-up time of these patients was 7 years (3 months to 21 years) after endocrine surgery. Twenty-seven patients had received pituitary surgery for Cushing's disease, three had undergone endocrine surgery for ectopic ACTH-production, and two for a cortisol-producing adrenal tumour. Remission was defined as absence of clinical symptoms of HC and negative bio-

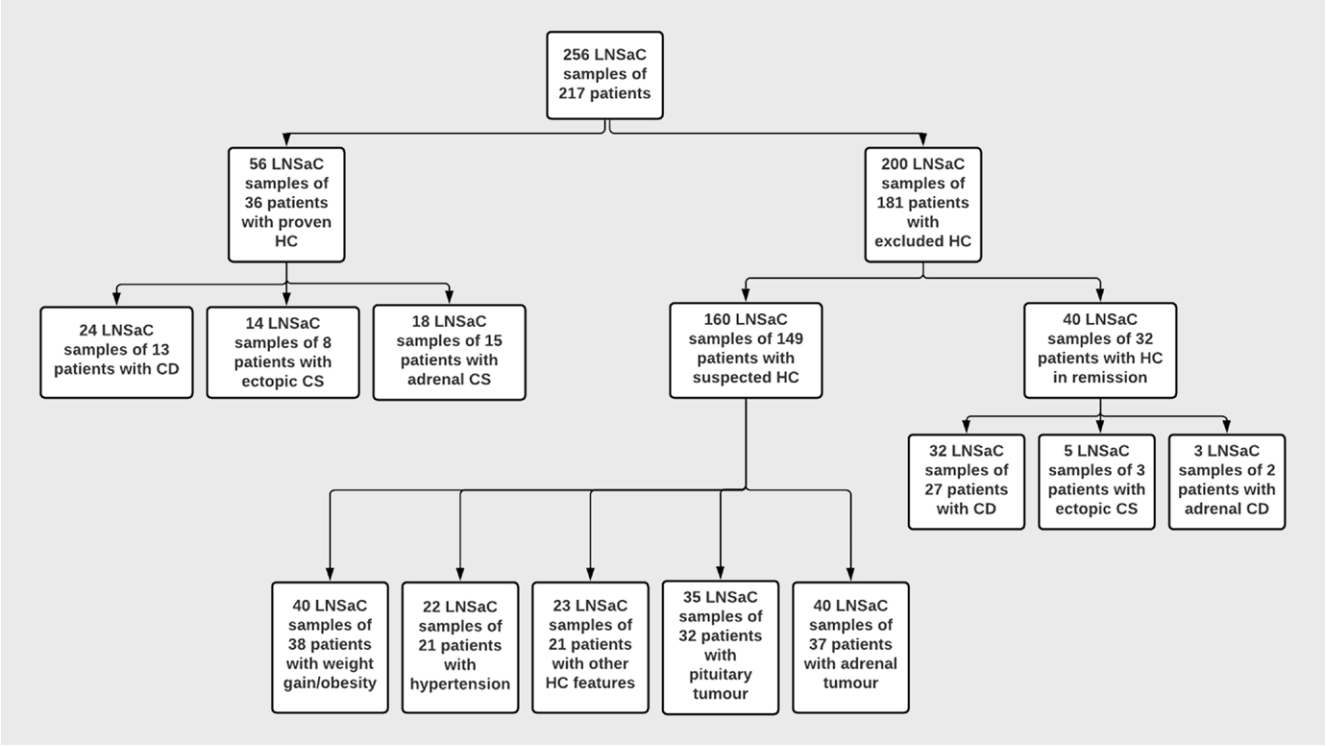
▶ **Table 1** Overview of cited studies considering cut-off values of LNSaC for the diagnosis of HC.

Ref.	Source of HC	Control group	Investigated groups	Cut-off	Sens.	Spec.	Assay
Amlashi et al. [20]	68 CD	89 CD in remission	CD vs. CD in remission	7.4 nmol/l (0.27 µg/dl)	75 %	95 %	ELISA
Lages et al. [19]	22 CD, 2 ectopic, 7 adrenal CS	57 healthy subjects	CS vs. control	2.8 nmol/l (0.10 µg/dl)	97 %	91 %	RIA
Raff et al. [11]	39 CS	39 pt. with HC features, 73 healthy subjects	CS vs. control	3.6 nmol/l (0.13 µg/dl)	92 %	100 %	RIA
Carrasco et al. [23]	18 CD	50 CD in remission	CD vs. CD in remission	5.5 nmol/l (0.20 µg/dl)	100 %	98 %	RIA
Putignano et al. [10]	41 CS	33 Pseudo-CS, 199 obese pt., 27 healthy	CS vs. control	9.7 nmol/l (0.35 µg/dl)	93 %	93 %	RIA
Ceccato et al. [24]	52 CD, 13 ectopic CS, 17 adrenal CS	73 pt. with HC features, 104 healthy subjects	HC vs. HC with features HC vs. healthy subjects HC vs. adrenal tumour	14.2 nmol/l (0.52 µg/dl) 14.5 nmol/l (0.52 µg/dl), 13.7 nmol/l (0.50 µg/dl)	96 % 96 % 98 %	95 % 97 % 98 %	RIA
Papanicolauo et al. [9]	98 CD; 12 ectopic CS, 13 adrenal CS	23 pt. with HC features	CS vs. control	15.2 nmol/l (0.55 µg/dl)	93 %	100 %	RIA
Sturmer et al. [26]	9 CS	65 pt. with HC features	CS vs. control	1.9 nmol/l (0.07 µg/dl)	100 %	92 %	LC-MS/MS
Zerikly et al. [16]	38 CS	52 pt. with HC features, 18 healthy subjects	CS vs. control	2.95 nmol/l (0.11 µg/dl)	92 %	92 %	LC-MS/MS
Erickson et al. [25]	47 CS	202 pt. with HC features	CS vs. control	2.1 nmol/l (0.08 µg/dl)	75 %	90 %	LC-MS/MS
Antonelli et al. [8]	25 CS	91 healthy subjects	CS vs. control	2.4 nmol/l (0.09 µg/dl)	100 %	98 %	LC-MS/MS
Bäcklund et al. [15]	22 CS	155 pt. with not more than obesity, hypertension, diabetes	CS vs. control	3.6 nmol/l (0.13 µg/dl)	90 %	96 %	LC-MS/MS
Mészáros et al. [17]	38 CS	185 pt. with HC features, 52 healthy subjects	CS vs. control	5.1 nmol/l (0.18 µg/dl) LC-MS/MS 7.3 nmol/l (0.26 µg/dl) CLIA	95 % 97 %	94 % 92 %	LC-MS/MS + CLIA
Aberle et al. [12]	34 CD	83 pt. with BMI ≥ 35 kg/m^2, 40 healthy subjects with BMI < 25 kg/m^2	CD vs. healthy subjects CD vs. pt. with BMI > 35 kg/m^2	8.3 nmol/l (0.30 µg/dl) 12.3 nmol/l (0.45 µg/dl)	85 % 68 %	88 % 78 %	CLIA
Belaya et al. [22]	40 CD, 1 ectopic CS, 4 adrenal	78 obese pt., 98 healthy subjects	CS vs. control CS vs. healthy subjects	9.4 nmol/l (0.34 µg/dl) 7.0 nmol/l (0.25 µg/dl)	84 % 91 %	98 % 97 %	CLIA
Cecatto et al. [21]	47 CS	117 pt. with HC features + 117 healthy subjects	CS vs control CS vs. features	16.0 nmol/l (0.58 µg/dl) 21.9 nmol/l (0.79 µg/dl)	97 % 92 %	84 % 77 %	CLIA

▶ **Table 1** Continued.

Ref.	Source of HC	Control group	Investigated groups	Cut-off	Sens.	Spec.	Assay
van Baal et al. This work	13 CD, 14 adrenal, 8 ectopic	149 pt. with HC features + 32 HC in remission	CS vs. control CD vs. ectopic	10.1 nmol/l (0.35 µg/dl) 109.0 nmol/l (1.97 µg/dl)	94 % 50 %	84 % 92 %	CLIA

HC: Hypercortisolism; CD: Cushing's disease; CS: Cushing syndrome; Ectopic: Ectopic ACTH-production; ELISA: Enzyme-linked immunosorbent assay; RIA: Radioimmunoassay; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; CLIA: Chemiluminescent immunoassay; Sens: Sensitivity; Spec: Specificity; pt: Patients; BMI: Body mass index.



▶ **Fig. 1** Flow chart showing late-night salivary cortisol (LNSaC) samples and patient flow. CD: Cushing's disease; HC: Hypercortisolism; CS: Cushing's syndrome.

chemical testing results in each of our follow-up investigations (median of 7 follow-up investigations with testing of urinary free cortisol at least two times each, 1 mg dexamethasone suppression test and awake midnight serum cortisol).

Collection of salivary cortisol samples

Salivary cortisol samples were collected between 11 – 12 PM. Samples were taken using Salivette sampling devices (Sarstedt, Nümbrecht, Germany). Patients were instructed not to smoke or brush teeth 60 minutes before collection and to chew gently on the cotton role and to transfer it to the sampling device without using fingers. Samples were not tested for the presence of blood. If two or more samples from the same patient were taken within five days, the highest LNSaC was used for each patient (24/36 patients in the group of proven HC, 11/149 patients in the group of suspected HC and 8/32 patients in the group of HC in remission, ▶ **Fig. 1**).

LNSaC assay

LNSaC was measured using an automated chemiluminescence immunoassay (CLIA, IDS-iSYS Salivary Cortisol, Immunodiagnostic Systems Holdings, UK). The assay is commercially available and worldwide established since 2016. According to the manufacturer, the reference interval is 0.55–9.38 nmol/l (0.02–0.34 µg/dl) (08:00 PM–12:30 AM). The limit of quantification is 0.55 nmol/l (0.02 µg/dl) and the assay range is 0.55–82.76 nmol/l (0.02–3.00 µg/dl). The range was calculated by investigation of 124 healthy subjects. Cross-reactivity with endogenous steroids was tested according to CLSI EP7-A2 guidelines. Cortisone and corticosterone demonstrated a cross-reactivity of 16 and 14 %, respec-

tively. Also according to the manufacturer, precision was evaluated in accordance with a modified protocol based on CLSI EP05-A3, "Evaluation of Precision of Quantitative Measurement Procedures". Six saliva samples were assayed using three lots of reagents in duplicate twice per day for 20 days on three systems. Total coefficient of variation for mean concentration levels between 0.073 and 1.940 µg/dl ranges from 6.6–13.8 %. The IDS-iSYS Salivary Cortisol assay was compared by the manufacturer against the commercially available quantitative Salivary Cortisol ELISA (RE52611) provided by IBL International, following CLSI EP09-A3, "Measurement Procedure Comparison and Bias Estimation Using Patient Samples". A total of 125 samples, selected to represent a wide range of cortisol concentrations (0.01–2.62 µg/dl), was assayed by each method. Linear and Passing–Bablok regression analyses were performed on the comparative data: IDS-iSYS Salivary Cortisol = 1.005 × IBL Salivary Cortisol ELISA + 0.056 µg/dl; correlation coefficient (r) = 0.97.

Statistical analysis

Results are expressed as median and range. GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) was used for statistical and receiver operating characteristic (ROC) analysis. Thresholds were established by ROC analysis. LNSaC below and above the detection limit were set to the lower or higher detection limit, respectively. Comparison between data following a Gaussian approximation was performed by using Student's *t*-test, otherwise Mann–Whitney U-test was performed. A p-value of < 0.05 was considered statistically significant. The quality of diagnostic tests was expressed as the area under ROC curve. The cut-off value with optimal sensitivity and specificity was calculated using the Youden's J index. Positive and negative likelihood ratios for the sensitivity and specificity are provided. A very high positive likelihood ratio (LR +) is defined by a value > 10, high LR + is suggested by values between 5 and 10, weak LR + is defined by values between 2 and 5 and very weak LR + is defined by values between 1 and 2. A very high negative likelihood ratio (LR–) is reflected by values < 0.1, high LR–is defined by values between 0.1 and 0.2, weak LR–is demonstrated by values between 0.2 and 0.5 and very weak LR–is suggested by values between 0.5 and 1. Probability analysis was done by calculating the relative risk (RR), including a 95 % CI.

Results

Median LNSaC level was significantly higher in patients with proven HC than in patients with suspected HC and in patients with HC in remission (▶ **Table 2**, ▶ **Fig. 2**).

ROC analysis between patients with proven HC and the control group (suspected HC + HC in remission) revealed an optimal threshold of 10.1 nmol/l (0.37 µg/dl) for the diagnosis of HC with high sensitivity and specificity (AUC 0.9431, $p < 0.0001$) (▶ **Table 3**). An identical threshold with identical sensitivity and a slightly higher specificity of 88 % as well as high LR + and LR–was calculated for patients with proven HC versus suspected HC (AUC of 0.9458; $p < 0.0001$). If a high clinical sensitivity of ≥ 95 % for diagnosis of HC is essential, a threshold of 6.5 nmol/l (0.24 µg/dl) was calculated, but this threshold decreased the specificity to 78 %. A higher clinical specificity ≥ 95 % was achieved at a cut-off of 21.0 nmol/l (0.76 µg/dl), but then sensitivity was decreased to 80 %.

ROC analysis for patients with Cushing's disease and patients with Cushing's disease in remission demonstrated a lower cut-off of 9.2 nmol/l (0.34 µg/dl) for the diagnosis of Cushing's disease with high sensitivity and moderate specificity (▶ **Table 3**). Probability analysis revealed a RR of 6.23 (95 % CI 2.49 – 15.60, p = 0.0001).

The median LNSaC concentration in patients with ectopic ACTH-production was significantly higher than in patients with Cushing's disease (▶ **Fig. 3**). ROC analysis between patients with ectopic ACTH-production and patients with Cushing's disease showed a cut-off of 109.0 nmol/l (3.95 µg/dl) for the diagnosis of ectopic ACTH-production with low sensitivity but high specificity (▶ **Fig. 3**, ▶ **Table 3**). Probability analysis revealed a RR of 3.79 (95 % CI 1.36–10.58, p = 0.0109). By using a cut-off of 11.0 nmol/l (0.40 µg/dl) a high clinical sensitivity of ≥ 95 % for the diagnosis of ectopic ACTH-production can be achieved, but specificity is decreased to 50 %. However, a high clinical specificity of ≥ 95 % can be achieved by using a cut-off of 284.4 nmol/l (10.31 µg/dl), but sensitivity is decreased to 7 %. Median LNSaC concentration in patients with a cortisol producing adrenal tumour was in between LNSaC levels of patients with Cushing's disease and ectopic ACTH-production.

Considering individual comorbidities of patients with suspected but excluded HC, the following results are demonstrated in ▶ **Table 2**: median LNSaC level in patients with adrenal tumours was significantly higher than in patients with suspected HC due to weight gain/obesity (p = 0.0116) and also than in patients with pituitary tumours (p = 0.0385). Moreover, median LNSaC level of patients with hypertension was also significantly higher than in patients with weight gain/obesity (p = 0.0436). No significant difference of the median LNSaC levels among the other subgroups could be demonstrated.

ROC analysis to distinguish between the individual comorbidities of patients with suspected HC and patients with proven HC revealed the following thresholds: For patients with hypertension or adrenal tumours an identical cut-off value of 9.8 nmol/l (0.36 µg/dl) for the diagnosis of HC was calculated, but with varying sensitivities and specificities as well as LR + and LR–(▶ **Table 3**). Furthermore, ROC analysis between patients with Cushing's disease and patients with pituitary adenomas showed a cut-off value of 9.5 nmol/l (0.35 µg/dl) with high sensitivity and specificity as well as very high LR + and LR–. In patients with suspected HC due to weight gain/obesity the cut-off value was 9.1 nmol/l (0.33 µg/dl) with high sensitivity, specificity, LR + and very high LR–(AUC 0.9853; $p < 0.0001$). Probability analysis revealed a RR of 34.83 (95 % CI 5.02–241.56, p = 0.0003). An isolated investigation of the 21 patients with other features leading to suspicion of HC revealed a cut-off value of 10.1 nmol/l (0.365 µg/dl) for the diagnosis of HC (AUC 0.9367; $p < 0.0001$; sensitivity = 87 %, specificity = 86 %, high LR + and weak LR–).

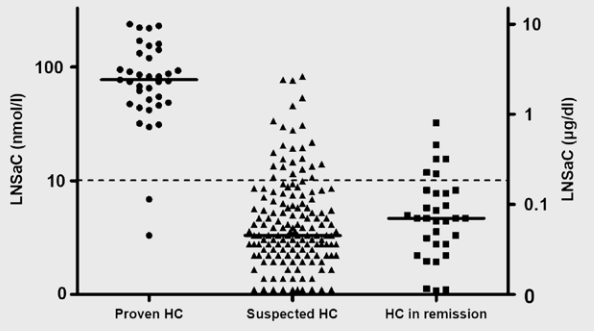
Discussion

Different groups have reported cut-off levels ranging from 2.8–12.3 nmol/l (0.10–0.45 µg/dl) with different sensitivities and specificities for the diagnosis of HC (▶ **Table 1**) [8–12, 15–17, 19–26]. Almost all cited studies used at least partially or solely healthy subjects as control group [8, 12, 16, 17, 19–23, 27]. This will very like-

▶ **Table 2** Clinical characteristics and LNSaC levels.

Aetiology	Subjects (n)	Gender (f/m)	Age (years)	LNSaC (nmol/l)
Proven HC	**36**	**25/11**	**45.50 (23–70)**	**65.8 (3.3–331.0)** *
Cushing's disease	13	11/2	43.50 (23–63)	57.0 (6.3–237.8) * *
Ectopic ACTH-production	8	1/7	50.00 (30–68)	107.4 (11.3–331.0) * *
Adrenal Cushing's syndrome	15	13/2	42.00 (30–70)	60.0 (3.3–331.0)
Suspected HC	**149**	**99/50**	**48.50 (19–78)**	**3.3 (0.6–82.8)** *
Weight gain/obesity	38	22/16	39.00 (20–78)	2.5 (0.6–14.1)
Hypertension	21	12/9	47.00 (19–68)	5.0 (1.1–76.4)
Pituitary tumour	32	26/6	46.00 (20–62)	2.8 (0.6–20.4)
Adrenal tumour	37	27/10	57.00 (31–78)	4.7 (0.8–82.8)
Others	21	12/9	37.00 (22–64)	4.1 (0.6–33.7)
HC in remission	**32**	**22/10**	**57.00 (26–79)**	**5.1 (0.6–75.9)** *
Cushing's disease	27	19/8	57.50 (26–79)	5.1 (0.8–54.3)
Ectopic ACTH-production	3	1/2	32.00 (26–42)	11.6 (1.4–75.9)
Adrenal Cushing syndrome	2	2/0	56.50 (50–63)	3.0 (2.8–3.6)

Age and LNSaC values are expressed as median as well as range in parentheses. HC: Hypercortisolism; f: Female; m: Male; LNSaC: Late night salivary cortisol; ACTH: Adrenocorticotropic hormone. * Median LNSaC of patients with proven HC is significantly higher than in patients with suspected HC ($p < 0.0001$) and in patients with HC in remission ($p < 0.0001$). * * Median LNSaC of patients with ectopic ACTH-production is significantly higher than in patients with Cushing's disease ($p = 0.0004$) and in patients with HC in remission.



▶ **Fig. 2** Scatter plot of the three different groups. The cut-off value for the diagnosis of hypercortisolism (HC) is shown as dotted black line (10.1 nmol/l, sensitivity 94 %, specificity 84 %). Median LNSaC of each group is shown as short solid black line.

ly limit the use of the calculated cut-offs in daily clinical work because healthy subjects will not be undergoing HC testing. In this context, a unique feature of our single-centre study is an inclusion of the so far largest clinically meaningful control group with a wide range of comorbidities associated with HC, in whom however HC was excluded by testing (▶ **Table 2**) over a time period of three years and in which LNSaC was measured by CLIA. Our study demonstrated that patients with proven HC had a significantly higher median LNSaC level than the control group comprising patients with suspected but subsequently excluded HC and patients in remission of HC. The calculated cut-off value for the diagnosis of HC was 10.1 nmol/l (0.37 µg/dl) with high sensitivity (94 %) and specificity (84 %). Specificity might be limited because of falsely elevated LNSaC levels in patients with suspected but excluded HC or HC in remission due to blood contamination in saliva [28, 29]. Although it has been demonstrated that a minor to moderate blood contamination as a result of vigorous tooth brushing does not influence LNSaC [7]. A possible effect of gingivitis or oral sores or injury has not been fully elucidated [2]. Our salivary samples were not tested for the presence of blood.

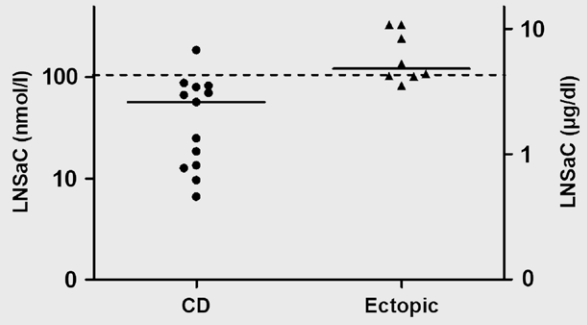
Furthermore, a recent study implied to prefer LNSaC sampling at individual bedtimes rather than 11–12 PM, because normal bedtime sampling yields equivalent or even better unstressed LNSaC values [30]. Moreover, Raff et al. demonstrated that the upper limit of normal of LNSaC for bedtime samples was lower than the previously published upper limit of normal of LNSaC for sampling between 11 and 12 PM. This might also explain false-positive results in our group of patients with suspected HC and HC in remission, because samples were taken between 11–12 PM, independent of individual bedtimes.

Although we could demonstrate a high sensitivity for our LNSaC cut-off, two patients with Cushing's disease presented with a false negative result. Both patients were female. One patient demonstrated a reproducible UFC within the reference range, non-suppressible serum cortisol during a 1 mg dexamethasone suppression test, elevated late-night serum cortisol levels two times and a high ACTH. The other patient demonstrated an elevated UFC two times, non-suppressible serum cortisol after 1 mg dexamethasone, a late-night serum cortisol within the reference range two times and a high ACTH level. Both patients underwent pituitary surgery after inferior petrosus sinus sampling. An ACTH-producing pituitary ad-

▶ **Table 3** Overview of each cut-off value evaluated in this study.

ROC-Analysis	Cut-off for diagnosis of HC in nmol/l (µg/dl)	Sens. (%)	Spec. (%)	LR+	LR–	RR
Proven HC vs. Controls (suspected HC + HC in remission)	**10.1 (0.37)**	**94**	**84**	**6.04**	**0.06**	**10.68**
Cushing's disease vs. Cushing's disease in remission	9.2 (0.34)	100	74	3.78	0.01	6.23
Ectopic ACTH-production vs. Cushing's disease	109.0 (3.95)	50	92	7.00	0.54	3.79
Cushing's disease vs. pituitary adenoma	9.5 (0.35)	100	97	33.00	0.00	29.54
Proven HC vs. hypertension	9.8 (0.36)	95	88	7.60	0.07	6.61
Proven HC vs. weight gain/obesity	9.1 (0.33)	95	98	38.00	0.06	34.83
Proven HC vs. adrenal tumour	9.8 (0.36)	95	77	4.18	0.12	6.99

HC: Hypercortisolism; Sens: Sensitivity; Spec: Specificity; ACTH: Adrenocorticotropic hormone; LR+: Positive likelihood ratio; LR–: Negative likelihood ratio; RR: Relative risk.



▶ **Fig. 3** Scatter plot of patients with Cushing's disease (CD) and ectopic ACTH-production (ectopic). The cut-off value for the diagnosis of ectopic ACTH-production is shown as dotted black line (109.0 nmol/l, sensitivity 50 %, specificity 92 %). Median LNSaC of each group is shown as short solid black line.

enoma was proven immunohistochemically. In both patients' signs and symptoms of HC were resolved postoperatively. Patients were followed-up for 10 and 4.7 years, respectively. A relapse was excluded until now. These are not unusual findings in the diagnostic process of HC, because variable hormonogenesis with fluctuation of LNSaC, UFC and late-night serum cortisol even within the normal range occur considerable frequently [31, 32].

An aim of this study was the evaluation of a cut-off value of LNSaC for the diagnosis of HC and to investigate the impact of individual comorbidities and different aetiologies of HC on this cut-off. Our ROC analysis showed a significantly different cut-off value of 109.0 nmol/l (3.95 µg/dl) with moderate sensitivity (50 %), but high specificity (92 %) and a RR of 3.79 for the diagnosis of ectopic ACTH-production. Until now no comparable study has elucidated a cut-off value of LNSaC for this target. Distinguishing Cushing's disease and ectopic ATCH-production remains challenging and hence invasive methods like inferior petrosus sinus sampling remained the gold standard for the differential diagnosis between the two conditions. Commonly ectopic ACTH-production is associated with more severe HC [33]. However, this is not a reliable criterion for differential diagnosis. Therefore, our cut-off value could be considered as a confirmation test, due to its high specificity, resulting in a low amount of false positive results. A specificity of ≥ 95 % to distinguish ectopic ACTH-production from Cushing's disease can be obtained using a cut-off value of 284.4 nmol/l (10.31 µg/dl). However, use of a high clinical specificity may be limited by decreased sensitivity as demonstrated. These findings should be evaluated in larger cohorts, in particular if LNSaC might be considered as an additional test to resolve the cause of ACTH-depended HC.

Furthermore, we could demonstrate that individual comorbidities lead to only slightly different cut-off values, which are within a range of 1.0 nmol/l compared to our main cut-off of 10.1 nmol/l (▶ **Table 3**). Considering the inter-assay coefficient of variation, it is of note that the calculated cut-off values are also within the range of this coefficient. On the other hand, several studies demonstrated the influence of individual comorbidities on LNSaC levels [10, 20, 34, 35]. Aberle et al. described a higher threshold of LNSaC to exclude patients with HC from obese patients [12]. A notable difference between Aberle et al. and our study is the fact, that Aberle et al. only investigated patients with a BMI > 35 kg/m^2, while we also included patients with significant weight gain (median 18 kg in 12 months). Elevated LNSaC levels in association with increasing BMI have also been described [3, 36]. Two other studies demonstrated higher LNSaC levels in patients with multiple chronic diseases [34, 35].

Another interesting aspect is the investigation of LNSaC levels in patients with Cushing's disease in remission. There is some evidence that LNSaC is a sensitive parameter to differentiate between remission and recurrence of HC. Until today only two other studies focused on the role of LNSaC in the identification of remission or recurrence in Cushing's disease [20, 23]. Amlashi et al. [20] proposed that recurrence should be considered by a LNSaC level of

7.4 nmol/l (0.27 µg/dl, sensitivity: 75 %; specificity: 95 %), whereas Carrasco et al. [23] proposed a LNSaC level of 5.5 nmol/l (0.20 µg/dl, sensitivity: 100 %, specificity: 98 %). We calculated a threshold of 9.2 nmol/l (0.34 µg/dl) to distinguish between Cushing's disease in remission and proven Cushing's disease with high sensitivity (100 %), but moderate and lower specificity (74 %) and a RR of 6.23. The variation between the cut-off values of the citied studies and our threshold could be due to differences in population-size, but more likely due to the different follow-up periods and the used assays. Amlashi et al. used an ELISA, Carrasco et al. an RIA. Median follow up was 53.5 months in the study of Amlashi et. al and 45 months in the study of Carrasco et al. The strength of our study is the long median follow-up period of 84 months in our patients with Cushing's disease in remission.

In summary, except for ectopic ATCH-production all remaining thresholds considering individual comorbidities and aetiologies of HC demonstrated values within the range of our main threshold and might only be useful if minor deviations of LNSaC levels from the main threshold are present.

A limitation of our evaluation was that the minority of patients, particularly with suspected hypercortisolism, had two or more measurements of LNSaC. In accordance with the current guideline, we cannot recommend LNSaC as the only parameter for the diagnosis of HC, confirmatory testing with 1 mg dexamethasone suppression test and/or measurement of urinary free cortisol is necessary [2].

Cut-off values also depend on the analytic method, for example, radio immunoassays, enzyme linked immunosorbent assays, chemiluminescence assays or liquid chromatography-tandem mass spectrometry (LC-MS/MS) (▶ **Table 1**) [25, 37, 38]. RIAs represent some practical disadvantages: they are a possible health hazard, a well-defined waste storage is mandatory, kits have limited shelf lives, because of the limited half-life of radioactive isotopes and measurement of radioactivity requires expensive instrumentation [39].

In our study, LNSaC was measured by a CLIA. LNSaC measurement by CLIA is widely spread and established in endocrine laboratories now, so cut-off levels determined by RIA may become outdated. The assay, which was used in our study, is commercially available since 2016. Nevertheless, up until today and to our best knowledge this assay was used only in one other study [12]. Cecatto et al. used a comparable control group and measured LNSaC also by a CLIA, but with a different assay [21]. Today this assay is less frequently used in comparison to the assay, which was used in our study. Cecatto et al. calculated a higher cut-off level for the diagnosis of HC of 21.9 nmol/l (0.79 µg/dl) with comparable sensitivity (92 %), but lower specificity (77 %).

A limitation of LNSaC measurement by immunoassays is an impaired specificity, most likely due to antibody-cross-reactivity with cortisone [40, 41]. The cross-reactivity results from the structural similarity of cortisone and cortisol [42]. Cortisone levels in saliva are known to be four to nine times higher than cortisol levels due to the activity of beta-hydroxysteroid dehydrogenase type 2, which rapidly converts cortisol to cortisone after diffusion into saliva [43]. Our salivary samples were not measured for cortisone. Therefore, we cannot rule out a potential cross-reactivity resulting in falsely elevated salivary cortisol levels. Measurement of salivary cortisone has been discussed as a possible alternative parameter for the diagnosis of HC, because it is unaffected by the activity of beta-hydroxysteroid dehydrogenase type 2 and also by the concentration of cortisol binding globulin [44, 45]. On the other hand the diagnostic value of salivary cortisone is limited due to lack of assessment of its daily variability in healthy individuals [46]. Moreover and in contrast to salivary cortisol, the validity of salivary cortisone in states of chronic glucocorticoid excess is still not fully elucidated [45]. Therefore, measurement of salivary cortisone is still not recommended in the current guidelines [2].

LC-MS/MS was investigated as an alternative measurement method of LNSaC over the past decade (▶ **Table 1**). In contrast to immunoassays, the number of studies, which analysed the diagnostic performance of LC-MS/MS in clinical practice is however limited up until today. The substantially lower cut-offs in the studies of Antonelli et. al., Erickson et al. and Sturmer et al. are most likely due to the missing cross-reactivity in LC-MS/MS [8, 25, 26]. The slightly higher sensitivity in the study of Antonelli et al. [8] might be due to the fact, that healthy volunteers were used to establish the cut-off value. Sturmer et al. however, used patients with features suggestive for HC as control group, but only nine patients with proven HC were investigated [8]. This will very likely decrease sensitivity of the threshold and it is a high risk of random errors due to the small population size. Therefore, the impact on daily clinical work is limited. In this context it is notable, that Erickson et al., which used a comparable study population described a significantly lower sensitivity (83 %) with equal specificity (84 %) compared to our cut-off value [8]. The impaired sensitivity of LC-MS/MS has also been described in other studies [27, 47]. On the other hand, while other endogenous or exogenous (systemic or topical) steroids, drugs or herbal medication demonstrated a relevant cross-reactivity for cortisol immunoassays [45, 48], Raff et al. demonstrated that LC-MS/MS helps to identify use of topical or oral hydrocortisone by measurement of salivary cortisone and calculation of cortisol-to-cortisone ratio, because salivary cortisone is unaffected by oral or topical hydrocortisone [49]. In that regard a very high cortisol-to-cortisone ratio in case of elevated LNSaC demonstrated a strong evidence, that patients underwent topical or oral hydrocortisone treatment. Nevertheless, this issue is not of relevance for our study, because patients treated with systemic or topical steroids were excluded from the study and therefore an influence on our results is not given.

However, LC-MS/MS is a laborious and time-consuming method and only in a very few, specialized centres available. Further studies are necessary to elucidate whether LC-MS/MS offers greater diagnostic accuracy compared to immunoassays. In summary, immunoassays for LNSaC measurement are reliable and convenient and therefore offer an alternative to LC-MS/MS.

In conclusion, determination of LNSaC, measured by CLIA, with a calculated cut-off value of 10.1 nmol/l (0.37 µg/dl) with high sensitivity and specificity is a reliable parameter for the diagnosis of HC. Except for ectopic ACTH-production with a significantly higher threshold, cut-off values considering different indications for evaluation of HC were only slightly different from this threshold. Therefore, they might only be useful if LNSaC results are near the cut-off value of 10.1 nmol/l. Our study emphasizes that further investigations of the role of LNSaC are necessary, because LNSaC

seems to be of important clinical use in different topics considering the diagnosis of HC.

Conflict of Interest

The authors declare that they have no conflict of interest.

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