

## RESEARCH

# Late-night salivary cortisol and cortisone should be the initial screening test for Cushing's syndrome

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## Abstract

Endogenous Cushing's syndrome (CS) poses considerable diagnostic challenges. Although late-night salivary cortisol (LNSC) is recommended as a first-line screening investigation, it remains the least widely used test in many countries. The combined measurement of LNSC and late-night salivary cortisone (LNS cortisone) has shown to further improve diagnostic accuracy. We present a retrospective study in a tertiary referral centre comparing LNSC, LNS cortisone, overnight dexamethasone suppression test, low-dose dexamethasone suppression test and 24-h urinary free cortisol results of patients investigated for CS. Patients were categorised into those who had CS (21 patients) and those who did not (33 patients). LNSC had a sensitivity of 95% and a specificity of 91%. LNS cortisone had a specificity of 100% and a sensitivity of 86%. With an optimal cut-off for LNS cortisone of >14.5 nmol/L the sensitivity was 95.2%, and the specificity was 100% with an area under the curve of 0.997, for diagnosing CS. Saliva collection is non-invasive and can be carried out at home. We therefore advocate simultaneous measurement of LNSC and LNS cortisone as the first-line screening test to evaluate patients with suspected CS.

### Key Words

- ▶ salivary cortisol
- ▶ salivary cortisone
- ▶ Cushing's syndrome
- ▶ screening

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## Introduction

Cushing's syndrome (CS) is caused by prolonged and inappropriate exposure of tissues to glucocorticoids (1). Endogenous CS often poses considerable diagnostic challenges. Most guidelines recommend two different tests to screen for CS including late-night salivary cortisol (LNSC), overnight dexamethasone suppression test (ODST), low-dose dexamethasone suppression test (LDDST) and 24-h urinary free cortisol (UFC) (1, 2, 3). A hallmark of CS is the disruption of the circadian rhythm of cortisol secretion whereby, in the evening, cortisol secretion is higher in CS patients than in normal subjects (4). Therefore, assessment of LNSC, without the impracticality of hospitalisation and disruption of a normal routine, would be the ideal

screening test for CS. Collection of LNSC is convenient for patients, it is non-invasive and avoids the need to obtain unstressed late-night blood samples, which is impractical in most circumstances (4). Despite the fact that LNSC has been shown to have high diagnostic sensitivity and specificity and that it has been shown to be cost-effective, it still remains the least widely used biochemical screening tool for CS (5, 6).

Salivary glands express 11- $\beta$ -hydroxysteroid dehydrogenase (11B-HSD2) which converts salivary cortisol to cortisone. The amount of salivary cortisone is significantly greater than salivary cortisol and an increased cortisol/cortisone ratio reflects exposure to both

endogenous and exogenous cortisol. Salivary cortisone can be reliably measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (7). There is no evidence of 11B-HSD2 activity in collected saliva. Therefore, measurement of salivary cortisone is particularly useful when contamination with topical or oral corticosteroids is suspected (8). In this study, we compare the diagnostic accuracy of LNSC and LNS cortisone to the diagnostic accuracy of other commonly used tests for the diagnosis of CS.

The aim of this retrospective study was to assess the reliability of LNSC and LNS cortisone in diagnosing CS compared to other commonly used methods in a single tertiary referral centre.

## Methodology

This study was approved by the Imperial College Healthcare NHS Trust governance team who confirmed that we are reporting on routinely collected non-identifiable clinical audit data, therefore, no approval from a research ethics committee was required under the UK policy framework for Health and Social Care. Data were collected from patients who underwent LNSC testing between 2017 to March 2021 at Imperial College Healthcare NHS Trust which is a regional tertiary referral centre. Patients with a high pre-test probability for CS were included in the study. Patients who were suspected to have cyclical CS, subclinical CS, pseudo-CS due to disrupted circadian rhythm or patients who were known non-compliant with the collection protocol were excluded from the study. The diagnosis of pituitary Cushing's disease (CD) was confirmed by positive histology. The diagnosis of adrenal CS was established by suppressed adrenocorticotrophic hormone (ACTH) levels in the context of hypercortisolaemia and histology in keeping with a cortisol-producing adenoma/adrenocortical carcinoma.

Patients were advised to collect saliva between 23 h and midnight. Saliva was collected with Salivette® tubes (Sarstedt, Germany). Patients were asked to collect saliva before brushing their teeth and no earlier than 30 min after eating or drinking. Patients were advised to avoid smoking, drinking alcohol or coffee for 2 h before sample collection. Steroid-containing skin creams were to be avoided, and patients were advised to wash their hands before collection. Some patients gave their saliva samples while having other tests (such as cortisol day curve, overnight dexamethasone suppression test (ODST) and LDDST) as an inpatient. Collected samples were stored in the patient's refrigerator

and subsequently delivered to the pathology department. Salivary cortisol and cortisone analysis was carried out by liquid chromatography and tandem mass spectroscopy (LC-MS/MS).

Results are presented as mean ± s.d. for quantitative variables. Sensitivity, specificity, area under the curve (AUC), positive likelihood ratio (LR), negative LR, positive predictive value (PPV), negative predictive value (NPV), of LNSC, LNS cortisone, ODST, LDDST and UFC were calculated. Cut-off values of <2.6 nmol/L for LNSC, <18 nmol/L for LNS cortisone and less than the upper limit of normal for 24-h UFC (270 nmol/24 h) were considered normal based on assay-specific reference ranges. Serum cortisol of less than 50 nmol/L for ODST and LDDST was considered normal (3).

## Results

Out of 76 patients, 54 were eligible for the study (45 were females and nine were males). The mean age was 44.8 years. CS was diagnosed in 21 patients and CS was excluded in 33 patients (Table 1). Ten patients were diagnosed with CD first presentation, six with recurrent CD, two with CS due to adrenal adenomas, two with CS due to adrenocortical cancers and one patient had CS due to an ectopic ACTH source. The latter patient underwent inferior petrosal sinus sampling to exclude a central source of ACTH with a confirmed ACTH source from diffuse idiopathic pulmonary neuroendocrine cell hyperplasia. Table 2 summarises the investigations performed.

LNSC had a sensitivity of 95%, a specificity of 91%, a PPV of 87% and a NPV of 97%. The AUC was 0.931, the positive LR was 10.5 and the negative LR was 0.05. LNSC had a specificity of 100%, with a PPV of 100%. However, sensitivity (86%) and NPV (92%) were lower than for LNSC. 36 patients underwent ODST. Although the sensitivity

**Table 1** Patients' gender and their final diagnosis.

	Number
Gender	
Male	09
Female	45
Diagnosis	
Cushing's disease first presentation	10
Recurrent Cushing's disease	6
Adrenal CS	4
Ectopic ACTH-producing CS	1
CS excluded	33

ACTH, adrenocorticotrophic hormone; CS, Cushing's syndrome.

**Table 2** Types of diagnostic tests done in confirmed cases of Cushing's syndrome.

Diagnosis	Diagnostic investigations	Confirmatory test
Cushing's disease (first presentation)		
1	LNSC, OST, LDDST	Positive histology, Post op AI
2	LNSC, LNS cortisone, ODST, UFC	Positive histology
3	LNSC, LNS cortisone, LDDST, ODST	Positive histology
4	LNSC, LNS cortisone, LDDST, ODST, UFC	Positive histology
5	LNSC, LNS cortisone, LDDST, UFC,	Positive histology, Post op AI
6	LNSC, LNS cortisone, LDDST, ACTH	Positive histology, Post op AI
7	LNSC, LNS cortisone, LDDST, ODST, UFC	Positive histology, Post op AI
8	LNSC, LNS cortisone, LDDST, ODST	Positive histology, Post op AI
9	LNSC, LNS cortisone, LDDST, ODST, UFC	Positive histology, Post op AI
10	LNSC, LNS cortisone, LDDST, ODST, UFC	Positive histology, Post op AI
Recurrent Cushing's disease		
11	LNSC, LNS cortisone, LDDST	Positive histology
12	LNSC, LNS cortisone, ODST	Positive histology
13	LNSC, ODST	Positive histology
14	LNSC, LNS cortisone, ODST	Positive histology
15	LNSC, LNS cortisone, ODST	Positive histology
16	LNSC, LNS cortisone, LDDST, ODST	Positive histology
Arenal Cushing's syndrome		
17	LNSC, LNS cortisone, ODST	Adrenocortical cancer
18	LNSC, LNS cortisone, LDDST, UFC	Adrenocortical cancer
19	LNSC, LNS cortisone, LDDST, ODST	Cortical adenoma
20	LNSC, LNS cortisone, LDDST	Cortical adenoma
Ectopic ACTH-secreting Cushing's syndrome		
21	LNSC, LNS cortisone, LDDST, ODST	DIPNECH with ectopic ACTH

LNSC cortisone, late-night salivary cortisone; LNSC, late-night salivary cortisol; LDDST, 48-h low-dose dexamethasone suppression test; ODST, overnight dexamethasone suppression test; UFC; 24-h urinary free cortisol.

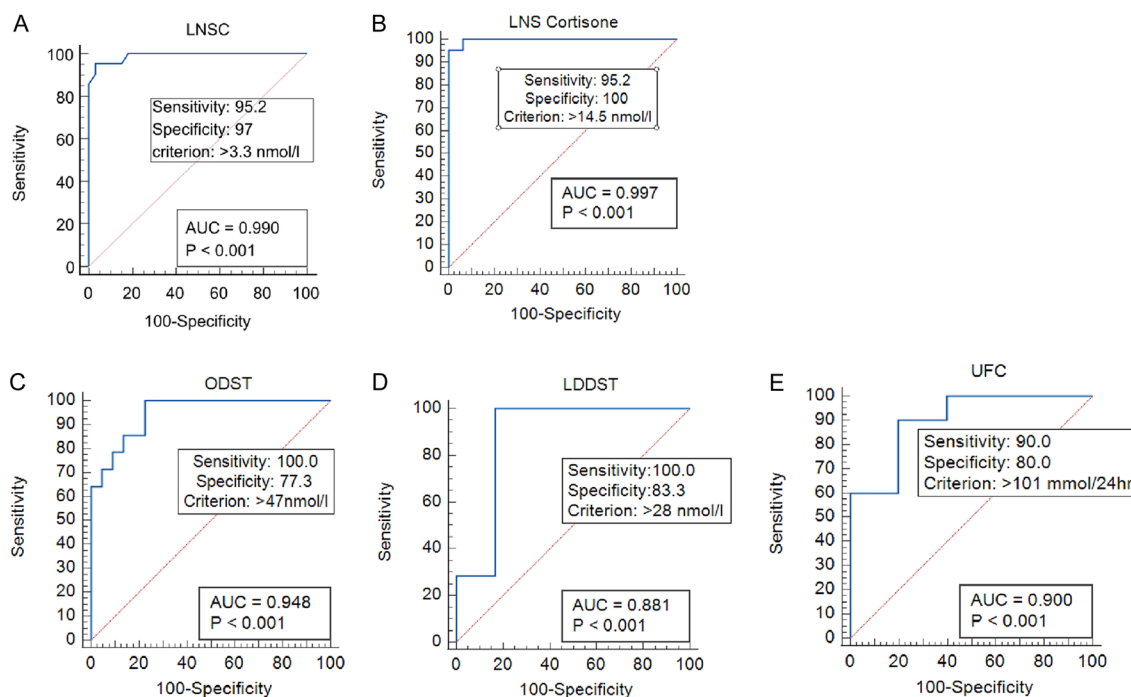
and the NPV of ODST were 100%, the specificity and the PPV were 77 and 74% respectively. Like ODST, LDDST had a sensitivity and a NPV of 100% but specificity (83%) and PPV (87%) were higher than with ODST. Fifteen patients underwent UFC as a screening test for CS. UFC was 100% specific with a PPV of 100% but had showed the lowest sensitivity (60%) and NPV (56%) of all investigations (Table 3). Receiver operating characteristic (ROC) curves were generated for LNSC, LNS cortisone, ODST, LDDST and UFC levels and showed area under the curve of 0.931,

0.929, 0.886, 0.917 and 0.800, respectively. Figure 1 shows optimal cut-offs derived by ROC analysis for LNSC, LNS cortisone, ODST, LDDST and UFC. LNSC value of >3.3 nmol/L provided a sensitivity of 95.2% and a specificity of 97% and LNSC cortisone value of >14.5 nmol/L provided a sensitivity and specificity of 95.2 and 100%, respectively, in diagnosing CS. Moreover, ODST value of 47 nmol/L while LDDST value of >28 nmol/L provided a sensitivity and specificity of 100 and 83.3%, respectively. AUC for

**Table 3** Comparison of diagnostic accuracy of investigations for Cushing's syndrome.

	LNSC	LNS cortisone	LDDST	ODST	UFC
Sensitivity	95% (76–99%)	86% (64–97%)	100% (75–100%)	100% (77–100%)	60% (26–88%)
Specificity	91% (76–98%)	100% (89–100%)	83% (52–98%)	77% (55–92%)	100% (48–100%)
AUC	0.931 (0.83–0.98)	0.929 (0.83–0.98)	0.917 (0.74–0.99)	0.886 (0.74–0.97)	0.8 (0.52–0.96)
Positive likelihood ratio	10.5 (3.6–31)		6 (1.7–21.3)	4.4 (2–9.5)	
Negative likelihood ratio	0.05 (0.01–0.36)	0.14 (0.05–0.41)	0	0	0.4 (0.19–0.86)
Positive predictive value	87% (70–95%)	100%	87% (65–96%)	74% (56–86%)	100%
Negative predictive value	97% (82–98%)	92% (85–99%)	100%	100%	56% (37–73%)

AUC, area under the curve; LNS cortisone, late-night salivary cortisone; LNSC, late-night salivary cortisol; LDDST, 48-h low-dose dexamethasone suppression test; ODST, overnight dexamethasone suppression test; UFC, 24-h urinary free cortisol.



**Figure 1**

ROC curve for (A) LNSC, (B) LNSC cortisone, (C) ODST, (D) 48-h LDDST and (E) 24-h UFC. AUC, area under the curve; LNSC, late-night salivary cortisol; LDDST, low-dose dexamethasone suppression test; ODST, overnight dexamethasone suppression test; ROC, receiver operating characteristics; UFC, urinary free cortisol.

LNSC, LNS cortisone, ODST, LDDST and UFC levels with optimal cut-offs by ROC analysis showed 0.990, 0.997, 0.948, 0.881 and 0.900, respectively.

Sub-analysis of LNSC for CD with first presentation ( $n=10$  patients) and recurrent CD ( $n=6$ ) had same sensitivity (100%), specificity (91%), NPV (100%), positive LR (10), negative LR (0) and AUC (0.960) except PPV with 77 and 67%, respectively. LNS cortisone for CD had sensitivity of 90%, NPV 97%, negative LR 0.1 and AUC of 0.950. Patients with recurrent CD had sensitivity of 83%, NPV 97%, negative LR 0.17 and AUC of 0.920 without affecting specificity (100%) or PPV (Table 4). Figure 2 also shows the same data.

## Discussion

Tandem mass spectrometry reliably measures salivary cortisol without cross-reactivity between cortisol and synthetic steroids (9, 10). Jones *et al.* showed LC-MS/MS method demonstrated excellent imprecision and accuracy for salivary cortisol and salivary cortisone. The assay was shown to be very specific for both cortisol and cortisone following the analysis of 29 structurally related steroids at supraphysiologic doses. Only 0.3% interference from

prednisolone was observed for salivary cortisol assay and 1% interference from prednisone for salivary cortisone. Salivary cortisol assay was linear up to a concentration of 2293 nmol/L and salivary cortisone was linear up to 3676 nmol/L. The lower limit of quantification was found to be 0.75 nmol/L for cortisol and 0.5 nmol/L for cortisone (11).

ERCUSYN data suggest that LNSC is not frequently used in the diagnostic workup for suspected CS despite having been shown to have excellent sensitivity and specificity (6). Our data confirm that LNSC has high sensitivity and high NPV when used in a tertiary referral setting. By contrast, UFC had very low sensitivity and NPV, thus making it unreliable as a screening tool for CS. A recent guideline update on CS diagnosis and management suggests LNSC has 97% sensitivity and 97.5% specificity in diagnosing CS and 75–90% sensitivity and 93–95% specificity in diagnosing recurrent CD. Moreover, UFC has 91% sensitivity and 81.5% specificity in diagnosing CS and 68% sensitivity and 100% specificity in diagnosing recurrent CD (3). Despite that, UFC remains the first-line screening test in many countries which can be explained by its historic use and therefore wider availability. We strongly recommend that ERCUSYN should reassess whether there has been any change in the use of diagnostic tests for CS across Europe over the past 5 years.

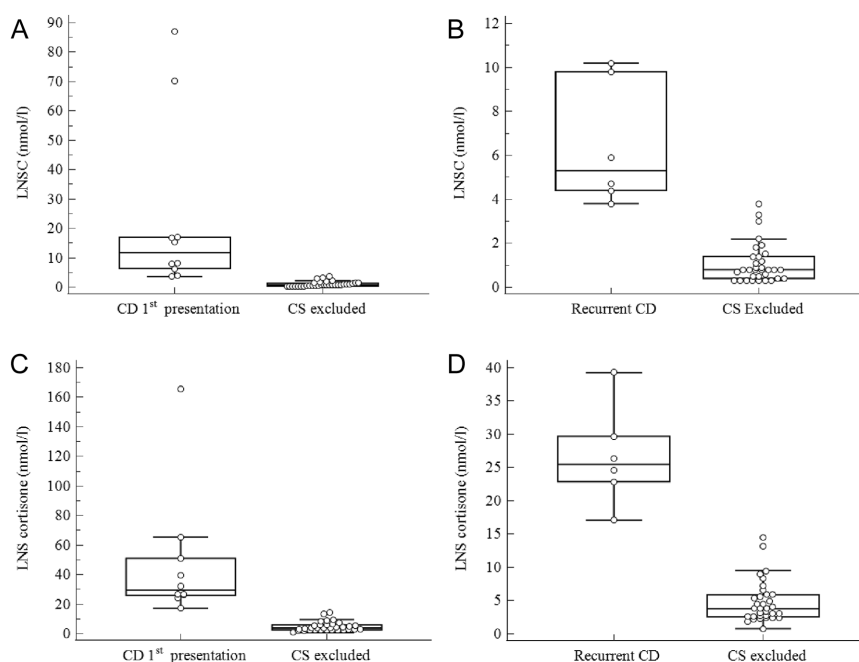
**Table 4** Diagnostic test accuracy for the diagnosis of Cushing's disease first presentation and recurrent Cushing's disease with late-night salivary cortisol and late-night salivary cortisone.

	CD first presentation + recurrent CD	CD first presentation	Recurrent CD
<b>Late-night salivary cortisol</b>			
Sensitivity	100% (79–100%)	100% (69–100%)	100% (54–100%)
Specificity	91% (76–98%)	91% (76–98%)	91% (76–98%)
AUC	0.96 (0.85–0.99)	0.96 (0.84–0.99)	0.96 (0.84–0.99)
Positive likelihood ratio	11 (3.7–32.4)	11 (3.7–32.4)	11 (3.7–32.4)
Negative likelihood ratio	0	0	0
Positive predictive value	84% (64–94%)	77% (53–91%)	67% (40–86%)
Negative predictive value	100%	100%	100%
<b>Late-night salivary cortisone</b>			
Sensitivity	88% (62–99%)	90% (55–99%)	83% (36–99%)
Specificity	100% (89–100%)	100% (89–100%)	100% (89–100%)
AUC	0.94 (0.83–0.99)	0.95 (0.84–0.99)	0.92 (0.78–0.98)
Positive likelihood ratio			
Negative likelihood ratio	0.13 (0.03–0.46)	0.1 (0.02–0.64)	0.17 (0.03–0.99)
Positive predictive value	100%	100%	100%
Negative predictive value	94% (81.8–98%)	97% (84–99%)	97% (85–99%)

AUC, area under the curve; CD, Cushing's disease.

LNSC is not affected by BMI or cortisol binding globulin and therefore accurately reflects serum cortisol concentrations. There is conflicting evidence regarding LNSC levels in polycystic ovarian syndrome. Ozkaya *et al.* found cortisol not to be affected by a PCOS phenotype (12). However, Basu *et al.* reported higher LNSC levels in PCOS patients compared to controls (13). Several studies found that there appears to be an age-related increase in LNSC (14, 15). It also appears that LNSC detects recurrent CD earlier than UFC thus making it the ideal test to follow up patients after successful pituitary surgery for CD (6, 11,

13). Our results are comparable to data published by others which confirm high sensitivity and specificity of LNSC (16, 17). A meta-analysis by Carroll *et al.* concluded that LNSC is a robust and convenient test to screen and diagnose CS. Similar to our own analysis, the authors found that LNSC had a diagnostic sensitivity of 92% and a specificity of 96% (18). Despite the strong evidence base and the practical advantages of the test, various reasons such as experience of a given centre, availability of adequate assays, individual diagnostic choices or delayed adaptation of evidence-based medicine into clinical practice may be



**Figure 2** Box and whisker plot demonstrating (A) late-night salivary cortisol in Cushing's disease first presentation; (B) late-night salivary cortisol in recurrent Cushing's disease; (C) late-night salivary cortisone in Cushing's disease first presentation; (D) late-night salivary cortisone in recurrent Cushing's disease; median value showed by horizontal line; box indicates 25–75th percentile, whisker indicates 2.5–97.5th percentiles of the data. *P* values are from Mann–Whitney rank sum tests.



responsible that LNSC is not yet the preferred first-line investigation for the evaluation of CS (6).

More than 90% of the variability in salivary cortisone can be accounted for by changes in serum cortisol (19). Recently published data show that when an 23 h upper reference limit of salivary cortisol and cortisone of 3.6 nmol/L and 13.5 nmol/L were used, the sensitivities and specificities of LNSC were 90 and 96%, and for LNS cortisone 100 and 95%, respectively (20). In our study, we used 18 nmol/L as the upper reference range for cortisone and calculated a sensitivity of 86% and specificity of 100%. When the upper reference limit was 14.5 nmol/L based on optimal cut-off by ROC analysis, sensitivity rose to 95.2% without affecting specificity. Normal LNSC: cortisone ratio is 0.2 with LC-MS/MS (6). Contamination of the salivary sample with topical hydrocortisone should be suspected when LNSC results are markedly increased, particularly when they are out of proportion with other biochemical results or clinical findings. In this instance, contamination with topical hydrocortisone would be suggested by a normal salivary cortisone concentration and high cortisol to cortisone ratio (21). LNSC and LNS cortisone can conveniently be analysed together with LC-MS/MS from a single saliva sample which appears to be one of the advantages compared to the immunoassay. However, many centres do not have access to LC-MS/MS. There is also the option of measuring LNSC by immunoassay which is much easier and cheaper with similarly excellent results (22).

A recently published study examining 1453 LNSC samples from 705 patients received by a diagnostic laboratory requested by both primary care physicians and specialists as part of clinical care showed that a majority of patients who had at least one positive LNSC sample did not have CS (22). A majority of samples in this study which was carried out in the United States were sent from primary care physicians following the publication of clinical practice guidelines. It is not known whether sampling conditions were accurately followed and there is no information about pre-test probability. In our study, the majority of the patients who had a positive LNSC had CS. There are several reasons for this discrepancy. Our centre is a tertiary referral centre, and, therefore, many of the patients investigated at our centre have a high pre-test probability. In keeping with our own data, Raff *et al.* confirmed that LNSC has excellent sensitivity and specificity in a tertiary referral setting when used in combination with a high pre-test probability (4). In addition, we excluded patients with so-called 'pseudo-Cushing's' and those with subclinical CS in our study. Crucially, an experienced clinician's assessment is

the most important factor to correctly diagnose CS. For example, conditions like obstructive sleep apnoea result in hypoxia-induced stress and subsequent ACTH-driven hypercortisolaemia thus mimicking ACTH-dependent CS biochemically. However, results of investigations for hypercortisolism often fully normalise once obstructive sleep apnoea has been treated. Multiple sequential LNSC measurements are very useful to investigate diagnostically challenging patients with cyclic Cushing's disease. However, the clinical assessment of an experienced endocrinologist remains key to make a correct diagnosis (23).

Sandouk *et al.* demonstrated that there can be significant fluctuations in salivary cortisol levels in CD. In newly diagnosed CD this seems to only rarely impair the diagnostic ability of LNSC, whereas in patients with recurrent or persistent disease after pituitary surgery, LNSC was frequently within the reference range, with the potential to cause false-negative results (20). In contrast with those findings, in our cohort, patients with proven recurrent CD all had elevated LNSC. In this study, only LNSC was measured by LC-MS/MS and measuring both LNSC and LNS cortisone using this method might improve its sensitivity as cortisol elevations maybe modest in recurrent disease. Garrahy *et al.* showed that a combination of LNSC and LNS cortisone had a sensitivity of 94% compared to LNSC and LNS cortisone alone which had a sensitivity of 92 and 87%, respectively. The previously mentioned guideline update on CS diagnosis and management recommends only using LNSC if cortisone concentrations can also be reported if an adrenal tumour is suspected (3). We fully agree that LNS cortisone should be routinely included in CS workup where available.

Limitations to our study were its retrospective nature, the small number of patients with CS and the fact that the selection of screening tests other than LNSC was based on the clinicians' preferences. Nevertheless, our study adds further evidence to the existing literature confirming that LNSC in combination with LNS cortisone represents the ideal screening test for patients with suspected CS referred to a tertiary centre following a thorough clinical assessment by an experienced physician.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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## References

- 1 Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, Stewart PM & Montori VM. The diagnosis of Cushing's syndrome: an Endocrine Society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 1526–1540. (<https://doi.org/10.1210/jc.2008-0125>)
- 2 Guignat L & Bertherat J. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline: commentary from a European perspective. *European Journal of Endocrinology* 2010 **163** 9–13. (<https://doi.org/10.1530/EJE-09-0627>)
- 3 Fleseriu M, Auchus R, Bancos I, Ben-Shlomo A, Bertherat J, Biermasz NR, Boguszewski CL, Bronstein MD, Buchfelder M, Carmichael JD, *et al.* Consensus on diagnosis and management of Cushing's disease: a guideline update. *Lancet: Diabetes and Endocrinology* 2021 **9** 847–875. ([https://doi.org/10.1016/S2213-8587\(21\)00235-7](https://doi.org/10.1016/S2213-8587(21)00235-7))
- 4 Raff H, Raff JL & Findling JW. Late-night salivary cortisol as a screening test for Cushing's syndrome. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 2681–2686. (<https://doi.org/10.1210/jcem.83.8.4936>)
- 5 Raff H. Cushing syndrome update on testing. *Endocrinology and Metabolism Clinics of North America* 2015 **44** 43–50. (<https://doi.org/10.1016/j.ecl.2014.10.005>)
- 6 Valassi E, Franz H, Brue T, Feelders RA, Netea-Maier R, Tsagarakis S, Webb SM, Yaneva M, Reincke M, Droste M, *et al.* Diagnostic tests for Cushing's syndrome differ from published guidelines: data from ERCUSYN. *European Journal of Endocrinology* 2017 **176** 613–624. (<https://doi.org/10.1530/EJE-16-0967>)
- 7 Raff H & Phillips JM. Bedtime salivary cortisol and cortisone by LC-MS/MS in healthy adult subjects: evaluation of sampling time. *Journal of the Endocrine Society* 2019 **3** 1631–1640. (<https://doi.org/10.1210/je.2019-00186>)
- 8 Perogamvros I, Keevil BG, Ray DW & Trainer PJ. Salivary cortisone is a potential biomarker for serum free cortisol. *Journal of Clinical Endocrinology and Metabolism* 2010 **95** 4951–4958. (<https://doi.org/10.1210/jc.2010-1215>)
- 9 El-Farhan N, Rees DA & Evans C. Measuring cortisol in serum, urine and saliva – are our assays good enough? *Annals of Clinical Biochemistry* 2017 **54** 308–322. (<https://doi.org/10.1177/0004563216687335>)
- 10 Monaghan PJ, Keevil BG, Stewart PM & Trainer PJ. Case for the wider adoption of mass spectrometry-based adrenal steroid testing, and beyond. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 4434–4437. (<https://doi.org/10.1210/jc.2014-2258>)
- 11 Jones RL, Owen LJ, Adaway JE & Keevil BG. Simultaneous analysis of cortisol and cortisone in saliva using XLC-MS/MS for fully automated online solid phase extraction. *Journal of Chromatography: B, Analytical Technologies in the Biomedical and Life Sciences* 2012 **881–882** 42–48. (<https://doi.org/10.1016/j.jchromb.2011.11.036>)
- 12 Ozkaya HM, Keskin FE, Tuten A, Korkmaz E, Oktay HZ & Kadioglu P. Late-night salivary cortisol is unaltered in patients with polycystic ovarian syndrome (PCOS), irrespective of disease phenotype, and in obese women, irrespective of the presence of PCOS. *Endocrine Practice* 2017 **23** 1045–1052. (<https://doi.org/10.4158/EP171887.OR>)
- 13 Basu BR, Chowdhury O & Saha SK. Possible link between stress-related factors and altered body composition in women with polycystic ovarian syndrome. *Journal of Human Reproductive Sciences* 2018 **11** 10–18. ([https://doi.org/10.4103/jhrs.JHRS\\_78\\_17](https://doi.org/10.4103/jhrs.JHRS_78_17))
- 14 Coelli S, Farias CB, Soares AA, Crescente GM, Hirakata VN, Souza LB, Czepielewski MA, Camargo JL & Silveiro SP. Influence of age, gender and body mass index on late-night salivary cortisol in healthy adults. *Clinical Chemistry and Laboratory Medicine* 2017 **55** 1954–1961. (<https://doi.org/10.1515/cclm-2016-1100>)
- 15 Raff H, Raff JL, Duthie EH, Wilson CR, Sasse EA, Rudman I & Mattson D. Elevated salivary cortisol in the evening in healthy elderly men and women: correlation with bone mineral density. *Journals of Gerontology: Series A, Biological Sciences and Medical Sciences* 1999 **54** M479–M483. (<https://doi.org/10.1093/gerona/54.9.m479>)
- 16 Raff H. Utility of salivary cortisol measurements in Cushing's syndrome and adrenal insufficiency. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 3647–3655. (<https://doi.org/10.1210/jc.2009-1166>)
- 17 Elias PCL, Martinez EZ, Barone BFC, Mermejo LM, Castro M & Moreira AC. Late-night salivary cortisol has a better performance than urinary free cortisol in the diagnosis of Cushing's syndrome. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 2045–2051. (<https://doi.org/10.1210/jc.2013-4262>)
- 18 Carroll T, Raff H & Findling JW. Late-night salivary cortisol for the diagnosis of Cushing syndrome: a meta-analysis. *Endocrine Practice* 2009 **15** 335–342. (<https://doi.org/10.4158/EP09023OR>)
- 19 Harrison RF, Debono M, Whitaker MJ, Keevil BG, Newell-Price J & Ross RJ. Salivary cortisone to estimate cortisol exposure and sampling frequency required based on serum cortisol measurements. *Journal of Clinical Endocrinology and Metabolism* 2019 **104** 765–772. (<https://doi.org/10.1210/jc.2018-01172>)
- 20 Sandouk Z, Johnston P, Bunch D, Wang S, Bena J, Hamrahian A & Kennedy L. Variability of late-night salivary cortisol in Cushing disease: a prospective study. *Journal of Clinical Endocrinology and Metabolism* 2018 **103** 983–990. (<https://doi.org/10.1210/jc.2017-02020>)
- 21 Raff H & Singh RJ. Measurement of late-night salivary cortisol and cortisone by LC-MS/MS to assess preanalytical sample contamination with topical hydrocortisone. *Clinical Chemistry* 2012 **58** 947–948. (<https://doi.org/10.1373/clinchem.2012.182717>)
- 22 Kannankeril J, Carroll T, Findling JW, Javorsky B, Gunsolus IL, Phillips J & Raff H. Prospective evaluation of late-night salivary cortisol and cortisone by EIA and LC-MS/MS in suspected Cushing syndrome. *Journal of the Endocrine Society* 2020 **4** bvaa107. (<https://doi.org/10.1210/jendso/bvaa107>)
- 23 Raff H. Late night salivary cortisol in the diagnosis of neoplastic hypercortisolism (including cyclic Cushing's syndrome). *Pituitary* 2022 [epub]. (<https://doi.org/10.1007/s11102-022-01214-2>)

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