

## CLINICAL STUDY

# Limitations of nocturnal salivary cortisol and urine free cortisol in the diagnosis of mild Cushing's syndrome

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## Abstract

**Objective:** Cushing's syndrome (CS) is difficult to diagnose due to its nonspecific presentation. Diagnostic tests like 24-h urine free cortisol (UFC) and the overnight 1 mg dexamethasone suppression test (DST) lack sufficient sensitivity and specificity. Measurement of nocturnal salivary cortisol (NSC) is an accurate and reproducible test with a high sensitivity for CS. However, its performance in mild CS has not been reported. We present 11 cases of CS with normal or mildly elevated UFC in whom NSC was helpful in making a diagnosis.

**Design and methods:** All patients had at least one collection of 24-h UFC and NSC and eight had an overnight 1 mg DST. The number of NSC measurements per patient was determined by the clinical index of suspicion and the results of initial testing. Imaging studies included magnetic resonance imaging (MRI) of pituitary or computer tomography scan of abdomen.

**Results:** Only four out of eleven patients had elevations in UFC and none were > 2 times the upper limit of normal. Seven out of eight had an abnormal DST. All patients had some elevated NSCs (14–100%). Out of eleven patients, six had an abnormality in the pituitary gland found by MRI and two out of eleven had adrenal masses. The remaining three had normal pituitary MRI but had inferior petrosal sinus (IPS) sampling indicating Cushing's disease. All patients had appropriate surgery, and histopathology of all except one was suggestive of either a cortisol-producing adrenal adenoma or an ACTH-secreting pituitary adenoma.

**Conclusion:** Neither a normal UFC nor a normal NSC excludes mild CS. Multiple samples (urine/saliva) and DST are needed to make the diagnosis of mild CS.

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## Introduction

The diagnosis of Cushing's syndrome (CS) and the differentiation of its causes are among the most challenging problems in clinical endocrinology. The features of endogenous hypercortisolism (especially, when mild) are protean and coincide with many common clinical conditions like the dysmetabolic syndrome (1, 2). Screening studies in high-risk populations have discovered unsuspected CS in as many as 2–5% of patients with diabetes mellitus (3–7) and suggest that mild CS is more common than previously appreciated.

Historically, elevation of 24-h urine free cortisol (UFC) to 2–3 times the upper limit of normal has been considered the gold standard for the diagnosis of spontaneous CS (8). In addition, the overnight 1 mg dexamethasone suppression test (DST) has been used as a screening test; however, it may lack adequate sensitivity and specificity to be a stand-alone test and needs to be complemented (9).

Since the earliest biochemical abnormality in patients with CS may be the failure to fully decrease cortisol secretion to its nadir at night, the measurement of serum cortisol at midnight yields a high sensitivity and specificity for the diagnosis of CS (10, 11). The measurement of nocturnal salivary cortisol (NSC), a surrogate for midnight serum cortisol, has proven to be a more practical means of screening for endogenous hypercortisolism and has excellent sensitivity and specificity (1, 12–16).

We present a series of 11 patients with surgically proven mild CS in whom the measurement of 24-h UFC was usually normal or only slightly elevated. In these patients, the use of NSC measurements in combination with the overnight 1 mg DST provided biochemical evidence of a pathological state of hypercortisolism.

## Subjects and methods

The 11 patients reported here were referred to our center for either first consultation or a second opinion.

Medical records were retrospectively reviewed (Table 1) with permission from the Aurora St Luke's Medical Center Institutional Review Board. None of these patients had florid symptoms or signs of CS; however, they had a few clinical features suggestive of CS that prompted the work-up. Two of the eleven patients were referred for evaluation because of an incidentally discovered adrenal mass. The remaining nine were referred to for evaluation due to excessive weight gain, truncal obesity, and/or uncontrolled diabetes mellitus. UFC (1–4 per patient) and NSC sampled between 2300 h and midnight (2–15 per patient) were measured in all patients. We typically assess at least two NSCs two nights in a row as a screening test for CS. The time between serial NSC measurements ranged from 1 to 23 months; and the time between serial UFC measurements ranged from 1 to 17 months. In some patients, large number of NSCs were assessed because of the high clinical index of suspicion and/or because at least one of the two initial NSCs sampled was abnormal or at the upper end of the reference range. The reference range for NSC is  $<4.3$  nmol/l and is based on 73 healthy, non-obese subjects (35 male/38 female), ages  $37 \pm 11$  (s.d.) (17, 18). Eight patients had an overnight 1 mg DST. A serum cortisol at 0800 h  $<50$  nmol/l (1.8 mcg/dl) was considered normal suppression. The two patients with adrenocorticotropin (ACTH)-independent CS had computerized tomography (CT) scans of the abdomen. Both had their scans before the clinical diagnosis of CS was made; one for back pain and one for presumed aortic aneurysm. Magnetic resonance imaging (MRI) of the pituitary was performed in the remainder of the nine patients in whom ACTH-dependent CS was suspected. Six patients (six out of nine) also had IPS sampling (IPSS) for ACTH to differentiate between Cushing's disease and ectopic ACTH syndrome (19). CT scans and MRI scans were performed either at the referring institution or at our institution. All radiographs were reviewed by J W Findling at the time of consultation.

## Laboratory evaluation

UFC was measured by high performance liquid chromatography (HPLC) at our reference laboratory (ARUP) or by a variety of methods from the referring physician. To account for this, UFC data were normalized by dividing the patient's UFC value by the upper limit of the assay reference range (17). Serum cortisol was measured by chemiluminescence immunoassay (Siemens Centaur, Tarrytown, NY, USA). Salivary cortisol was measured by using ELISA (18). The intra-assay imprecision (coefficient of variation, CV) was 5.2% at 3.1 (s.d., 0.2) nmol/l ( $n=10$ ) and 2.6% at 10.4 (0.2) nmol/l ( $n=10$ ). Interassay (total) imprecision (CV) was 11% at 2.8 (0.3) nmol/l ( $n=10$ ), 11% at 10.1 (1.1) nmol/l ( $n=10$ ), and 6.9% at 25.0 (1.7) nmol/l ( $n=10$ ). Plasma ACTH was measured in samples from IPSS by immunoradiometric assay (IRMA) (20). All

patients did not have a similar work-up because they were evaluated by different physicians and specialists at various centers during the course of disease. We report the results of those patients for whom we had the minimal required information.

## Results

All patients were female (25–79 years old) and their baseline clinical features are presented in Table 1. Of 11 patients, 7 had consistently normal UFC and the remaining 4 had elevations in UFC between 1–2 times the upper limit of normal (Table 1 and Fig. 1A). None had UFC  $>2$  times the upper limit of normal. Seven of eight patients who had overnight DST were found to have abnormal overnight 1 mg dexamethasone suppression suggestive of CS. All of the patients had at least some significantly elevated NSC levels; however, 5 out of 11 also had some NSC levels within the normal range. The percentage of abnormal salivary cortisol levels ranged from 14 (patient #3) to 100% (patients #4–8 and 11; Table 2).

The two patients with adrenal gland lesions had suppressed ACTH levels (3.0–6.2 pg/ml (0.7–1.3 pmol/l)) and underwent adrenalectomy. Histopathology confirmed the presence of adrenocortical adenomas in these patients and both had secondary adrenal insufficiency after surgery. In six out of nine patients who had MRI, there were unequivocal pituitary adenomas and they underwent pituitary surgery. Five of these six patients had pituitary tumor removal with development of post-operative adrenal insufficiency. The remaining patient (patient #7) had a hemihypophysectomy and was found to have a pituitary adenoma which was, however, negative for ACTH by immunohistochemistry. However, she developed prolonged secondary adrenal insufficiency post-operatively. The remaining three of nine patients (#2, 6, and 10), who underwent MRI imaging had no pituitary lesions. These patients had IPSS for ACTH which indicated Cushing's disease. All three of these patients underwent pituitary surgery with resection of pituitary microadenoma that stained for ACTH. IPSS was also performed in three patients in whom there were known pituitary lesions (#3, 7, and 9) either at treating physician's discretion or large/undefined pituitary lesions which are atypical for ACTH-secreting adenomas.

## Discussion

We describe 11 patients with mild spontaneous CS with either normal or only slightly elevated levels of UFC. Establishing the diagnosis of hypercortisolism in this group of patients required a high index of clinical suspicion and multiple measurements of NSC and UFC, in addition to low-dose DST in some patients. The

**Table 1** Clinical, laboratory and post-operative information

Gender/Age Subject #	Clinical presentation	NSC (nmol/l; normal <4.3)	Serum cortisol after 1 mg DST (nmol/l <sup>a</sup> ; normal <44)	24 h UFC nmol/d (upper limit of normal) <sup>b</sup>	Lesion	Surgery and pathology	Post-operative course
F/39 #1	wt ↑, facial rounding and easy bruising, headache	1.8, 5.1, 4.4, 3.8, 5.1, 4.1, 6.6, 4.3, 5.7	39	152 (<138) 121 (<124)	MRI: equivocal hypodense 9 mm lesion with cystic area in pituitary gland	ACTH-secreting microadenoma	Adrenal insufficiency post-operatively
F/43 #2	wt ↑, metabolic syndrome, DM, menstrual irregularities, hypokalemia, facial rounding and easy bruising	1.1, 1.1, 15.6, 5.9, 1.4, 1.1, 1.3	579	52 (<94) 177 (<124)	MRI: negative IPSS: peak IPS:P ACTH gradient after CRH >120	ACTH-secreting pituitary adenoma	No adrenal insufficiency post-operatively, eucortisolemic; lost weight
F/50 #3	wt ↑ in truncal region, facial rounding, depression, easy bruising, fatigue, loss of muscle strength	4.0, 2.6, 4.0, 4.7, 3.2, 3.1, 7.9, 2.7, 3.1, 2.4, 3.4, 3.4, 3.7, 2.0, 2.5, 3.0	127	25 (<116)	MRI: large 12 mm cystic lesion in the pituitary gland IPSS: peak IPS:P ACTH gradient after CRH >100	ACTH-secreting pituitary adenoma	Adrenal insufficiency post-operatively 1 mg DST normal after surgery
F/48 #4	wt ↑, central obesity, back pain led to CT scan which led to 3–4 cm adrenal mass	4.8, 6.1	Not done	221, 88, 55, 28 (<138)	CT abdomen: 3.2×3.4 cm mass in left adrenal gland	Macro-nodular adrenocortical hyperplasia with a dominant nodule	Adrenal insufficiency post-operatively
F/79 #5	Incidental large left-sided nodule, osteoporosis	9.4, 11.6	190	69 (<124)	MRI abdomen: 4.7 cm left adrenal mass	Left-sided adrenalectomy Benign adrenocortical adenoma of 4.8 cm	Adrenal insufficiency post-operatively
F/49 #6	Poor glycemic control, cushingoid, irregular menses	6.3, 5.6, 5.2	188	146 (<386)	MRI: negative IPSS: peak IPS:P ACTH gradient after CRH >8	ACTH-producing microadenoma	Menses resumed regularly
F/52 #7	Facial rounding, hirsutism, uncontrolled diabetes, easy bruising	8.2, 8.1, 7.0, 4.5	Not done	116 (<141) 110 (<138)	MRI: diffusely enlarged pituitary gland (1.4 cm in largest dimension) with suprasellar extension IPSS: peak PPS: P ACTH gradient after CRH >11	Hemihypophysectomy but pathology was negative for ACTH immunostaining	Adrenal insufficiency post-operatively, lost 70 pounds
F/54 #8	Weight gain of 35 lbs despite diet and weight training, easy bruising	5.0, 7.7, 8.5, 12.6, 6.7, 8.7, 16.3, 22.7	193	36, 119 (<124)	MRI: 6 mm right-sided pituitary microadenoma	ACTH-producing microadenoma	No post-operative adrenal insufficiency, no weight loss, salivary cortisol remain elevated, being considered for radiation therapy
F/57 #9	Metabolic syndrome, severe insulin resistance	9.4, 10.6, 10.3, 19.6, 8.9, 1.1, 2.1	601	69 (<124)	MRI: 12×5×9 mm right sided pituitary tumor IPSS: peak IPS:P ACTH gradient after CRH >100	ACTH-producing adenoma	Adrenal insufficiency post-operatively

Table 1 Continued

Gender/Age Subject #	Clinical presentation	NSC (nmol/l; normal < 4.3)	Serum cortisol after 1 mg DST (nmol/l <sup>a</sup> , normal < 44)	24 h UFC nmol/d (upper limit of normal) <sup>b</sup>	Lesion	Surgery and pathology	Post-operative course
F/41 #10	Uncontrolled blood sugars, morbid obesity, referred for bypass, fatigue, depression, proximal muscle weakness	6.3, 17.8, 1.9, 3.0, 2.0, 2.9	Not done	127, 157 (<116) 182 (<215)	MRI: normal IPSS; peak IPS:P ACTH gradient after CRH > 100	ACTH-producing microadenoma	Lost 35 pounds and significant improvement in glycaemic control post operatively
F/25 #11	Weight gain, facial rounding, plethora, easy bruising, hirsutism, irregular periods hypertension, muscle weakness	13.2, 13.0	326, 350	72 (<124)	MRI: 8 mm pituitary microadenoma	ACTH-producing microadenoma	Adrenal insufficiency post-operatively, weight loss post surgery

NSC, Nocturnal salivary cortisol (presented in chronological order); DST, dexamethasone suppression test; UFC, urine free cortisol; F, Female; wt, weight; MRI, magnetic resonance imaging; CT, computer tomography scan; IPSS, inferior petrosal sinus sampling; IPS, inferior petrosal sinus; P, peripheral; ACTH, adrenocorticotropin; CRH, corticotrophin-releasing hormone.

<sup>a</sup>To convert nmol/l to mcg/dl, divide by 27.59.

<sup>b</sup>To convert nmol/d to mcg/24 h, divide by 2.759.

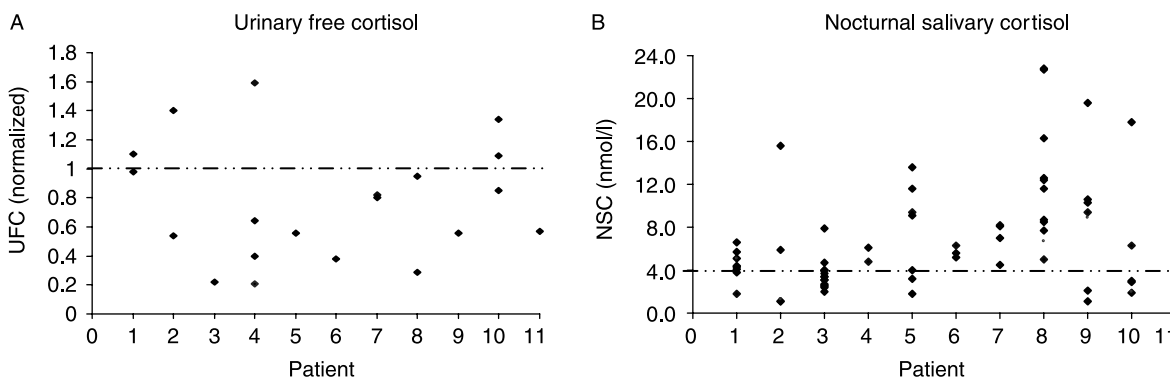
measurement of UFC in a 24-h collection has been considered the gold standard for the diagnosis of CS. However, UFC may not accurately reflect the cortisol secretory state in patients with even the modest impairment of renal function (8). In addition, most of the cortisol secreted during a 24-h period is between 0400 h and 1600 h. Subtle increases in nighttime secretion, as may be seen in mild CS, may not be detected or only intermittently detected in a 24-h urine collection (21, 22). Other potential problems with UFC are the different analytical methods used and differing potential interfering substances (23–25). Since repeated measurements are usually needed to determine the presence or absence of mild or intermittent hypercortisolism, the use of 24-h urine collections can be impractical.

Seven of the eleven patients reported here did not have an elevation of UFC. The high false negative rate in our series may be because we did not obtain as many UFC as NSC samples. This reflects the practical aspects of clinical medicine. If the only measurement available in these patients had been UFC, the diagnosis would probably have been overlooked in many, when a normal UFC was measured the first time.

NSC is a sensitive and specific means of determining the presence or absence of endogenous hypercortisolism. Numerous studies have validated this approach which yields a remarkable 93% sensitivity at 100% specificity (17, 26–29). The test is useful in cyclic CS and in children (30, 31), and may be able to distinguish pseudo-Cushing states from CS with 95% diagnostic accuracy (16, 32). Although NSC measurement is not without fault, the ease with which it can be repeated makes it ideal in certain situations.

Even though a majority of these patients had NSC levels above the upper limit of normal (4.0 nmol/l (0.15 mcg/dl)), six had NSC levels in the normal range on several occasions. For example, patient #3 actually had 14 NSC samples measured over 2 years, because several of the initial NSCs were at or slightly above the upper limit of the reference range. Careful examination by an experienced endocrinologist revealed subtle but specific symptoms and signs of CS, which made it hard to ignore these slightly abnormal NSCs even though UFC was normal. Although most of the NSCs were < 4.0 nmol/l (0.15 mcg/dl), the majority of the levels were > 3.0 nmol/l (0.11 mcg/dl), suggesting the potential for very mild, but significant hypercortisolism. Patient #2 had a similar NSC profile. We do not know the reason for these variable NSC measurements, but can only speculate that it might be secondary to cyclic CS (22) or normal biological variability around a mildly elevated set point. Obviously, both of these studies, NSC and UFC, may need to be performed several times before the suspected diagnosis of endogenous hypercortisolism can be correctly identified.

The overnight 1 mg DST is also widely used for screening test (1, 33), although some patients with CS retain their ability to suppress the cortisol. Due to high



**Figure 1** (A) Twenty-four-hour urine free cortisol (UFC) in 11 patients with surgically proven Cushing's syndrome. The data were normalized by the dividing UFC of the subject by the upper limit of the UFC assay reference range (indicated by the dotted horizontal line). (B) Nocturnal salivary cortisol (NSC) levels. The dotted line indicates the upper limit of the reference range (4.2 nmol/l (0.15 mcg/dl)). To convert nmol/l to mcg/dl, divide by 27.59.

sensitivity, it still remains one of the important tests in the Cushing's diagnostic armamentarium. Unfortunately, all the patients presented in this series did not have the DST and hence it is impossible to evaluate its full performance in the current series. However, we identified seven out of eight patients in whom it was performed.

It is difficult to distinguish between CS and pseudo-CS, especially in mild cases like those presented here. The dexamethasone suppression–corticotrophin-releasing hormone (CRH) stimulation test has been recommended to confirm the presence or absence of CS and help to distinguish it from pseudo-Cushing's conditions (psychiatric disorders, anorexia nervosa, and alcoholism) (34, 35). Four of our patients (#4, 6, 8, and 10) in whom dexamethasone–CRH testing was performed had an abnormal serum cortisol response of >38.6 nmol/l (1.4 mcg/dl). Patient #10 had two dexamethasone–CRH tests 2 years apart; the first was normal and the second was abnormal with a serum cortisol of 58 nmol/l (2.1 mcg/dl) 15 min after CRH injection. Newer studies have questioned the validity of dexamethasone–CRH suppression test as the final 'gold standard' to distinguish between CS and

pseudo-CS (36, 37). NSC, when performed properly, may be an important tool to distinguish pseudo-Cushing's from Cushing's disease.

Despite contradictory test results, the diagnosis in these patients shows the importance of the clinical exam and evaluation with tests that complement each other. It is also certain that, due to difficulty in making a diagnosis, several cases may be missed if clinical suspicion is not high enough and only one test is used to exclude CS. None of our patients had overt clinical features or unequivocal elevation of UFC, as recommended by a consensus statement of expert endocrinologists (8). Frequent measurement of NSC in our patients, complemented in some by overnight DST, provided the best evidence of mild hypercortisolism leading to appropriate differential diagnostic testing and surgical intervention. We acknowledge that we have incomplete data for some patients; however, we want to emphasize that this is a case series and not a prospective study. Our intention, in this report, is to illustrate the difficulty in establishing the diagnosis of mild hypercortisolism in patients without dramatic increases in UFC secretion. It also highlights the complementary nature of the three commonly performed initial screening studies. It is clear that none of these tests are ideal and one or two measurements of normal NSC, DST, or UFC should not be used to exclude mild CS in patients with a reasonable index of clinical suspicion. The reliability and simplicity of NSC makes it a reasonable choice for initial screening test in patients with suspected hypercortisolism. Patients with either consistent or frequent elevations of NSC of >3.0–4.0 nmol/l (0.11–0.15 mcg/dl) may have CS. Additional and persistent diagnostic testing including low-dose DST testing should also be employed in these patients in order to provide more diagnostic certainty. Further studies are needed to see whether these patients have a long-term benefit from diagnosis and treatment of mild CS.

**Table 2** Percentage of positive results with salivary cortisol and urine free cortisol in 11 patients with surgically proven Cushing's syndrome

Patient no.	% Abnormal urine free cortisol	% Abnormal nocturnal salivary cortisol
1	50	78
2	50	29
3	0	14
4	25	100
5	0	100
6	0	100
7	0	100
8	0	100
9	0	71
10	66	33
11	0	100

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