Simultaneous assay of cortisol and dexamethasone improved diagnostic accuracy of the dexamethasone suppression test

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Abstract

Objectives: The overnight dexamethasone (DXM) suppression test (DST) has high sensitivity, but moderate specificity, for diagnosing hypercortisolism. We have evaluated if simultaneous measurement of S-DXM may correct for variable DXM bioavailability and increase the diagnostic performance of DST, and if saliva (sa) is a feasible adjunct or alternative to serum.

Design and methods: Prospective study of DST was carried out in patients with suspected Cushing's syndrome (CS) ($n=49$), incidentaloma ($n=152$) and healthy controls ($n=101$). Cortisol, cortisone and DXM were assayed by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Results: Three hundred and two subjects underwent DST; S-cortisol was ≥ 50 nmol/L in 83 patients, of whom 11 had CS and 27 had autonomous cortisol secretion. The lower 2.5 percentile of S-DXM in subjects with negative DST ($n=208$) was 3.3 nmol/L, which was selected as the DXM cut-off level. Nine patients had the combination of low S-DXM and positive DST. Of these, three had been misdiagnosed as having autonomous cortisol secretion. DST results were highly reproducible and confirmed in a replication cohort ($n=58$). Patients with overt CS had significantly elevated post-DST sa-cortisol and sa-cortisone levels compared with controls; 23 of 25 with autonomous cortisol secretion had elevated sa-cortisone and 14 had elevated sa-cortisol.

Conclusions: Simultaneous measurement of serum DXM and cortisol reduced false-positive DSTs by 20% and improved the specificity. S-DXM >3.3 nmol/L is sufficient for the suppression of cortisol <50 nmol/L. Measurement of glucocorticoids in saliva is a non-invasive and easy procedure and post-DST sa-cortisone was found particularly useful in the diagnosis of CS.

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Introduction

Over the last decades, an increasing number of patients are referred to medical centers for the assessment of hypercortisolism. A major indication is adrenal

incidentalomas detected by the widespread use of CT and MRI scans. Another is the obesity epidemic, as overweight is associated with manifestations reminiscent to Cushing's

syndrome such as dyslipidemia, hypertension and diabetes. The workup of hypercortisolism has been complicated by the recognition of autonomous cortisol secretion as an intermediate entity between normal physiology and overt Cushing's syndrome, for which treatment is still under debate (1). In this context, diagnostic tests need to be highly sensitive, but also specific in order to avoid unnecessary and expensive workup. The assessment of hypercortisolism may be difficult and requires the combination of several biochemical tests. Except for the introduction of late-night salivary cortisol measurements, the diagnostic workup has remained virtually unchanged for decades. Moreover, the cut-off levels that discriminate healthy subjects from those with hypercortisolism have been based on immunoassays that are not traceable to international validated reference materials and are no longer in use, thus questioning their validity (2).

A widely used screening test for hypercortisolism is the 1 mg overnight dexamethasone suppression test (DST). Recent guidelines recommend that serum cortisol levels >50 nmol/L should be interpreted as a positive test (3). This cut-off level provides a high diagnostic sensitivity (95%), but a moderate specificity (80%). Hence, false-positive DSTs are a significant problem, causing a burden to the healthcare system and to the patients (2). The lack of specificity in DST could be due to variable gastrointestinal absorption of DXM, inter-individual differences in the metabolism of DXM or decreased hypothalamic or pituitary sensitivity for DXM (4). False-positive tests are also common in subjects with increased cortisol-binding globulin (CBG) levels (e.g. women on oral contraceptives) as serum cortisol measurements usually report the sum of free and bound hormones (5). Some reports also claim that kidney failure and obesity increase the risk for a false-positive DST (2, 4, 6, 7, 8, 9, 10, 11).

Only two studies have investigated S-DXM as a marker of DXM bioavailability in the setting of DST (12, 13). The study by Meikle showed that the plasma DXM level above 5.6 nmol/L was necessary for adequate suppression of cortisol. However, this study used an immunological assay that is prone to cross-reactivity between steroids (2), has limited availability (14) and is not well standardized. Liquid chromatography-mass spectrometry (LC-MS/MS) offers highly specific simultaneous measurement of multiple steroids within one analytical run. LC-MS/MS is less influenced by the sample matrix, enables easier standardization and is now recognized as the gold standard for steroid analysis. Steroids can be assayed in serum, urine and saliva. Sa-cortisol and sa-cortisone correlate well with

serum cortisol (15) and may have advantages over serum samples because of its independence of cortisol-binding globulin (CBG) levels (16).

The aim of the current study was to investigate if LC-MS/MS measurements of DXM, cortisol and cortisone in the serum and in the saliva could improve the diagnostic accuracy of the DST and to estimate if the cut-off for S-cortisol at 50 nmol/L after DST is valid also for the LC-MS/MS method in the evaluation of individuals with suspected Cushing's syndrome and incidentalomas.

Subjects and methods

Subjects and study design

From November 2011 to June 2015, we consecutively included patients referred for the evaluation of hypercortisolism to the tertiary endocrine specialist centers at the Haukeland and Akershus University Hospitals. Healthy subjects were recruited from the hospital and university staffs and patients from the obesity clinic of the Haukeland University Hospital. These groups were not different in terms of median post-DST S-cortisol or S-DXM and were combined to one group named controls (Wilcoxon rank, $P=0.24$ for S-cortisol and $P=0.7$ for S-DXM). The Regional Committee for Medical and Health Ethics approved the study protocol, and all participants gave their written informed consent. A total of 302 participants, including 152 patients with adrenal incidentalomas, 49 with clinical suspected Cushing's syndrome and 101 controls, were enrolled (Table 1).

All underwent routine diagnostic workup for Cushing's syndrome according to the Endocrine Society's guidelines of 2008 (2), involving collection of two late-night salivary cortisol samples on different days and a DST. If abnormal test results were obtained, further workup with conventional DST and/or 24-h urine cortisol measurements was performed, depending on the pre-test probability of autonomous cortisol secretion or CS. The study subjects were categorized as having Cushing's syndrome, autonomous cortisol secretion or no hypercortisolism (2). The diagnosis of autonomous cortisol secretion was based on a positive DST, low morning plasma ACTH (2, 3, 17) and at least one comorbidity (hypertension, osteoporosis, obesity or type 2 diabetes mellitus). The DST was repeated in 44 participants (16 patients and 28 healthy) to assess reproducibility. The results were validated in a replication cohort ($n=58$) from the St. Olav's University Hospital (18).

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Table 1 Characteristics of patients and controls. Categorical data are given as *n* (%) and continuous data as median (range).

	Incidentalomas	Suspected CS	Controls
Patients (<i>n</i>)	152	49	101
Women (<i>n</i> (%))	94 (62)	38 (76)	64 (63)
Age (years, range)	62 (29–86)	43 (17–77)	49 (23–81)
Hypertension (<i>n</i> (%))	74 (49)	12 (24)	19 (19)
Diabetes (<i>n</i> (%))	19 (13)	13 (26)	9 (9)
Smokers (<i>n</i> (%))	57 (38)	6 (12)	7 (7)
Oral estrogen use (<i>n</i> (%))	7 (5)	8 (20)	2 (2)
BMI (kg/m ² , range)	28 (16–43)	31 (19–56)	25 (18–62)
Creatinine (μmol/L, range)	73 (40–971)	64 (47–91)	61 (47–97)
S-DXM (nmol/L) ^a	7.8 (0–24)	7.5 (0–21)	7.0 (3–19)
DXM-levels ≤3.3 nmol/L (<i>n</i> (%))	10 (7)	2 (4)	3 (3)
S-cortisol after DST (nmol/L, range) ^a	42.6 (13–577)	22.7 (9.9–289)	22.2 (8.4–103)
Inadequate suppression (<i>n</i> (%)) ^a	61 (41)	9 (22)	2 (2)

^aOvert CS excluded.

Sample collection and handling

All participants had 1 mg DXM administered orally at 23:00 h. The serum and saliva samples were obtained at 08:00 h the next day. The saliva samples were collected from 131 patients with incidentaloma, 43 with clinical suspicion of Cushing's syndrome and 90 healthy subjects. Saliva was collected by chewing a cylindrical cotton swab (Salivette, Sarstedt, Germany) for approximately 2 min. At least 1 h before the collection, subjects were told not to eat or brush their teeth. All samples were centrifuged and stored at –80°C.

Assay of glucocorticoids

Cortisol, cortisone and DXM were quantitatively determined by ultrahigh pressure liquid chromatography mass spectrometry (UPLC–MS/MS) (19). In serum, the precision was below 7.4% relative standard deviation (RSD) for all steroids, and accuracy ranged from 97 to 102%. For salivary cortisol, cortisone and DXM, the limit of quantifications were 46, 69 and 23 pmol/L respectively. The precision ranged from 3.6 to 8.1% RSD, and the accuracy ranged between 96% and 114% for all steroids.

Statistical analysis

The subject characteristics are reported as median (range). The Kruskal–Wallis test or the Wilcoxon rank test were used to compare groups, and the Wilcoxon rank test with Bonferroni correction was used for *post hoc* analysis. As the data were not normally distributed (Supplementary Fig. 1, see section on supplementary data given at the end of this article), reference range

and cut-off for S-DXM after DST were obtained using nonparametric statistics. The lowest level of S-DXM with 90% confidence interval (CI) (20) necessary to suppress cortisol was defined as the lower 2.5th percentile of all individuals suppressing serum cortisol below 50 nmol/L (*n*=208). The upper S-DXM cut-off level was defined as the 97.5th percentile in the control group (*n*=101). The salivary cortisol and cortisone upper normal levels were obtained from the 97.5th percentile of S-cortisol in control subjects who are not on oral estrogens. Segmented regression model was used to confirm the lower cut-off for S-DXM after DST. To assess within-person reproducibility of the DST, the test was repeated in 44 subjects. The Spearman correlation coefficient was used to describe the associations between hormone measurements. Within-person reproducibility for S-cortisol and S-DXM was also assessed by the determination of intraclass correlation coefficient (ICC) (95% CI) using ln-transformed data and a random-effects mixed model with participant identification as the random variable. An ICC <0.40 is considered as poor reproducibility, 0.40–0.75 as fair-to-good reproducibility and ≥0.75 as an excellent reproducibility (21). The statistical significance level was set at *P* < 0.05.

Results

S-DXM after DST

To define a cut-off value for S-DXM identifying low DXM bioavailability, we studied the S-DXM levels in all subjects with adequate cortisol suppression after DST (*n*=208). The median (range) S-DXM level after DST was 7.3 (2.3–24) nmol/L. According to the

Figure 1

Segmented regression model with breakpoint estimation for the relationship between post-DST S-cortisol and S-DXM (CS and autonomous cortisol secretion excluded). Estimated breakpoint for S-DXM at 3.6 nmol/L (95% CI: 3.15–4.1). The horizontal dotted line represents S-cortisol 50 nmol/L.

study by Reed *et al.*, there were no obvious outliers (22). The 2.5th percentile for S-DXM was 3.3 nmol/L (90% CI: 3.12–3.82) (20). The segmented regression model for post-DST S-cortisol and S-DXM, applied to all patients who were not diagnosed with overt CS or autonomous cortisol secretion, showed a breakpoint for S-DXM at 3.6 nmol/L (95% CI: 3.15–4.1) (Fig. 1). There was no correlation between DXM and cortisol above this threshold.

A total of 14 patients (5%) had S-DXM levels below 3.3 nmol/L. Of these, five had adequate suppression of cortisol (≤ 50 nmol/L) after DST. However, nine patients (3% of the total study subjects) had both inadequate S-DXM levels and elevated S-cortisol. Three of these nine patients (33%) were initially misdiagnosed as having autonomous cortisol secretion, one of whom was unilaterally adrenalectomized. The other six patients were diagnosed as having normocortisolemia based on the results of supplementary hormonal workup (Supplementary Table 1). The main difference between normocortisolemic subjects and the three diagnosed with autonomous cortisol secretion was suppressed or low-normal p-ACTH levels and abnormal responses on repeated DST or conventional DST in the latter (Supplementary Table 1). Seven of the nine patients with inadequate S-DXM levels and elevated S-cortisol levels repeated the DST. Three subjects achieved S-DXM > 3.3 nmol/L after a second 1-mg overnight DST, three after a conventional DST (0.5 mg 4 times daily for 48 h) and one subject needed an 8-mg single dose. Six of these seven patients showed adequate cortisol suppression when S-DXM level was > 3.3 nmol/L.

The upper limit for S-DXM (nmol/L) was 13.8 (90% CI: 12.41–18.46) nmol/L ($n=101$). Fifteen study participants had S-DXM values above 13.8 nmol/L, and all showed cortisol suppression below 50 nmol/L. The S-DXM levels did not differ between the various groups. Moreover, subgroup analysis showed no sex difference and no impact of smoking (Table 2). There was a positive correlation between S-DXM and kidney function measured as S-creatinine in the complete study cohort (Table 3). Subgroup analyses revealed such correlation for the incidentaloma group, but not for the controls or the group with suspected CS. The S-DXM did not correlate with BMI ($P=0.27$).

Table 2 Post-DST S-cortisol and S-DXM levels.

Subgroup	n	S-cortisol (nmol/L), median (range)	s-DXM (nmol/L), median (range)
Controls	94	23 (8–103)	7.0 (3.0–18.5)
Cushing's syndrome	11	266 (120–780)	6.3 (4.3–14.1)
Autonomous cortisol secretion	27	90 (62–329)	8.4 (0.0–14.9)
Estrogen users ^a	16	67 (22–179)	7.77 (4.2–15.5)
Incidentaloma ^a	152	43 (13–577)	7.8 (0.0–23.9)
Suspected Cushing's syndrome ^a	49	29 (10–780)	7.3 (0.0–20.7)
S-DXM $< 3.3^a$	14	208 (38–577)	1.7 (0.0–3.2)
Women ^a	187	31 (11–577)	7.4 (0.0–24.0)
Men ^a	104	26 (8–289)	7.3 (0.0–16.8)
Smokers ^a	67	54 (13–577)	7.4 (0.0–23.9)
Non-smokers ^a	225	25 (8–524)	7.3 (0.0–20.7)

^aOvert CS excluded.

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Table 3 Correlation coefficients between post-DST S-DXM, and creatinine, age and BMI.

	Post DST S-DXM			Post DST S-cortisol ^a		
		P value	n		P value	n
Creatinine						
- All	0.28	<0.01	127	0.07	0.93	126
- Incidentalomas	0.47	<0.01	72	0.04	0.77	71
- Suspected CS	-0.025	0.9	30	-0.37	0.08	23
- Controls	-0.14	0.5	24	0.31	0.53	23
Age						
- All	0.24	<0.01	303	0.39	<0.01	302
- Incidentalomas	0.12	0.16	152	0.27	0.01	150
- Suspected CS	0.37	0.01	50	-0.01	0.99	40
- Controls	0.16	0.12	101	0.48	0.08	101
BMI						
- All	0.067	0.27	289	-0.18	<0.01	289
- Incidentalomas	0.08	0.35	143	-0.30	<0.01	141
- Suspected CS	-0.02	0.91	46	-0.23	0.18	37
- Controls	0.10	0.34	100	-0.22	0.03	100

^aOvert CS and women on oral estrogens excluded.

Post-DST cortisol levels

Altogether, 83 of 302 patients had a positive DST defined as S-cortisol >50nmol/L. Overt CS was found in 11 patients (13%), whereas autonomous cortisol secretion was diagnosed in 27 patients (33%). Furthermore, 10 patients (12%) had low levels of DXM, 10 were on oral estrogens (12%), and in 30% we could not determine the cause (Fig. 2).

Of 11 patients who were diagnosed with overt CS, 10 patients were diagnosed with Cushing's syndrome, and one with adrenal CS. All had elevated late-night

salivary cortisol, positive conventional DST with post-DST S-cortisol above 50nmol/L and increased secretion of cortisol in 24-h urine samples. In 27 patients who were diagnosed with autonomous cortisol secretion, the median post-DST S-cortisol value was 90nmol/L (range: 62–329). Nine patients had suppressed p-ACTH, and in another 18 patients, the median p-ACTH was 1.5pmol/L (range: 1.3–2.4).

Median cortisol value after DST (Table 1) was nearly doubled in the incidentaloma group compared to the control subjects and those with suspected CS, when patients finally diagnosed with CS ($P<0.01$) were excluded. Even after eliminating patients diagnosed with autonomous cortisol secretion from the incidentaloma group, median S-cortisol remained higher than that in the healthy subjects ($P<0.01$). The median p-ACTH value in this group was 3.7pmol/L (range: <1.1–14.8), which is lower when compared with the normocortisolemic subjects (p-ACTH 4.5pmol/L, range: 1.2–15.8, $n=43$). However, there were more patients with inadequate cortisol suppression in the group with initially suspected CS (finally diagnosed CS excluded) compared with controls.

Assessment of the 97.5th percentile for cortisol after DST in controls (excluding estrogen users) yielded a value of 43.6nmol/L (90% CI: 37.1–63.0). This is close to the established cut-off of 50nmol/L used to define a positive DST. For CS, the sensitivity of DST was 100% and the specificity was 75% using S-cortisol 50nmol/L as a cut-off for positive test. After inclusion of subjects with autonomous cortisol secretion, the specificity increased to 82%. The specificity for CS was 77% when patients with

Figure 2

Distribution of positive and negative DSTs (left circles) and causes of positive DSTs (right circles). The whole study population (A), the subgroups of incidentalomas (B), suspected CS (C) and controls (D). White: negative DST, black: positive DST, red: Cushing's syndrome, pink: autonomous cortisol secretion, yellow: low s-DXM bioavailability, green: oral estrogens and blue: unknown.

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Table 4 Diagnostic performance of serum and saliva glucocorticoids in DST for CS (left), and for CS and autonomous cortisol secretion (right).

	CS			CS+autonomous cortisol secretion		
	Cut-off (nmol/L)	Sensitivity (%)	Specificity (%)	Cut-off (nmol/L)	Sensitivity (%)	Specificity (%)
S-cortisol						
S-cortisol	50	100	75	50	100	82
S-cortisol (DXM <3.3 nmol/L excluded)	50	100	77	50	100	84
Based on 97.5th percentile						
Saliva cortisone	2.7	100	74	2.7	94	81
Saliva cortisol	0.55	100	85	0.55	67	89
Saliva cortisone (DXM <3.3 nmol/L excluded)	2.7	100	86	2.7	91	82
Saliva cortisol (DXM <3.3 nmol/L excluded)	0.55	100	80	0.55	71	90

S-DXM <3.3 nmol/L were excluded, and the specificity was 84% for the patients who were diagnosed with either CS or autonomous cortisol secretion. The sensitivity and specificity for the serum and saliva cortisol after DST, with and without excluding patients with low S-DXM are summarized in Table 4. Receiver-operating characteristic (ROC) analysis is shown in Supplementary Table 2.

Serum creatinine was not significantly correlated with post-DST S-cortisol overall or in the subgroups (Table 3). Age was positively correlated with post-DST cortisol except in the group with suspected CS, whereas BMI was inversely correlated with post-DST cortisol (Table 3). Women had higher post-DST cortisol than men, both when CS patients were included ($P=0.015$) and excluded ($P=0.018$). However, when excluding women using estrogens, the differences were not significant ($P=0.076$) (Table 2). Smokers had higher post-DST cortisol than nonsmokers ($P<0.01$). Alcohol consumption was overall low with a mean intake of 2 units per week and did not influence the results (data not shown).

Correlation between post-DST cortisol and dexamethasone

Post-DST S-DXM was not correlated with S-cortisol in the whole study population (CS excluded) ($\rho=0.05$, $P=0.43$), but a strong inverse correlation was observed in 14 patients with low S-DXM (≤ 3.3 nmol/L; $\rho=0.65$, $P=0.012$) (Supplementary Fig. 2).

Reproducibility of DST

DST was repeated in 44 participants, 28 healthy subjects and 16 patients. The time interval between the first (test 1) and second (test 2) tests was 279 (124 and 1057) days (range). S-cortisol was similar between the two time points (Wilcoxon rank, $P=0.13$). Post-DST

S-cortisol levels for tests 1 and 2 were strongly positively correlated ($\rho=0.87$, $P<0.01$; Supplementary Fig. 3A). The within-subject reproducibility in terms of ICC was 0.94 (0.91 in healthy subjects and 0.79 in patients). The corresponding correlation and reproducibility of S-DXM were $\rho=0.35$ ($P=0.03$) (Supplementary Fig. 3B) and 0.70 respectively.

Replication cohort

A total of 58 post-DST serum samples from patients with incidentaloma were obtained in the replication cohort described by Åsvold *et al.* (18). Thirty-five patients had suppression of cortisol below 50 nmol/L, whereas 23 patients did not. Median S-DXM in the total group was 7.8 nmol/L (range 0–24). Five patients (9%) had S-DXM <3.3 nmol/L, and they all had post-DST cortisol >50 nmol/L. Thus, low levels of S-DXM explained 22% of the positive DSTs

Saliva-DST test

Both sa-cortisol (Spearman $\rho=0.74$; $P<0.01$) and sa-cortisone ($\rho=0.84$; $P<0.01$) were strongly correlated with S-cortisol after DST (Supplementary Fig. 4A/B). The cut-off level for sa-cortisol and sa-cortisone was 0.55 nmol/L (90% CI: 0.34–1.1) and 2.7 nmol/L (90% CI: 2.2–3.3) respectively. All 11 patients with overt CS had elevated post-DST sa-cortisol and sa-cortisone levels compared with the levels in healthy subjects ($P<0.01$). For patients with autonomous cortisol secretion, 13 of 25 had elevated post-DST sa-cortisol levels and 23 had elevated sa-cortisone levels. Sixteen subjects in the study used oral estrogens, 10 had inadequate suppression of S-cortisol after DST of whom eight had available saliva samples. Of eight, two had elevated sa-cortisol levels and six had elevated sa-cortisone levels. Correlation between

S-DXM and sa-DXM was moderate ($\rho=0.48$, $P<0.01$) (Supplementary Fig. 4C).

Discussion

We demonstrate that approximately one in 10 positive DSTs can be explained by the inadequate serum levels of DXM. Measuring DXM simultaneously with cortisol using LC–MS/MS might reduce the need for retesting and further investigations for Cushing's syndrome, depending on the pre-test level of suspicion. Our data confirm 50nmol/L as the cut-off limit for S-cortisol after DST using LC–MS/MS and that this cut-off provides reasonable diagnostic sensitivity and specificity for the diagnosis of CS. Moreover, we show that salivary cortisone has a potential to replace S-cortisol, and hence, further simplify the workup.

An S-DXM >3.3 nmol/L is needed to obtain adequate suppression of endogenous cortisol levels. The cut-off level was validated in an independently collected cohort from another tertiary endocrine center, including patients with incidentalomas and was compared to incidentaloma group of this study. This showed that the established S-DXM cut-off is valid and that low S-DXM levels were responsible for a similar proportion of false-positive tests. Six of seven patients with low DXM levels and inadequate S-cortisol suppression showed normal suppression when retested at DXM levels elevated above the cut-off. This indicates that insufficient DXM levels most likely explained the initial false-positive DST.

Reduced S-DXM after DST has been attributed to a CYP 3A4 polymorphism causing rapid metabolism of steroid hormones (23, 24) or to use of drugs that induce these enzymes (25, 26, 27). However, in our study, this is unlikely as concomitant drug use was recorded. Other potential causes of low S-DXM include variable and low gastrointestinal absorption (28) and increased distribution due to low albumin binding (29). Elimination could also be affected by reduced liver and kidney functions. It has been postulated from animal studies that chronic exposure to glucocorticoids can increase the clearance of DXM, but this has not been verified in humans (4, 13, 30, 31). Our data do not support such a hypothesis, as S-DXM levels were similar between CS, autonomous cortisol secretion and control subgroups.

The upper cut-off for S-DXM after DST was estimated from data deriving from controls. One may argue that 15 participants in the study that had S-DXM values over 13.8 nmol/L after DST should be evaluated for potentially

false-negative test. In contrast, the total DXM dose is larger in the conventional DST that uses the same S-cortisol cut-off level. Furthermore, we found no correlation with post-DST S-cortisol of DXM levels above the cut-off at 3.3 nmol/L in patients not diagnosed with overt CS or autonomous cortisol secretion.

Gender-specific differences in the DXM metabolism or physiological response to DXM could possibly impact the DST, but we found no gender differences that could not be explained by oral estrogens. Therefore, the S-DXM cut-off level identified in the present study should be valid for both sexes. Age correlated positively with post-DST cortisol in the whole study population and the subgroup of patients with incidentaloma. Age effects on S-cortisol may reflect age-related impaired negative feedback in the corticotrophin axis, giving a larger proportion of non-suppressors among older people (32). Lack of significant association between age and S-cortisol in controls and subjects with suspected CS may be explained by a small number of old subjects in these groups.

We found no correlation between BMI and S-DXM in the whole study population, neither in any of the subgroups of patients nor in healthy controls. Rather, there was a weak negative correlation between BMI and post-DST cortisol. A possible explanation may be that overweight individuals exhibit lower CBG levels (33). Obese subjects assessed for hypercortisolemia do not need higher DXM doses, as advocated by others (34).

S-DXM was positively correlated to serum creatinine concentration in the whole cohort and in the subgroup of patients with incidentaloma. This suggests that reduced renal function decreases DXM elimination. However, post-DST cortisol levels were not correlated with S-creatinine, a finding that opposes previous reports (4, 6, 35). This discrepancy may be due to the fact that our study did not include patients with moderate-to-serious kidney failure.

Smoking is known as an inducer of Cyp3A (36) and may as such increase the risk of a false-positive test. We found similar S-DXM levels in smokers and nonsmokers, which is in agreement with a previous pharmacokinetic study (36). Altered DXM pharmacokinetics therefore does not explain the higher post-test cortisol levels that we observed in smokers compared with nonsmokers. Collectively, these data suggest that smoking is a risk factor for a false-positive test, although not linked to altered DXM metabolism.

With the increasing adoption of LC–MS/MS over immunoassays, it has been speculated that the cut-off level for cortisol is not optimal. In the present study, 97.5% of the controls, excluding those on oral estrogens, had

post-DST S-cortisol less than 43.6 nmol/L (90% CI, 37.1–63.0). This is close to the well-established S-cortisol cut-off of 50 nmol/L after DST and gives support to maintain this cut-off also for LC–MS/MS-based assays.

The higher post-DST cortisol in the incidentaloma group compared to that in controls may reflect a slight autonomous production of cortisol from adenomas or hyperplasia. Worth noting is that when patients finally diagnosed with both CS and autonomous cortisol secretion from the incidentaloma group were excluded, the post-DST median S-cortisol still increased compared to that in the control groups. These data might suggest that some subjects with adrenal incidentalomas have a certain autonomous cortisol production, even though they are not diagnosed with autonomous cortisol secretion.

The post-DST S-cortisol demonstrated excellent within-person reproducibility both in healthy subjects and in patients. The reproducibility was more moderate for S-DXM, which may be explained by variable absorption of DXM both within and between individuals (28). The excellent within-person reproducibility of post-DST S-cortisol implies that if you have enough S-DXM to suppress, you suppress cortisol to similar level despite different S-DXM levels from test to test.

The use of saliva samples is attractive because glucocorticoids in saliva are thought to reflect the free circulating levels. Indeed, we found that post-DST S-cortisol showed strong correlation with sa-cortisol and even stronger with sa-cortisone. Interestingly, sa-cortisone was the best single marker to discriminate between healthy subjects and patients with CS as well as with autonomous cortisol secretion. This finding is in line with the observation that sa-cortisone correlates better to S-cortisol than to sa-cortisol (37).

In oral estrogen users, sa-cortisol has the potential to increase the diagnostic accuracy compared with S-cortisol as the levels are less dependent on CBG (38). Our data, although limited by few participants, indicate that some healthy women on oral estrogen may still have increased salivary cortisol and cortisone levels. This might be because women on oral estrogens have an increased free fraction of cortisol as well as an increased CBG-bound fraction (16, 39, 40).

In conclusion, we demonstrate that the 50 nmol/L cut-off for cortisol is still valid using LC–MS/MS and that S-DXM can be used to reduce the number of false-positive DSTs. An S-DXM level of 3.3 nmol/L was found to be sufficient to expect adequate suppression of cortisol. Salivary cortisone was found to be a promising

novel biomarker with high diagnostic sensitivity for autonomous cortisol secretion.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-17-0078>.

Declaration of interest

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

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