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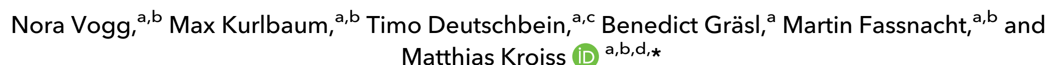
Method-Specific Cortisol and Dexamethasone Thresholds Increase Clinical Specificity of the Dexamethasone Suppression Test for Cushing Syndrome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BACKGROUND: The dexamethasone suppression test (DST) is the recommended first-tier test for suspected Cushing syndrome (CS). Missed dexamethasone intake or insufficient dexamethasone serum exposure may yield false positive results. Quantification of serum dexamethasone in DST samples may therefore improve test performance.

METHODS: Simultaneous quantification of dexamethasone and cortisol by liquid chromatography-tandem mass spectrometry in 400 DST serum samples (100 overt CS, 200 excluded CS, 100 adrenal incidentalomas with (possible) autonomous cortisol secretion, AI-ACS) randomly selected within the indication groups. The 2.5th percentile of dexamethasone in patients with excluded CS was considered the lower limit of normal (LLN).

RESULTS: Serum dexamethasone varied from undetectable to 20.2 ng/mL with a median of 4.8 ng/mL (95% CI 4.5-5.1 ng/mL). Dexamethasone was undetectable in only 16 patients (4%), suggesting non-compliance. The dexamethasone LLN was 1.8 ng/mL (4.6 nmol/L). Decreased glomerular filtration rate and diabetes mellitus were associated with higher serum dexamethasone concentration, while body mass index, sex, age, nicotine, and oral contraceptives had no significant effect. By excluding the 27 samples with dexamethasone <LLN and applying the method-specific cortisol cutoff of 2.4 μg/dL (66 nmol/L) to samples with suspected CS, the clinical specificity for CS increased from 67.5% to 92.4% while preserving 100% clinical sensitivity. Among 100 AI-ACS samples (defined by immunoassay), 4 samples had dexamethasone <1.8 ng/mL and 14 samples had cortisol <2.4 μg/dL, which excluded autonomous cortisol secretion.

CONCLUSIONS: Quantification of dexamethasone and method-specific cortisol cutoffs in DST samples may reduce the false positive rate and lower the proportion of patients requiring further workup.

Introduction

Cushing syndrome (CS) is a rare disease characterized by hypercortisolism and associated with relevant morbidity and impaired overall survival (1–4). The low-dose overnight dexamethasone (Dex) suppression test (DST) is the most widely used laboratory test for the diagnosis of CS and recommended first-tier test if CS is suspected (5–9). Following oral administration of 1 mg Dex at 11:00 PM, serum cortisol concentration is determined in a blood sample collected the next morning between 8.00 AM and 9:00 AM. Serum cortisol suppression to 1.8 μg/dL (50 nmol/L) or lower excludes autonomous cortisol secretion with high clinical sensitivity (10). In addition, the test has been recommended to stratify further workup of adrenal incidentaloma patients in current guidelines (11). For patients without clinical signs of CS but with insufficient cortisol suppression after Dex, the term possible autonomous cortisol secretion has been suggested when cortisol is in the range of 1.8–5.0 μg/dL. Autonomous cortisol secretion (ACS) is suggested for a cortisol concentration >5.0 μg/dL (11). However,

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there is an ongoing debate about the best test strategy in this setting (12).

Given the high clinical sensitivity of DST as a first-tier test, the low cutoff value chosen to exclude hypercortisolism leads to a relatively low clinical specificity (13). Positive DST results require further diagnostic workup by using late night salivary cortisol or 24 h urinary free cortisol measurement (5). Various reasons may lead to insufficient Dex exposure and thereby also to false-positive tests. Among those, missed Dex ingestion by the patient is frequently suspected but can rarely be ascertained. Variable absorption, distribution, metabolism, and elimination of Dex may confound test results (14, 15). Known examples are food or drug interactions through enzyme induction (e.g., phenytoin, rifampicin).

The quantification of Dex in serum in addition to cortisol may provide information about possibly insufficient Dex exposure and may help to identify false positive tests. Dex in DST samples has historically been measured by radioimmunoassay (16–19) which is associated with lack of analytical specificity and cross-reactivity with structurally similar compounds (20, 21). More analytically specific and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods with the ability to measure several analytes simultaneously have recently been developed (22–26). The impact of Dex measurement by LC-MS/MS on DST performance was evaluated in 2 studies with a total case number of 502 patients, but including only 27 patients with overt CS and 27 patients with ACS (23, 26). Even if earlier studies suggested cutoff values between 1.3 ng/mL (3.3 nmol/L) and 1.8 ng/mL (4.6 nmol/L) to verify sufficient Dex exposure allowing for adequately suppressed serum cortisol (23–26), the value of concomitant Dex quantification and the threshold to apply are still under discussion.

We here report the development and validation of an LC-MS/MS method for the simultaneous determination of Dex and cortisol in the same DST serum sample. We applied this method to 400 DST samples of patients in whom CS was suspected. The aim of our study was to evaluate whether Dex quantification reduced the proportion of false positive test results in a large number of pathological DST samples, to establish method-specific Dex and cortisol cutoff concentrations, and to investigate factors that could possibly influence serum Dex exposure during DST.

Materials and Methods

PATIENTS AND SAMPLES

The retrospective study was approved by the ethics committee of the university of Würzburg (correspondence 20200930 01) and individual patient informed consent waived. DST blood samples of patients in whom endogenous CS was suspected or who were diagnosed with an adrenal incidentaloma at the University Hospital Würzburg between February 2008 and November 2019 were collected in an S-Monovette (Serum-Gel, Sarstedt AG & Co. KG). After 30 min resting at room temperature, samples were centrifuged for 5 min at 4000 x g and stored at -20 °C.

Patients with cortisol concentrations >1.8 mg/dL post Dex underwent further workup according to current guidelines (5, 11).

400 samples were included in the study: 100 from patients with overt endogenous CS, 200 samples from patients in whom CS was excluded, and 100 samples from patients with adrenal incidentalomas with (possible) autonomous cortisol secretion (AI-ACS) (Supplemental Fig. 1). The sample size for the control cohort used for Dex threshold development as lower limit of the reference range was determined by the availability of biomaterial and considering applicable guidelines (27, 28). The sample size for the method-specific cortisol threshold adaption can be justified by the precision of estimates of diagnostic accuracy. Analyses were performed in an unblinded fashion.

Clinical and further biochemical data were obtained from patients' records. Renal function was evaluated by the estimated glomerular filtration rate (eGFR) according to the Modification of Diet in Renal Disease formula with eGFR > 90 mL/min/1.73 m^2 considered normal and chronic kidney disease stages mild (60–89 mL/min/1.73 m^2), moderate (30–59 mL/min/1.73 m^2), and severe (<30 mL/min/1.73 m^2).

MEASUREMENT OF SERUM CORTISOL

Routine measurements of cortisol were performed immediately after blood sampling with a standard immunoassay (Immulite® 2000 XPi, Siemens Healthcare GmbH) following the manufacturer's instructions. Limit of detection of the immunoassay was 0.2 µg/dL with a quantification limit of 1.0 µg/dL and a precision <9.4%. The LC-MS/MS method for cortisol measurement is described below.

STANDARDS AND REAGENTS

Cortisol, cortisol-d4, dexamethasone, acetic acid, and ammonium acetate were purchased from Sigma-Aldrich Chemie GmbH. Dexamethasone-d5 was purchased from Toronto Research Chemicals Inc. MS-grade water and methanol were from VWR International GmbH, and acetonitrile from Merck KGaA.

SAMPLE PREPARATION

200 µL sample (calibrator, QC or unknown patient sample) were mixed for 30 s with 200 µL precipitation reagent [(methanol: acetonitrile (1:1) containing deuterated internal standards at 30 ng/mL dexamethasone-d5 and 50 ng/mL cortisol-d4, stored at -20 °C]. After centrifugation at

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21 382 x g for 10 min, 200 µL supernatant were diluted with 100 µL mobile phase A (see below) and centrifuged again. 150 µL supernatant were transferred into an HPLC vial for further analysis.

LC-MS/MS CONDITIONS

An Agilent 1290 Infinity HPLC system (Agilent Technologies Germany GmbH & Co. KG) was used for chromatography. Mobile phases consisted of LC-MS-grade water with 2 mM ammonium acetate and 0.04% (V/V) acetic acid adjusted to pH = 3.8 (mobile phase A) and LC-MS-grade methanol with 2 mM ammonium acetate and 0.04% (V/V) acetic acid (mobile phase B). 25 µL prepared sample were injected onto an Oasis HLB 15 µm 2.1 x 20 mm online solid phase extraction column (Waters GmbH) with valve position to waste and after 1-min run time switching to the analytical column. Analytes were separated chromatographically on an XBridge BEH C18, 2.5 µm, 3.0 x 75 mm analytical column during a total run time of 5.35 min and additional column auto-equilibration of 1 min before each injection. Retention times were at 2.92 min for cortisol and 2.98 min for Dex.

For LC-MS/MS, a QTRAP 4500 MD (AB Sciex Germany GmbH) was used in electrospray ionization positive mode. Measurements were performed in the multiple-reaction monitoring mode with the following mass transitions (*m/z*) for cortisol (quantifier: 363.1→120.9, qualifier: 363.1 → 97.1), dexamethasone (393.1→355.1), cortisol-d4 (367.1→120.9), and dexamethasone-d5 (398.1→360.1). Methodological details are provided in the Supplemental Tables (Chromatography: online Supplemental Table 1; Mass spectrometry: Supplemental Table 2) and a representative chromatogram in Supplemental Fig. 2.

Linearity of quantification was assessed for Dex from 1.0 to 60.0 ng/mL and for cortisol from 1.0 to 60.0 µg/dL in water as surrogate matrix. Quality controls (QC) were prepared by spiking standard solution into plasma with low QC (QC1) containing 2.0 ng/mL Dex and 9.4 µg/dL cortisol and high QC (QC2) containing 10.0 ng/mL Dex and 12.9 µg/dL cortisol.

Concentrations were calculated with Analyst Software (1.6.3, Sciex) via 6-point calibration and 1/x weighting. Correctness of quantification was verified for cortisol by measurement of commercial in vitro diagnostics quality controls (Mass*Chrom*® Steroids in Serum/Plasma, Chromsystems Instruments & Chemicals GmbH) and certified ring trial samples from the German Reference Institute for Bioanalytics (RfB).

METHOD VALIDATION

Method validation was oriented to the recommendations of the Center for Drug Evaluation and Research, May 2018 (29). Calibration curves of 9 independent runs were utilized to evaluate linearity. The coefficients of determination for Dex were >0.988 in all runs and >0.998 for cortisol in every calibration curve. Detailed linearity data are supplied in Supplemental Table 3. The limit of detection, defined by a signal-to-noise ratio >3, was 0.5 ng/mL for Dex and 0.5 µg/dL for cortisol and the lower limit of quantitation was the lowest calibration level at 1 ng/mL for Dex and 1 µg/dL for cortisol, both with a signal-to-noise ratio >10.

Intra-assay precision (percent coefficient of variation) and accuracy (percent relative error) were calculated by analyzing 10 QC samples of each level in one run. Inter-assay precision was determined by measuring concentrations of both QC levels in triplicate in 8 independent runs (n = 24 for each QC level). Intra-assay precisions were <8.8% and inter-assay precisions <13.8% for both analytes. Details for precision and accuracy are provided in Supplemental Table 4.

Matrix effects were evaluated by comparing slopes of a matrix calibration curve with a calibration curve in water. Ion enhancement of 112% for Dex and 110% for cortisol were detected. Recovery was found to be 101% for Dex and 83% for cortisol.

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS version 26 (IBM Corp.) and OriginPro 2020 b (OriginLab Corp.). Subject characteristics are given as median (range) for continuous data. Normal distribution of data was evaluated by Shapiro-Wilk test. Groups were compared by Mann-Whitney U test or Kruskal-Wallis test, with a *p*-value <0.05 considered statistically significant. Pearson coefficient was used to test for linear correlation. Test performance was evaluated by receiver operating characteristics (ROC) analyses. From a clinical perspective, we considered excellent clinical sensitivity more important than an optimized compromise between clinical sensitivity and specificity (Youden's index) and therefore aimed to maintain a 100% clinical sensitivity. We applied 3 different cortisol thresholds: 1.8 µg/dL as the commonly used threshold in the literature, but also the threshold with the highest clinical specificity while maintaining 100% clinical sensitivity and the threshold with the highest Youden's index. Positive predictive value was calculated as the ratio of true positives to total positives and negative predictive value results in the true negatives divided by the total negatives. Influences of clinical variables were assessed by multiple linear regression modelling with eGFR, body mass index (BMI), sex, age, nicotine consumption, use of oral contraceptives, and the diagnosis of diabetes mellitus as covariates.

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Results

CLINICAL CHARACTERISTICS

Dex and cortisol were quantified by LC-MS/MS in 400 DST samples tested for clinical suspicion of CS or during the endocrine workup of an adrenal incidentaloma. Demographic and clinical characteristics are listed in Table 1.

SERUM DEX CONCENTRATIONS IN DST SAMPLES

Serum Dex concentrations of the 400 study samples were highly variable and ranged from undetectable to 20.2 ng/mL with a median concentration of 4.8 ng/mL Dex. Overall, Dex was undetectable in only 16 of 400 samples (4%), indicating missed Dex administration. CS could be excluded in 10 of these patients, while the diagnosis of CS in the remaining 6 patients was supported by further diagnostic testing.

Comparison of serum Dex concentration in DST samples from patients with CS (median = 5.1 ng/mL), patients with excluded CS (median = 4.7 ng/mL), and AI-ACS-samples (median = 5.4 ng/mL) missed the prespecified significance level of 0.05 ($P = 0.059$, Kruskal-Wallis; Fig. 1). Samples with undetectable Dex were excluded prior to this analysis.

DEXAMETHASONE THRESHOLD DEVELOPMENT

To determine reference values for Dex after DST, the Dex concentrations in all 137 DST samples with a

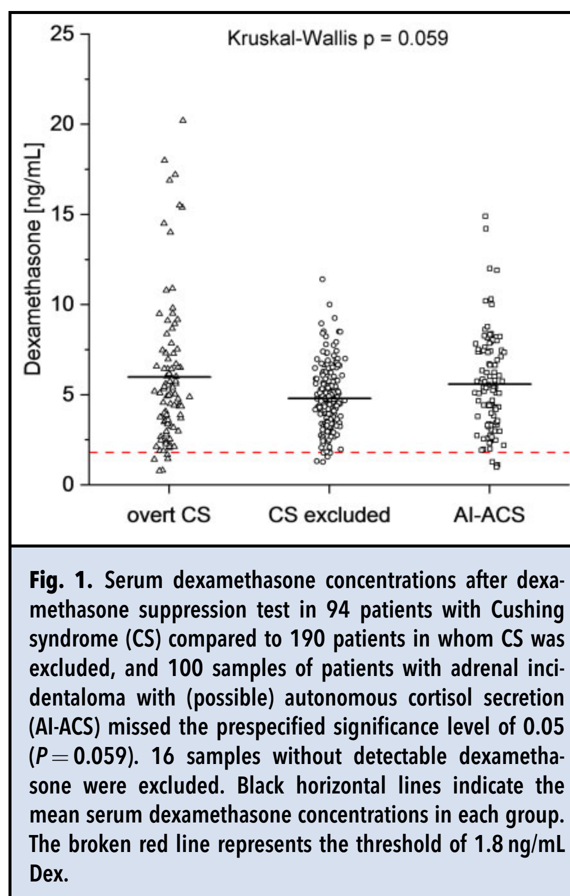

Fig. 1. Serum dexamethasone concentrations after dexamethasone suppression test in 94 patients with Cushing syndrome (CS) compared to 190 patients in whom CS was excluded, and 100 samples of patients with adrenal incidentaloma with (possible) autonomous cortisol secretion (AI-ACS) missed the prespecified significance level of 0.05 ($P = 0.059$). 16 samples without detectable dexamethasone were excluded. Black horizontal lines indicate the mean serum dexamethasone concentrations in each group. The broken red line represents the threshold of 1.8 ng/mL Dex.

Table 1. Demographic and clinical characteristics of dexamethasone suppression test samples.

	Overt CS	CS excluded	AI-ACS
Patient samples, n	100	200	100
Females, n	81	130	62
Age in years, median, (range)	52 (20-77)	52 (17-85)	65 (26-83)
BMI [kg/m^2], median, (range)	27.5 (18.9-57.4)	32.0 (11.6-62.5)	27.8 (18.9-50.4)
Diabetes mellitus, n	32	39	36
Smokers, n/Ex-smokers, n	21 /20	31 /33	32 /18
eGFR (MDRD) [mL/min/1.73m^2], median, (range)	91 (13-263)	86 (27–157)	76 (7-145)
Oral contraceptives, n	7	10	1
Serum cortisol concentration after Dex [µg/dL] (median, range)	12.1 (2.5-59.8)	1.5 (0.6*-43.0)	3.9 (1.7-17.4)
Serum dexamethasone in ng/mL, (median, range)	5.0 (0.0*-20.2)	4.6 (0.0*-11.4)	5.4 (1.0-14.9)
Serum dexamethasone concentration <1.80 ng/mL, n	11	16	4

*below LOD.
BMI, body mass index; MDRD, Modification of Diet in Renal Disease; CS, Cushing syndrome; AI-ACS, Adrenal incidentaloma with (possible) autonomous cortisol secretion.

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negative DST result (defined by serum cortisol <1.8 µg/dL during routine testing) were analyzed, resulting in a median serum Dex concentration of 4.8 ng/mL and a serum Dex range from 1.3 ng/mL to 11.4 ng/mL. The 2.5th percentile at 1.8 ng/mL was considered as lower limit of the reference range and hence set as the minimal Dex concentration leading to an adequate serum cortisol suppression. By applying the Dex cutoff and excluding 27 samples with Dex below 1.8 ng/mL among 100 samples with overt CS and 200 samples with excluded cortisol excess, test specificity increased from 67.5% to 71.7%.

IMPACT OF CLINICAL CHARACTERISTICS ON SERUM DEX CONCENTRATION

To investigate influences on serum Dex concentration, the following factors were investigated: eGFR, BMI, sex, age, nicotine consumption, use of oral contraceptives, and diagnosis of diabetes mellitus. DST samples with no detectable Dex (n = 16) that may therefore be false positive (e.g., due to non-compliance) were excluded.

Median Dex concentration increased from 4.5 ng/mL (range 0.8–16.9 ng/mL) in 165 patients with a normal renal function to 4.9 ng/mL (range 1.1–17.2 ng/mL) in 175 patients with a mild chronic kidney disease, and 7.2 ng/mL (range 2.3–20.2 ng/mL) in 38 patients with a moderate chronic kidney disease. Highest median Dex was measured in 6 patients with severe chronic kidney disease at 9.5 ng/mL (range 4.6–18.0 ng/mL) (Fig. 2, A). A moderate but significant correlation was found between eGFR and Dex concentration with a Pearson correlation coefficient r = -0.25 ($P = 2.2*10^{-5}$).

A significantly higher Dex concentration (0.8 ng/mL difference) was found in 104 patients with diabetes mellitus with a median of 5.5 ng/mL compared to 280 samples from patients without diabetes mellitus with a median of 4.7 ng/mL ($P = 0.009$) (Fig. 2, B).

BMI did not show any effect on the serum Dex concentration, neither did sex, age, nicotine consumption, or use of oral contraceptives. eGFR and diabetes mellitus retained statistically significant association with Dex concentration after multiple linear regression (Supplemental Table 5).

METHOD-SPECIFIC CORTISOL THRESHOLD

As recommended by the Endocrine Society Clinical Practice Guideline for the diagnosis of Cushing syndrome (5), a method-specific threshold for our LC-MS/MS assay was established. For this, only samples with Dex concentrations above 1.8 ng/mL were considered (i.e., 89 samples from patients with confirmed CS and 184 samples from patients with excluded CS) to exclude bias from insufficient Dex exposure. Positive predictive value and negative predictive value with 95% confidence intervals are listed in Table 2. Receiver operating characteristics analysis was performed using the clinical diagnosis based on routine endocrine workup for classification. Clinical sensitivity and specificity with 95% confidence intervals were calculated with adjusted

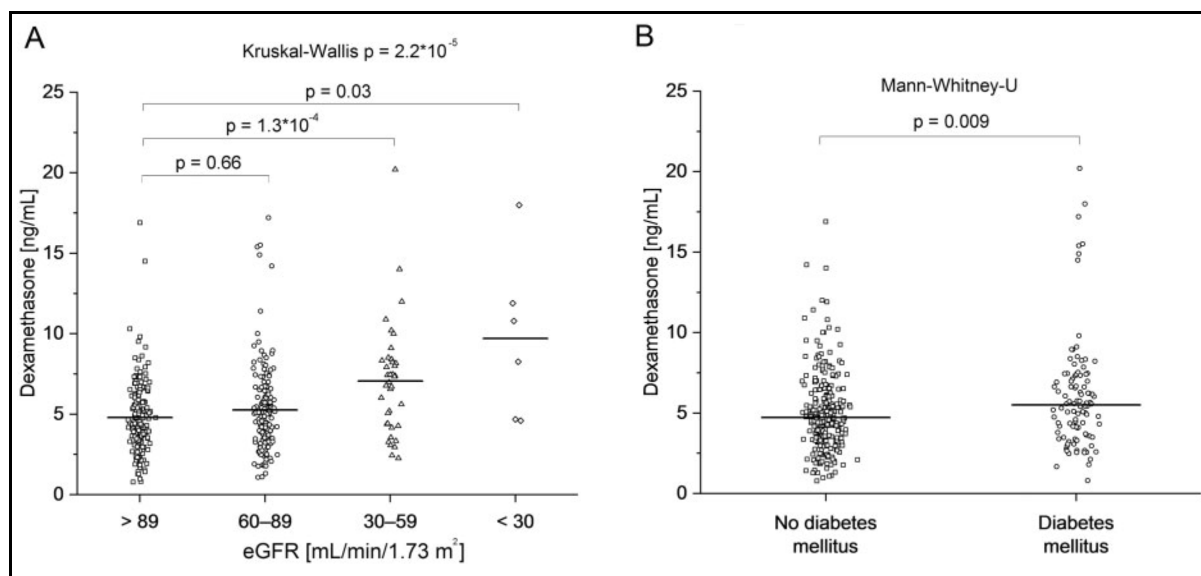

Fig. 2. A) A lower eGFR [mL/min/1.73 m^2] leads to a higher serum dexamethasone concentration. B) Patients with diabetes mellitus showed a significantly higher serum dexamethasone concentration than patients without diabetes mellitus.

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Table 2. 2x2 tables and calculated positive predicted value (PPV) and negative predictive value (NPV) for the cortisol cutoff concentrations 1.8 µg/dL, 2.4 µg/dL, and 3.1 µg/dL.

Cortisol cutoff [µg/dL]		CS	CS excluded	Total	PPV [%] (95% CI)	NPV [%] (95% CI)
1.8	Test positive	89	52	141	63.1(54.6–71.0)	100(96.5–100)
	Test negative	0	132	132		
	Total	89	184	**273**		
2.4	Test positive	89	14	103	86.4(77.9–92.1)	100(97.3–100)
	Test negative	0	170	170		
	Total	89	184	**273**		
3.1	Test positive	86	4	90	95.6(88.4–98.6)	98.4(94.9–99.6)
	Test negative	3	180	183		
	Total	89	184	**273**		

CS, Cushing syndrome.

cortisol cutoff values. Specificity increased from 71.7% at 1.8 µg/dL (Fig. 3, A) over 92.4% at 2.4 µg/dL (Fig. 3, B) to 97.8% at 3.1 µg/dL (Fig. 3, C). Even though the threshold at 3.1 µg/dL cortisol resulted in the best sum of clinical sensitivity and specificity (Youden index), we defined 2.4 µg/dL as the method-specific cortisol cutoff concentration, since it maintained 100% clinical sensitivity. The adaption of the cortisol threshold relies on consideration of diagnostic accuracy at 3 cortisol thresholds after receiver operating characteristics analysis of the 273 samples with i) ascertained diagnosis and ii) Dex >1.8 ng/mL (89 CS/184 CS excluded). The split between the groups does not represent prevalence in general population or a broader population with suspected CS but approximates proportions in a specialized center with many suspected cases. Under the assumption of a CS proportion of 33% in the available sample collection, with the reported sample size clinical specificity of 71.7% (cortisol cutoff at 1.8 µg/dL) can be estimated with a precision of 13.4%. Clinical specificity of 92.4% (cortisol cutoff at 2.4 µg/dL) can even be estimated with a precision of 8.3% and clinical sensitivity of 100% can be estimated with a precision of 5.2% (= width of the 95% CI according to the method of Score (Wilson)). Therefore, the sample size can be considered sufficient for a precise estimate of clinical sensitivity and specificity.

CLINICAL RELEVANCE OF DEX MEASUREMENT IN DST SAMPLES FROM PATIENTS WITH ADRENAL INCIDENTALOMAS WITH AUTONOMOUS CORTISOL SECRETION (AI-ACS)

After establishing the new thresholds, Dex and cortisol were quantified in 100 DST samples from patients with AI-ACS. The former diagnosis was re-evaluated applying the newly established cutoff values for Dex and cortisol (Fig. 4).

Appropriate test execution can be questioned in 4 samples for which the threshold for Dex of 1.8 ng/mL was not reached (thereby indicating possibly inadequate Dex exposure). In 14 samples, serum cortisol was below the adapted cortisol cutoff of 2.4 µg/dL. For these patients, the exclusion of autonomous cortisol secretion could be considered.

Discussion

Here we developed and applied an LC-MS/MS method for the simultaneous quantification of cortisol and Dex to a large population of patients, thereby demonstrating its diagnostic value for an improved interpretation of DST results. False positive tests due to non-compliance or insufficient Dex exposure (<1.8 ng/mL) can now be clearly identified and—by applying the method-specific cutoff for cortisol (2.4 µg/dL) – clinical specificity improved in a clinically relevant manner.

First, we found an extremely broad range of Dex concentrations after administration of 1 mg at 11:00 PM the previous day; this finding is similar to earlier reports (10, 16). This stresses the relevance of inter-individual variations in absorption, distribution, metabolism, and elimination. There was no association of Dex exposure with disease state. High variability of Dex exposure was also reported, using therapeutic Dex doses (30). Blood sampling in the morning after Dex administration (8 AM—9 AM) may not accurately assess real exposure, given that the peak Dex in a pharmacokinetics study was observed already after ~1 h (31). In addition, the rather low dose of only 1 mg Dex may contribute to

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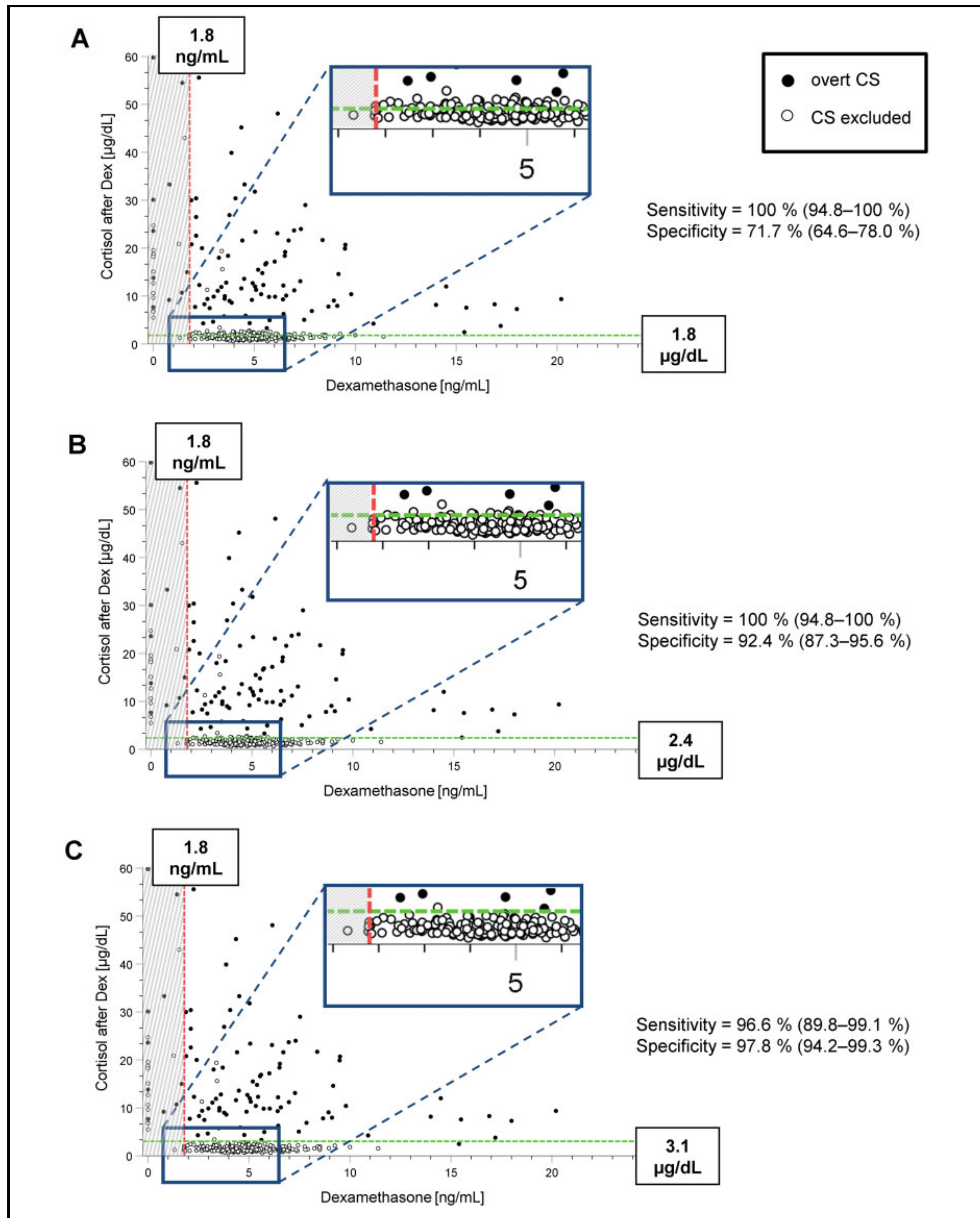

Fig. 3. Diagnostic specificity of the dexamethasone suppression test improves by adjusting the serum cortisol cutoff from 1.8 µg/dL (A) to 2.4 µg/dL (B) and 3.1 µg/dL (C, broken green line). To maintain clinical sensitivity, the method-specific cortisol cutoff of 2.4 µg/dL (B) was chosen. Samples in the gray-shaded area are excluded from receiver operating characteristics analysis due to insufficient dexamethasone (Dex) exposure (broken red line).

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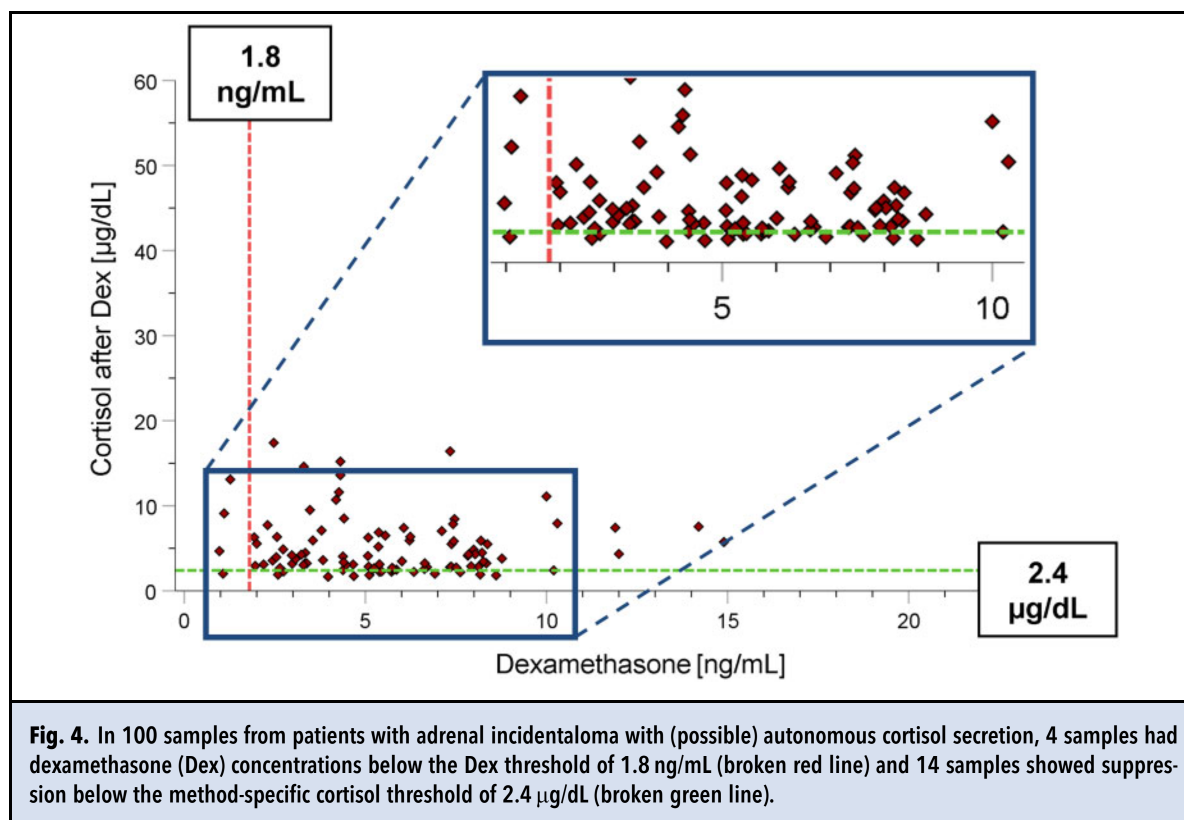

Fig. 4. In 100 samples from patients with adrenal incidentaloma with (possible) autonomous cortisol secretion, 4 samples had dexamethasone (Dex) concentrations below the Dex threshold of 1.8 ng/mL (broken red line) and 14 samples showed suppression below the method-specific cortisol threshold of 2.4 µg/dL (broken green line).

the variable exposure. An unusually high Dex exposure could cause false negative test results, even though this case might be extremely rare. Nevertheless, Dex concentrations have to be interpreted individually, and cortisol may be suppressed even below the Dex threshold due to the different sensitivity of hypothalamic CRH neurons. This is illustrated by the fact that 5 patients with Dex <1.8 ng/mL in our cohort still had suppressed cortisol.

Ueland et al. proposed a Dex cutoff value of 1.3 ng/mL (3.3 nmol/L) to verify a minimal concentration required for an adequate cortisol suppression (23). This cutoff value was later confirmed by Hawley et al. (24). In contrast, Ceccato et al. calculated a Dex threshold of 1.8 ng/mL (4.5 nmol/L) (26). The latter cutoff complies very well with our currently calculated threshold of 1.8 ng/mL.

Importantly, variabilities of Dex exposure are also relevant for other tests such as the 8 mg overnight Dex suppression test that is used for the differential diagnosis of corticotropin-dependent CS (32).

No impact on the serum Dex concentration was detected by age, sex, BMI, or nicotine consumption, confirming results from previously published studies (23, 26, 33). We could observe a negative correlation with kidney function, which was also described by Ueland et al. (23), whereas Ceccato et al. only detected this effect in their small CS cohort ($n = 16$) and not in the control group (26). Additionally, we found a significant effect by diabetes mellitus, also after multivariable adjustment.

The cutoff for cortisol after 1 mg DST of 1.8 µg/dL recommended by current guidelines has the aim of maximizing clinical sensitivity. This value was proposed in the early era of immunoassays (10). Although it is important to adapt cutoffs to the specific method applied, the limited number of CS patients severely hampers definition of own cutoffs by each center. An advantage of our study is that we were able to compare 100 patients with proven CS to 200 patients in whom CS was ruled out. The high clinical sensitivity of DST was not decreased when we adapted the cortisol threshold from 1.8 µg/dL to 2.4 µg/dL for our LC-MS/MS method, thereby increasing test specificity from 71.7% to 92.4%. This outcome is similar to the specificity of the more inconvenient-to-perform 2-day low dose DST which is not recognized as a first-tier test by most centers any more. While for many tests the aim is to find an appropriate compromise between clinical sensitivity and specificity that is reflected in the Youden index, the DST is used as a screening test and aims at maximizing sensitivity, which is why we rather accepted false positive results than false negative results.

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The high frequency of cross-sectional imaging leads to an increasing number of incidentally discovered adrenal tumors (11). While imaging criteria in combination with steroid mass spectrometry of 24 h urine samples have recently confirmed to enable reliable detection of malignancy (34, 35), the endocrine workup for clearly benign adrenal incidentalomas still poses a relevant clinical challenge. The identification of patients with subclinical or only mild CS has remained a matter of controversy and ongoing research. Clinically, the risk of unnecessary surgery needs to be balanced against the potentially deleterious effects of chronic tissue exposure to long-term glucocorticoid excess (11). Using our new cutoffs for Dex and cortisol, among the 100 DST samples of patients with AI-ACS, autonomous cortisol secretion was excluded in 14 patients. This is a clinically relevant proportion in whom the current practice of repeated testing (with its potential psychological disturbance) may be omitted.

Our study has potential limitations. First, the sample size is still limited due to the rarity of patients with overt CS. However, the number of patients in our study considerably exceeds that of previously published studies and appears sufficient for reliable statistical analyses. In addition, the proportion of patients with CS compared to those in whom CS was excluded may reflect the situation in a referral center and not that in general population or primary endocrine care. Moreover, since no external quality controls were available for Dex, a certified reference standard was used to prepare quality controls in our laboratory, ensuring the best possible level of analytical quality. Further, samples were collected for several years, resulting in a comparably long period of time between cortisol quantification by immunoassay and the LC-MS/MS analysis. However, cortisol degradation was considered marginal in view of the good comparability of cortisol concentrations between methods. Even though we developed thresholds based on a relatively large study, given the rarity of CS, the absolute number is still limited and the study is retrospective in nature. Consequently, independent validation is necessary.

In conclusion, the developed Dex threshold turns out to be a valuable tool to evaluate sufficient Dex exposure during DST. The patients with unsuppressed cortisol and insufficient Dex exposure should either perform a repeat DST in case of non-compliance or undergo another diagnostic testing procedure such as late-night salivary cortisol or 24 h-urinary free cortisol measurement. Applying method-specific cutoffs for Dex and cortisol significantly improved the specificity of DST from 67.5% to 92.4%, while preserving 100% sensitivity. Thus, our data clearly highlight the necessity to establish method-specific cutoffs, which is often neglected in clinical practice.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: DST, dexamethasone suppression test; CS, Cushing's syndrome; Dex, dexamethasone; LC-MS/MS, liquid chromatography-tandem mass spectrometry; AI-ACS, adrenal incidentaloma with (possible) autonomous cortisol secretion; eGFR, estimated glomerular filtration rate; m/z, mass-to-charge ratio; QC, quality control; ROC, receiver operating characteristics; LLN, lower limit of normal; RfB, Reference Institute for Bioanalysis; BMI, body mass index

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