Clinical Research Article

New Cutoffs for the Biochemical Diagnosis of Adrenal Insufficiency after ACTH Stimulation using Specific Cortisol Assays

Bradley R. Javorsky, Hershel Raff, Ty B. Carroll, Alicia Algeciras-Schimnich, Ravinder Jit Singh, Jessica M. Colón-Franco, and James W. Findling

1Endocrinology Center and Clinics, Froedtert & the Medical College of Wisconsin, Milwaukee, WI 53051, USA; 2Division of Endocrinology and Molecular Medicine, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI 53226, USA; Endocrine Research Laboratory, Aurora St. Luke’s Medical Center, Advocate Aurora Research Institute, Milwaukee, WI 53215, USA; 3Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN 55905, USA; and 4Department of Pathology, Medical College of Wisconsin, Milwaukee, WI 53226, USA

ORCID numbers: 0000-0001-5052-3043 (B. R. Javorsky); 0000-0002-5128-8476 (H. Raff); 0000-0002-1310-6563 (T. B. Carroll).

Abbreviations: ACTH, adrenocorticotropic hormone; AI, adrenal insufficiency; CST, synthetic ACTH[1–24] (cosyntropin) stimulation testing; HPA, hypothalamic-pituitary-adrenal; LC-MS/MS, liquid chromatography–tandem mass spectrometry; ROC, receiver operator characteristic curve.

Received: 1 February 2021; Editorial Decision: 10 February 2021; First Published Online: 18 February 2021; Corrected and Typeset: 19 March 2021.

Abstract

Context: The normal cortisol response 30 or 60 minutes after cosyntropin (ACTH[1–24]) is considered to be ≥18 μg/dL (500 nmol/L). This threshold is based on older serum cortisol assays. Specific monoclonal antibody immunoassays or LC-MS/MS may have lower thresholds for a normal response.

Objective: To calculate serum cortisol cutoff values for adrenocorticotropic hormone (ACTH) stimulation testing with newer specific cortisol assays.

Methods: Retrospective analysis of ACTH stimulation tests performed in ambulatory and hospitalized patients suspected of adrenal insufficiency (AI). Serum samples were assayed for cortisol in parallel using Elecsys I and Elecsys II immunoassays, and when volume was available, by Access immunoassay and LC-MS/MS.

Results: A total of 110 patients were evaluated. Using 18 μg/dL as the cortisol cutoff after ACTH stimulation, 14.5%, 29%, 22.4%, and 32% of patients had a biochemical diagnosis of AI using the Elecsys I, Elecsys II, Access, and LC-MS/MS assays, respectively. Deming regressions of serum cortisol were used to calculate new cortisol cutoffs based on the Elecsys I cutoff of 18 μg/dL. For 30-minute values, new cutoffs were 14.6 μg/dL for Elecsys II, 14.8 μg/dL for Access, and 14.5 μg/dL for LC-MS/MS. Baseline cortisol <2 μg/dL was predictive of subnormal stimulated cortisol values.
The accurate and swift diagnosis of adrenal insufficiency (AI) is imperative given the potential for life-threatening consequences if missed [1, 2]. Conversely, inappropriate assignment of AI to individuals has the potential for unnecessary glucocorticoid therapy [3]. Therefore, confirmation of the diagnosis of AI mandates precise biochemical testing, often requiring the assessment of adrenocorticotropic hormone (ACTH)-stimulated adrenal function to evaluate the integrity of hypothalamic-pituitary-adrenal (HPA) axis function.

ACTH (synthetic ACTH[1–24]; cosyn tropin; synacthen) stimulation testing (CST) is the most commonly performed dynamic test to assess the adequacy of adrenal function in patients with suspected secondary adrenal insufficiency [4]. CST assesses the maximum adrenocortical secretory response to a supraphysiologic dose of ACTH. Patients with primary AI always have an elevated plasma ACTH, so CST is typically not needed for confirmation of the diagnosis [5, 6]. Although insulin-induced hypoglycemia has previously been considered the gold standard test for decreased HPA axis function, it is very challenging to perform properly, labor intensive, and risky [7]. Accordingly, it has been abandoned in most clinical settings. The CST cortisol cutoff threshold for the diagnosis of AI 30 or 60 minutes after ACTH administration has evolved over the years, but it has become entrenched at 18 µg/dL (500 nmol/L) despite improved specificity of newer cortisol assays [5, 8-10]. Historically, immunoassays using polyclonal antibodies have been used to establish post-cosyntropin cortisol cutoff concentrations as high as 20 µg/dL [11, 12]. These assays had cross-reactivity with other serum steroids [13-15]. Newer-generation assays with greater specificity for cortisol have been developed and have already replaced polyclonal antibody assays in many institutions [13, 15, 16].

Basal morning serum cortisol concentrations are also used to either increase the suspicion for, or rule-out the diagnosis of AI [11, 12, 17-20]. Furthermore, basal morning serum cortisol concentrations ranging from 11 to 19 µg/dL have been cited as a criterion to rule-out AI. Conversely, it has been argued that very low cortisol values (ie, <3-6 µg/ dL) may establish biochemical AI and thus obviate the need for dynamic CST.

The Elecsys Cortisol generation II (Roche Diagnostics, City, CA) immunoassays utilize monoclonal antibodies to identify cortisol [16, 21]. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a non-antibody, structural assay highly specific for cortisol [22-25]. Serum cortisol concentrations are approximately 20% lower with the newer assays compared with the older assays in some studies [8, 13, 15, 26, 27]. However, other studies have actually suggested that LC-MS/MS yielded a higher peak cortisol cutoff after ACTH stimulation than cortisol measured by immunoassay [28, 29]. Considering these discrepancies in the literature, ACTH-stimulated cortisol threshold values using new, more-specific cortisol assays are needed to accurately diagnose AI and minimize overtreatment.

The purpose of the current study was to evaluate basal serum cortisol and the serum cortisol response to synthetic ACTH[1–24] stimulation in patients suspected of having AI and compare more-specific cortisol assays (2 monoclonal antibody assays and LC-MS/MS) with a polyclonal antibody cortisol assay in order to calculate new cutoffs for the cortisol response to CST.

Materials and Methods
The study was approved by the Medical College of Wisconsin/Froedtert Hospital Institutional Review Board.

Study Design
This study used residual serum samples (n = 110) from patients undergoing CST (ambulatory and hospitalized) ordered by any clinician as part of clinical care from January 8, 2017 to April 30, 2017. This time frame was dictated by the limited availability of reagents for the Elecsys Cortisol generation I (Roche Diagnostics, IN) assay which was being phased out by the vendor during the study. Samples were identified using laboratory order codes for CST.

CST was performed as follows: cosyntropin (250 mcg ACTH[1–24]) was administered intravenously. Blood samples for cortisol were obtained in serum separator tubes (SST) before injection (0 minutes) and at 30 minutes. If a clinician also ordered a blood sample for cortisol at 60 minutes, that was analyzed. Basal ACTH values in EDTA plasma were also obtained in some instances at the ordering provider’s discretion. All samples (n = 110 for the

Conclusion: To reduce false positive ACTH stimulation testing, we recommend a new serum cortisol cutoff of 14 to 15 µg/dL depending on the assay used (instead of the historical value of 18 µg/dL with older polyclonal antibody assays). Clinicians should be aware of the new cutoffs for the assays available to them when evaluating patients for AI.

Key Words: ACTH stimulation testing, cosyntropin, monoclonal cortisol immunoassay, LC-MS/MS
0- and 30-minute samples; n = 89 for the 60-minute samples) were run in parallel on the Elecsys I and II cortisol immunoassays. When specimens were available, de-identified samples were analyzed at the Mayo Clinical Laboratory using the Beckman Access Cortisol (Beckman Coulter, CA) immunoassay (n = 79 for the 0-minute sample, n = 78 for 30-minute sample, and n = 66 for 60-minute sample) and an LC-MS/MS assay (n = 79 for the 0-minute sample, n = 79 for the 30-minute sample, and n = 66 for the 60-minute sample).

Clinical data were obtained through chart review and entered into a password-protected Excel spreadsheet as follows: date, sampling times, cortisol, basal ACTH, age, sex, medical history related to potential adrenal insufficiency, relevant medications (eg, opioids, glucocorticoids), and inpatient vs outpatient setting.

Assays

The Elecsys Cortisol (referred to here as generation I) and Cortisol II electrochemiluminescence competitive immunoassays were run on the Cobas E170 (Roche Diagnostics, Indianapolis, IN) [14, 16]. The generation I cortisol assay used a polyclonal antibody and was standardized against the Enzymun test, which was standardized against the cortisol assay used a polyclonal antibody and was standardized against the IRMM/IFCC 451 Panel (ID-GC/MS).

Intraassay variability was 1.6% to 2.4% and interassay variability was 1.9% to 2.8% for concentrations ranging from 1.4 to 60.2 μg/dL. The Cortisol II assay uses a monoclonal antibody and is standardized against a reference material, the IRMM/IFCC 451 Panel (ID-GC/MS). The assay is more specific for cortisol than its predecessor and reduced all steroid cross-reactivities to under 10%, except for prednisolone (12% at 0.1 μg/mL). Intraassay variability was 1.0% to 1.7% for concentrations ranging from 4.7 to 31.4 μg/dL. Interassay variability was 2.2% to 2.8% for concentrations ranging from 4.5 to 12.4 μg/dL.

The Access Cortisol assay is a competitive binding immunoenzymatic assay run on the Beckman Coulter UniCel Dxi 800 (Beckman Coulter, Brea, CA) [21]. Relevant cross-reactivities for other steroids include 11-deoxycortisol (100 μg/dL), 17.8% and prednisolone (20 μg/dL), 23.9%. Intraassay variability was 3.4% to 4.7% and interassay variability was 4.1% to 5.7% for concentrations ranging from 4.4 to 35.3 μg/dL. The serum LC-MS/MS assay method has been described previously [30]. Intraassay variability was 4.8% to 7.5% for concentrations ranging from 1.1 to 20.7 μg/dL. Interassay variability was 8.7% to 9.8% for concentrations ranging from 2.2 to 21.0 μg/dL. Plasma ACTH was measured using the Roche Cobas platform immunometric assay [31, 32].

Statistical Analysis

All statistical analyses were performed used SigmaPlot 12.5 (Systat Software Inc., San Jose, CA). Deming regressions were used to calculate the slope and intercept (with 95% CI) of the relationship between Elecsys II, Access, or LC-MS/MS vs Elecsys I cortisol assay and then calculate the new cutoffs based on Elecsys I cutoffs. Bland-Altman plot bias data were analyzed by one-factor analysis of variance with all pairwise comparisons (Duncan multiple range test). Receiver operator characteristic curve (ROC) analysis was performed using standard algorithms with the Elecsys I data and its 18 μg/dL cutoff to assign the designation of biochemical adrenal insufficiency by maximizing both sensitivity and specificity [10, 11]. In patients with complete baseline and 30- and 60-minute post-cosyntropin data within each assay method, two-factor analysis of variance repeated on one factor (time) with all pairwise comparisons (Duncan’s multiple range test) was used to compare results against Elecsys I and within time. Mann-Whitney Rank Sum Test was used to compare baseline serum cortisol results separated by whether the serum cortisol response to ACTH stimulation was normal vs subnormal. Chi-square was used to evaluate proportional data.

Results

Figure 1 shows Deming regression of serum cortisol by Roche Elecsys II, Beckman Access, and LC-MS/MS compared with Elecsys I. Shown are baseline serum cortisol (left column) and 30- and 60-minute post-cosyntropin samples for results with a baseline Elecsys I cortisol <18 μg/dL (500 nmol/L; middle and right columns). There were excellent Deming correlation coefficients between cortisol assay methods. Notice that the N values were less for Access and LC-MS/MS because of insufficient serum samples. Also, there were fewer 60- vs 30-minute samples because not all clinicians requested the 60-minute sample.

The more-specific cortisol assays (Elecsys II, Access, and LC-MS/MS) resulted in lower cortisol concentrations and lower cutoffs as the Deming slopes were all <1.00 (0.61-0.78). Elecsys II and Access cortisol were similar. The slope of LC-MS/MS vs Elecsys I regression was lower than Elecsys II and Access cortisol leading to lower cutoffs. Table 1 shows a series of baseline cutoffs (to rule-out AI) calculated from commonly used baseline Elecsys I cutoffs using Deming regression data shown in Fig. 1.
Fig. 2 shows the Bland-Altman plots relative to Elecsys I (with the panels organized as in Fig. 1). The bias for baseline cortisol ranged from −2.9 to −4.3 μg/dL. The bias for 30-minute post-ACTH stimulation (with the cortisol baseline <18 μg/dL) ranged from −3.9 to −4.1 μg/dL and for 60-minute post-ACTH stimulation, −3.7 to −6.0 μg/dL. For the baseline cortisol data, the bias of LC-MS/MS was greater than for Elecsys II (P = 0.019). There were no differences in bias for the 30-minute post-ACTH stimulation results. For the 60-minute post-ACTH data, bias of LC-MS/MS was greater than Elecsys II (P = 0.009) and Access (P < 0.001).

To further explore the data, we performed ROC analyses with the assumption that Elecsys I and its associated cortisol cutoff of 18 μg/dL is the “gold standard” [10, 11]. The sensitivities and specificities of Elecsys II, Access, and LC-MS/MS were the same as one would expect considering the excellent correlations shown in Fig. 1. More importantly, the calculated post-ACTH ROC cortisol cutoffs were as follows: At 30 minutes post-ACTH, the ROC cutoffs were 14.4 μg/dL for Elecsys II, 15.5 μg/dL for Access, and 13.5 μg/dL for LC-MS/MS. At 60 minutes post-ACTH, the ROC cutoffs were 13.4 μg/dL for Elecsys II, 14.5 μg/dL for Access, and 11.5 μg/dL for LC-MS/MS.

Table 2 shows the new post-ACTH stimulation cortisol cutoffs for Elecsys II, Access, and LC-MS/MS interpolated

![Figure 1. Deming regressions of serum cortisol by Roche Elecsys II, Beckman Access, and LC-MS/MS, compared with Elecsys I. Cortisol cutoff values for the 30- and 60-minute samples after ACTH (cosyntropin) stimulation were calculated from patients with baseline cortisol <18 μg/dL (500 nmol/L) on the Elecsys I assay. Confidence limit lines are 95%.](image)

Table 1. Common Baseline Serum Cortisol Cutoffs Used to Rule-Out Adrenal Insufficiency (8–16 μg/dL)

| Elecsys I | Elecsys II | Access | LC-MS/MS |
|-----------|-----------|--------|----------|
| 8.0       | 6.6       | 6.9    | 6.6      |
| 10.0      | 8.1       | 8.4    | 7.8      |
| 12.0      | 9.7       | 9.9    | 9.0      |
| 14.0      | 11.3      | 11.3   | 10.2     |
| 16.0      | 12.8      | 12.8   | 11.5     |
| N         | 110       | 79     | 79       |
| Slope     | 0.78      | 0.73   | 0.61     |
| 95% CI    | [0.75-0.81] | [0.70-0.76] | [0.57-0.66] |
| Y-int     | 0.33      | 1.1    | 1.7      |
| 95% CI    | [-0.23-0.89] | [0.41-1.7] | [0.81-2.6] |
| r value   | 0.98      | 0.98   | 0.95     |

Cutoffs were generated using Deming regression data shown. [95% CIs for slope and intercept are shown in brackets]. Cortisol units are in μg/dL. To convert μg/dL to nmol/L, multiply by 27.6.

Abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; Y-int, y-axis intercept of the Deming regression.

Fig. 2 shows the Bland-Altman plots relative to Elecsys I (with the panels organized as in Fig. 1). The bias for baseline cortisol ranged from −2.9 to −4.3 μg/dL. The bias for 30-minute post-ACTH stimulation (with the cortisol baseline <18 μg/dL) ranged from −3.9 to −4.1 μg/dL and for 60-minute post-ACTH stimulation, −3.7 to −6.0 μg/dL. For the baseline cortisol data, the bias of LC-MS/MS was greater than for Elecsys II (P = 0.019). There were no differences in bias for the 30-minute post-ACTH stimulation results. For the 60-minute post-ACTH data, bias of LC-MS/MS was greater than Elecsys II (P = 0.009) and Access (P < 0.001).

To further explore the data, we performed ROC analyses with the assumption that Elecsys I and its associated cortisol cutoff of 18 μg/dL is the “gold standard” [10, 11]. The sensitivities and specificities of Elecsys II, Access, and LC-MS/MS were the same as one would expect considering the excellent correlations shown in Fig. 1. More importantly, the calculated post-ACTH ROC cortisol cutoffs were as follows: At 30 minutes post-ACTH, the ROC cutoffs were 14.4 μg/dL for Elecsys II, 15.5 μg/dL for Access, and 13.5 μg/dL for LC-MS/MS. At 60 minutes post-ACTH, the ROC cutoffs were 13.4 μg/dL for Elecsys II, 14.5 μg/dL for Access, and 11.5 μg/dL for LC-MS/MS.

Table 2 shows the new post-ACTH stimulation cortisol cutoffs for Elecsys II, Access, and LC-MS/MS interpolated
Again, the slopes were <1 resulting in new cortisol cutoffs < 0.001). Post-ACTH (cosyntropin) data, bias of LC-MS/MS cortisol was greater than Elecsys II (P = 0.019). There were no differences in bias for 30-minute post-ACTH (cosyntropin) results. For the 60-min result. Finally, the cortisol response to ACTH stimulation was used as the cutoff. For Elecsys II, 32 of 110 (29%) results would be considered subnormal if 18 μg/dL was used as the cutoff (P = 0.014 compared with Elecsys I). Using the LC-MS/MS assay, 25 of 78 (32%; P = 0.007 compared from the Deming regressions with Elecsys I from Fig. 1. Again, the slopes were <1 resulting in new cortisol cutoffs <18 μg/dL for the more-specific assays. Notice that Table 1 cannot be used to compare 30- vs 60-minute results because the data are for all patients, not only for those who had complete baseline, 30-minute, and 60-minute samples (repeated measure on all times points).

In order to accurately compare the 30- vs 60-minute response to ACTH stimulation, we then analyzed the data for only those patients who had complete 0 (baseline), 30-minute, and 60-minute results within each method (repeated measures) (Fig. 3). All the assays had statistically significantly lower values at every time point compared with the Elecsys I assay. More importantly, the 60-minute cortisol result for all methods was higher than the 30-minute result. Finally, the cortisol response to ACTH stimulation for the 3 more-specific assays were not different from each other.

When evaluating patients who had data for basal cortisol and at least one other time point (30- or 60-minutes post-ACTH stimulation), 16 of 110 (14.5%) Elecsys I ACTH-stimulated cortisol were subnormal if 18 μg/dL was used as the cutoff. For Elecsys II, 32 of 110 (29%) results would be considered subnormal if 18 μg/dL was used as the cutoff (P = 0.014 compared with Elecsys I). Using the LC-MS/MS assay, 25 of 78 (32%; P = 0.007 compared

---

**Figure 2.** Bland-Altman Plots of serum cortisol by Roche Elecsys II, Beckman Access, and LC-MS/MS compared with Elecsys I. Cortisol cutoff values for the 30- and 60-minute samples after ACTH (cosyntropin) stimulation were calculated from patients with baseline cortisol <18 μg/dL (500 nmol/L) on the Elecsys I assay. N values are the same as in Fig. 1. Bland-Altman parameters are shown in each panel. For the baseline data, the bias of LC-MS/MS cortisol was greater than for Elecsys II (P = 0.019). There were no differences in bias for 30-minute post-ACTH (cosyntropin) results. For the 60-min post-ACTH (cosyntropin) data, bias of LC-MS/MS cortisol was greater than Elecsys II (P = 0.009) and Access (P < 0.001).
with Elecsys I) would be considered subnormal if 18 μg/dL was used as the cutoff. Although not significantly different from the Elecsys I assay, 17 of 76 (22.4%; \( P = 0.239 \)) compared with Elecsys I) would be considered subnormal using the Access assay.

To better understand if the new calculated cutoff values would provide similar biochemical diagnoses of AI (compared with the historical cutoff of 18 μg/dL in the Elecsys I assay), we identified discrepancies between assays (Table 3). Eight of the 110 patients (7.3%) had abnormal CST results (both the 30- and 60-minute results were below the cutoff) on at least one assay, but normal results using the new cutoffs on another assay. Five were abnormal in 1 of the 4 assays, and 3 were abnormal in 2 of the 4 assays. Most of the abnormal values were close to the cortisol cutoff. On chart review, 3 of these 8 patients were clinically thought to have secondary AI. LC-MS/MS would have identified all 3 of these patients as having a biochemical diagnosis of AI using the newly derived cortisol cutoff, while 2 of 3 would have been identified with Elecsys II and none with Elecsys I or Access.

Nine of the patients had abnormal 30-minute cortisol results, but normal 60-minute cortisol results on at least one assay (using the new cutoffs) (Table 3). On chart review, only 1 of these 9 patients were clinically thought to have secondary AI (case 85). Three of the patients fell into both categories (discordant results between assays and 30-minute vs 60-minute discordance). Most of the CST were performed before 9 am (all but 3). Assay discordance could not be explained by obviously identifiable features of the clinical history including reason for CST, age, sex, time of day, inpatient (± critical illness) vs outpatient status, estrogen use, or ACTH level (Chi-square).

We further explored the data from Table 3 in only those samples with discordant post-CST results at 30 vs 60 minutes in the 8 patients in whom the clinical diagnosis of secondary AI was excluded (based on chart review). We did this analysis for the Elecsys II samples, as that is the monoclonal antibody assay currently in use for which we had complete data. In those 8 patients, 2 had a cortisol response at 30 minutes above the new cutoff whereas 7 had a cortisol response at 60 minutes above the new cutoff (\( P = 0.041 \)).

**Table 2.** New Cosyntropin-Stimulated 30- and 60-Minute Cortisol Cutoffs for Elecsys II, Access, and LC-MS/MS Around Commonly Used Values (17-20 μg/dL) for the Assessment of Adrenal Insufficiency

| 30-min post-cosyntropin (250 μg) | 60-min post-cosyntropin (250 μg) |
|----------------------------------|----------------------------------|
| **Elecsys I** | **Elecsys II** | **Access** | **LC-MS/MS** | **Elecsys II** | **Access** | **LC-MS/MS** |
| 17.0 | 13.9 | 14.0 | 13.7 | 13.9 | 13.8 | 13.0 |
| 18.0 | 14.6 | 14.8 | 14.5 | 14.7 | 14.5 | 13.7 |
| 19.0 | 15.4 | 15.5 | 15.2 | 15.5 | 15.3 | 14.5 |
| 20.0 | 16.2 | 16.3 | 16.0 | 16.3 | 16.1 | 15.2 |
| N 78 | 54 | 48 | 64 | 48 | 48 | 48 |
| Slope 0.78 | 0.77 | 0.77 | 0.81 | 0.78 | 0.77 | 0.74 |
| 95% CI [0.74-0.82] | [0.70-0.84] | [0.68-0.86] | [0.76-0.86] | [0.71-0.85] | [0.64-0.83] | [0.74-0.83] |
| Y-int 0.6 | 0.9 | 0.6 | 0.1 | 0.5 | 0.4 |
| 95% CI [−0.3 to 1.5] | [−0.7 to 2.4] | [−1.4 to 2.6] | [−1.1 to 1.3] | [−1.4 to 2.4] | [−2.1 to 2.9] | [−0.7 to 2.4] |
| \( r \) value 0.98 | 0.95 | 0.93 | 0.97 | 0.96 | 0.92 |

Cutoffs were generated using Deming regression data shown. [95% CIs for slope and intercept are shown in brackets]. Cortisol units are in μg/dL. To convert μg/dL to nmol/L, multiply by 27.6. The data are based on all patients with a baseline serum cortisol <18 μg/dL. The N values are different for 30 vs 60 minutes because these were not all paired results. Therefore, one cannot compare 30 vs 60 minutes in this Table.

Abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; Y-int, y-axis intercept.

\*18.0 μg/dL is the widely used cutoff.
Table 3. Discordant Cosyntropin Stimulation Test (CST) Results Between Assays and Discordant 30- vs 60-Minure Results Within Assays Using the New Cortisol Cutoffs

| Case | Discordant CST result | 30 Abn, 60 Nml | Elecsys I | Elecsys II | Access | LC-MS/MS | CST time | Inpt (I) | Outpt (O) | Basal ACTH | Clinical diagnosis | Pertinent Clinical History |
|------|-----------------------|----------------|----------|-----------|--------|----------|---------|---------|-----------|------------|---------------------|--------------------------|
| 2    | X                     | X              | 9.6      | 16.1      | 18.5   | 7.2      | 11.9    | 13.9    | 9.8       | 14         | 18       | M        | 12:37    | I        | NA      | No      | Syncope; on fludrocortisone for autonomic insufficiency; treated with GC 1 week prior |
| 3    | X                     | 8.9            | 20       | 23.7      | 6.9    | 15.3     | 18      | 7.8     | 17        | 21         | 5.4      | 12       | 13      | 58      | M       | 8:00    | O       | 28.6    | No |
| 13   | X                     | 13.1           | 12.9     | 24.1      | 10.7   | 10.4     | 18.7    | 20      | 17        | 86         | F        | 13:05    | I        | NA      | No      | 6 mm pituitary lesion; intraarticular GC injections 1–2 months prior |
| 14   | X                     | 12.2           | 23       | 9.8       | 14.5    |         |         |         |           |            |          |          |          |         | 65      | M       | 8:19    | O       | 35.8    | No |
| 20   | X                     | 24.2           | 25.9     | 13        | 14.3    | 19      | 19      | 11      | 12        | 85         | M        | 9:01     | I        | NA      | Yes     | Weakness, vomiting, weight loss; long-term GC tapered off just before stim test; chronic opioids |
| 23   | X                     | 22.8           | 32.6     | 39.9      | 14.6   | 21.9     | 26.8    | 12      | 77        | F          | 6:13     | I        | 13.1    | No      | Unilateral adrenalectomy; bilateral adrenal nodules with cortisol excess |
| 39   | X                     | 25.3           | 27.3     | 31.8      | 17.3   | 19.5     | 21.7    | 19      | 20        | 22         | 13       | 14       | 17      | 74      | M        | 7.02    | I        | 33      | No |
| 42   | X                     | 13.8           | 19.5     | 21.7      | 10.6   | 14.5     | 16      | 52      | F          | 8:57       | O        | 26.1     | No      | Unilateral adrenal nodule with cortisol excess s/p unilateral adrenalectomy |
| 47   | X                     | 15.4           | 18.2     | 20.5      | 11.7   | 13.9     | 14.8    | 12      | 14        | 16         | 11       | 13       | 12      | 55      | F        | 6:13    | I        | NA      | No |
| 59   | X                     | 10.2           | 15.2     | 18.1      | 8.9    | 13.3     | 15.1    | 28      | M          | 9:01       | O        | 38.8     | No      | Unilateral adrenalectomy; bilateral adrenal nodules with cortisol excess |
| 69   | X                     | 9.1            | 15.8     | 18.7      | 8.4    | 14.3     | 16.4    | 7.8     | 15        | 16         | 10       | 14       | 17      | 47      | F        | 13:20   | O        | 49.9    | No |
| 85   | X                     | 17.7           | 17.1     | 20.7      | 10.1   | 10       | 12.8    | 15      | 15        | 18         | 11       | 10       | 13      | 73      | M        | 6:00    | I        | 6.2     | Yes |
| 89   | X                     | 16             | 19.3     | 20.1      | 12.4   | 15.4     | 15.4    | 12      | 15        | 16         | 12       | 13       | 50      | M        | 5:44    | I        | NA      | Yes |
| 99   | X                     | 9.7            | 18.5     | 23        | 7.9    | 15       | 17.1    | 7.3     | 15        | 15         | 7.7      | 14       | 16      | 24      | F        | 7:55    | O        | 15.1    | No |

Note: CST time refers to time of day the test was performed. Cortisol units (columns 4-15) are µg/dL. To convert µg/dL to nmol/L, multiply by 27.6. ACTH units are pg/mL. To convert to pmol/L, multiply by 0.2202. For inpatients, reason for hospitalization is listed first in Pertinent Clinical History. None of the female patients were on estrogen. Patients were not critically ill unless specified. Abbreviations: Abn, abnormal; AI, adrenal insufficiency; c/b, complicated by; GC, glucocorticoids; Inpt, inpatient; NA, not available; Nml, normal; Outpt, outpatient; s/p, status-post.
Figure 4 shows the data from patients with baseline cortisol <8 μg/dL separated by whether they had a normal or subnormal response to CST. The baseline cortisol was statistically lower in the patients with a subnormal ACTH-stimulated cortisol response, but there was still considerable overlap with the baseline cortisol value in the patients with a normal response. Elecsys I baseline cortisol had to be very low (less than ~2 μg/dL) to reliably predict an abnormal post-ACTH stimulated cortisol. These differences were not accounted for by age, sex, inpatient or outpatient status, or baseline plasma ACTH (Chi-square).

Discussion

This study demonstrated that newer, more-specific cortisol assays have a lower threshold for the normal cortisol response to CST in patients with suspected AI. Although there was excellent correlation between these new assays and an older polyclonal antibody cortisol assay, the results were 22% to 39% lower than the less-specific Elecsys I assay. Accordingly, the new cortisol response to CST should be 14 to 15 μg/dL rather than 18 μg/dL. Not unexpectedly, there were differences in the 30- and 60-minute post-CST cortisol levels; however, these differences rarely caused diagnostic confusion and there was excellent correlation among all the specific assays at every time point.

The CST is the most commonly used dynamic test to probe for a decrease in HPA axis function. The advantage of CST is that it can be performed at any time of the day and elicits the maximal cortisol production from the adrenal glands in a short time. Both high dose (250 mcg) and low dose (1 mcg) CST have each been advocated, but meta-analysis and consensus statements have concluded that these doses yield similar diagnostic accuracy [4]. This is not surprising since the high dose and low dose cosyntropin generate plasma ACTH concentrations of 22,000 to 100,000 pg/mL and 2000 pg/mL, respectively, at 2 minutes after injection [33, 34]. The concentrations of plasma ACTH greatly exceed the maximum endogenous physiologic stimulus to the adrenal cortex [4]. In fact, plasma ACTH >100 pg/mL generates a near maximum cortisol response [4]. Since patients with primary AI always have elevated plasma ACTH, CST will stimulate very small increments in cortisol if at all. Accordingly, the increase in cortisol stimulated by cosyntropin in these patients is not a reliable criterion for the adequacy of adrenal function. Historically, a cortisol level at 30 or 60 minutes of >18 μg/dL has been considered normal [5, 9, 10, 35, 36].

Newer, more-specific cortisol assays with lower cross-reactivity to other endogenous steroids have replaced older, less-specific assays [8, 13, 15, 26, 27]. Cortisol concentrations are approximately 20% lower in these more-specific assays. We have shown that the threshold cortisol response to CST of 14 to 15 μg/dL in a large group of patients with suspected secondary adrenal insufficiency is comparable to the 18 μg/dL cutoff previously used. Grassi et al also found lower cortisol cutoff values of 12.7 and 13.3 μg/dL using Elecsys II and LC-MS/MS assays, respectively [37]. Cutoff values 30 minutes after ACTH stimulation have been proposed to be 14.9 μg/dL for LC-MS/MS and 16 μg/dL for Elecsys II based on the 2.5th centile in healthy controls [26]. In another study with healthy subjects, a new lower reference limit 30 minutes after ACTH stimulation has been suggested to be ~16.6 with the Access assay [27]. If the historically accepted cutoff of 18 μg/dL after ACTH stimulation were used for the newer, more-specific cortisol assays, approximately twice as many patients in our study would have been given the biochemical diagnosis of AI. This means many more patients may have been unnecessarily treated with glucocorticoids, putting them at risk of adverse complications.

We did not perform other studies of HPA function, such as insulin-induced hypoglycemia or metyrapone stimulation. These studies are cumbersome, difficult to execute in an inpatient setting, and therefore rarely used in clinical practice. Regardless, the cortisol response to insulin-induced hypoglycemia would be lower than 18 μg/dL with specific cortisol assays [13].
As expected, the 60-minute post-ACTH cortisol was higher than the 30-minute cortisol on each of the cortisol assays evaluated [26, 28, 38]. Most laboratories and guidelines do not distinguish between these different time points when recommending a cutoff value to exclude AI and some electronic medical records and laboratory information systems even fail to provide a normal cortisol threshold after ACTH stimulation. Nine of the 110 patients undergoing CST had normal 60-minute cortisol concentrations (when using new cutoff values), but subnormal 30-minute cortisol concentrations in at least one assay. Of note, only one of these 9 biochemically abnormal patients was assigned the clinical diagnosis of AI based on chart review. These data would support the notion that 30-minute cortisol values after high dose CST provide more sensitivity, but less specificity than 60-minute cortisol values [39]. However, it was not certain whether any of these patients had AI, since none had follow-up testing or other testing of HPA axis function. Since some of these patients were hospitalized, it is possible that low levels of cortisol binding proteins may have accounted for some of the impaired cortisol responses [40]. This has implications for clinical practice. In some institutions, it is standard to only obtain a post-ACTH stimulation sample at 30 minutes. This means that approximately 7% (8/110) of patients may have been assigned the biochemical diagnosis of AI when the diagnosis could have been excluded if a 60-minute sample had been obtained. Alternatively, it is possible that these patients may have been misassigned by the 60-minute result and that glucocorticoid support during stress may have been prudent.

As noted in Table 3, 8 of the 110 patients had a cortisol value after CST on one assay method that was discordant with one of the other assay methods. In most of these instances, the cortisol value was very close to the cutoff value. Discordant results may be accounted for in part by normal assay variability (up to 6% to 10% depending on the assay method). As with any endocrine test, no absolute cortisol cutoff for the ACTH stimulation test is perfect for the diagnosis of AI [4, 7, 11, 12]. It also serves as a reminder that re-
cordant results between different assays underscores the im-
portance of clinical judgment by an endocrinologist is still essential to establish the diagnosis of AI.

Varying cutoffs for basal cortisol levels have been sug-
gested to predict AI [11, 17-19, 41]. In this study, there
was a difference in low baseline cortisol values (defined as a cortisol of <8 µg/dL) in patients identified as having a subnormal cortisol response after CST (using the new cortisol cutoff values) vs those who had a normal response. However, there was considerable overlap in patients with cortisol values between 2 and 8 µg/dL. It is likely that basal cortisol values are not a reliable predictor of the cortisol response to ACTH unless they are <2 µg/dL. This is similar to results by Kalaria et al, who found that a cortisol <2.8 µg/dL was required to have 100% sensitivity for failed CST [20]. Age, male vs female, inpatient vs outpatient setting, and baseline ACTH value did not account for patients with low basal cortisol who had normal cortisol responses to ACTH stimulation.

Limitations of this study include lack of an independent endocrine test to determine if a patient had AI. Many of our patients did not have long-term follow-up available to confirm the diagnosis of chronic AI and the need for sustained glucocorticoid support. Patients in this study were from diverse clinical settings (eg, inpatient vs outpatient, relatively healthy vs critically ill, older vs younger). The study also illustrates the limitations of interpreting CST without an assessment of the pre-test probability of AI and the many clinical circumstances that might affect the results such as serum protein levels, concurrent medications that impact HPA axis function, and the proper timely execution of CST itself. Another limitation of this study was our inability to study a cohort of healthy subjects. Despite that, previous studies in healthy subjects using more-specific cortisol assays and baseline ACTH value did not account for patients with low basal cortisol who had normal cortisol responses to ACTH stimulation.

In summary, our study calculated new cortisol cutoff values for highly specific cortisol assays utilizing LC-MS/MS or a monoclonal antibody. To reduce the number of false positive results, we recommend a new cortisol cutoff of 14 to 15 µg/dL depending on the assay used (instead of the generic, historical value of 18 µg/dL derived from polyclonal antibody assays). It is important for clinicians to be aware of the new, assay-specific cutoff for the method available in their institution when evaluating patients for AI. Laboratories should consider providing these values when CST is performed.

Baseline cortisol values <2 µg/dL were consistently associated with stimulated cortisol values below threshold. Finally, discordant results between different assays underscores the importance of clinical judgment from an experienced physician when determining the diagnosis of AI.

Acknowledgments

Financial Support: This work was supported in part by the Advocate Aurora Research Institute.

Additional Information

Correspondence: Bradley R Javorsky, MD, Endocrinology Center and Clinics, Froedtert & the Medical College of Wisconsin, W129
N7055 Northfield Drive, Building A, Suite 203, Menomonee Falls, WI 53051, USA. E-mail: bradley.javorsky@froedtert.com.

**Disclosures:** Bradley Javorsky, consultant for Clarus Therapeutics; research investigator for Novartis Pharmaceuticals. Ty Carroll, consultant for Concept Therapeutics; research investigator for Novartis Pharmaceuticals. Jessica Colón-Franco, no disclosures. Ravinder Singh, no disclosures. Alícia Algèciras-Schimmich, no disclosures. James Findling, consultant for Concept Therapeutics; consultant and investigator for Novartis. Hershel Raff, consultant for Concept Therapeutics; consultant for Cerium Pharmaceuticals.

**Data Availability:** Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

**References**

1. Charmandari E, Nicolaiides NC, Chrousos GP. Adrenal insufficiency. *Lancet*. 2014;383(9935):2152-2167.

2. He X, Findling JW, Auchus RJ. Diagnosis and management of adrenal insufficiency. In: Levine A, ed. *Adrenal Disorders: Physiology, Pathophysiology and Treatment*. Philadelphia: Springer; 2017:199-215.

3. Mazzotti G, Formenti AM, Frara S, et al. MANAGEMENT OF ENDOCRINE DISEASE: Risk of overtreatment in patients with adrenal insufficiency: current and emerging aspects. *Eur J Endocrinol*. 2017;177(5):R231-R248.

4. Dorin RI, Qiao Z, Quals CR, UrbanFK 3rd. Estimation of maximal cortisol secretion rate in healthy humans. *J Clin Endocrinol Metab*. 2012;97(4):1285-1293.

5. Bornstein SR, Alloio B, Arlt W, et al. Diagnosis and treatment of primary adrenal insufficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2016;101(2):364-389.

6. Husseyre ES, Alloio B, Arlt W, et al. Consensus statement on the diagnosis, treatment and follow-up of patients with primary adrenal insufficiency. *J Intern Med*. 2014;275(2):104-115.

7. Oekkers W. Adrenal insufficiency. *N Engl J Med*. 1996;335(16):1206-1212.

8. Kline GA, Buse J, Krause RD. Clinical implications for biochemical diagnostic thresholds of adrenal insufficiency using a highly specific cortisol immunoassay. *Clin Biochem*. 2017;50(9):475-480.

9. Findling JW, Buggy BP, Gilson IH, Brummitt CF, Bernstein BM, Raff H. Longitudinal evaluation of adrenocortical function in patients infected with the human immunodeficiency virus. *J Clin Endocrinol Metab*. 1994;79(4):1091-1096.

10. Kloe M, Lange M, Rasmussen AK, et al. Factors influencing the adrenocorticotropic test: role of contemporary cortisol assays, body composition, and oral contraceptive agents. *J Clin Endocrinol Metab*. 2007;92(4):1326-1333.

11. Grinspoon SK, Biller BM. Clinical review 62: Laboratory assessment of adrenal insufficiency. *J Clin Endocrinol Metab*. 1994;79(4):923-931.

12. May ME, Carey RM. Rapid adrenocorticotropic hormone test in practice. Retrospective review. *Am J Med*. 1983;79(6):679-684.

13. Raverot V, Riche C, Morel Y, Raverot G, Borson-Chazot F. Establishment of revised diagnostic cut-offs for adrenal laboratory investigation using the new Roche Diagnostics Elecsys® Cortisol II assay. *Ann Endocrinol (Paris)*. 2016;77(5):620-622.

14. RRID:AB_2811288. Roche Cat# 11875116. https://scicrunch.org/resolver/AB_2811288

15. Vogeser M, Kratzsch J, Ju Bae Y, et al. Multicenter performance evaluation of a second generation cortisol assay. *Clin Chem Lab Med*. 2017;55(6):826-835.

16. RRID:AB_2802131. Roche Cat# 06687733. https://scicrunch.org/resolver/AB_2802131

17. Schmidt IL, Lahner H, Mann K, Petersen S. Diagnosis of adrenal insufficiency: Evaluation of the corticotropin-releasing hormone test and basal serum cortisol in comparison to the insulin tolerance test in patients with hypothalamic-pituitary-adrenal disease. *J Clin Endocrinol Metab*. 2003;88(9):4193-4198.

18. Hägg E, Asplund K, Lithner F. Value of basal plasma cortisol assays in the assessment of pituitary-adrenal insufficiency. *Clin Endocrinol (Oxf)*. 1987;26(2):221-226.

19. Karaca Z, Tanriverdi F, Atmaca H, et al. Can basal cortisol measurement be an alternative to the insulin tolerance test in the assessment of the hypothalamic-pituitary-adrenal axis before and after pituitary surgery? *Eur J Endocrinol*. 2010;163(3):377-382.

20. Kalaria T, Buch H, Agarwal M, et al. Morning serum cortisol is superior to salivary cortisone and cortisol in predicting normal adrenal function in suspected adrenal insufficiency. *Clin Endocrinol (Oxf)*. Published online December 8, 2020. doi:10.1111/cen.14388

21. RRID:AB_2802133. Beckman Coulter Cat# 33600. https://scicrunch.org/resolver/AB_2802133

22. Hawley JM, Owen LJ, Lockhart SJ, et al. Serum cortisol: an up-to-date assessment of routine assay performance. *Clin Chem*. 2016;62(9):1220-1229.

23. Keevil BG. LC-MS/MS analysis of steroids in the clinical laboratory. *Clin Biochem*. 2016;49:989-997.

24. Monaghan PJ, Keevil BG, Stewart PM, Trainer PJ. Case for the wider adoption of mass spectrometry-based adrenal steroid testing, and beyond. *J Clin Endocrinol Metab*. 2014;99(12):4433-4437.

25. Monaghan PJ, Keevil BG, Trainer PJ. The use of mass spectrometry to improve the diagnosis and the management of the HPA axis. *Rev Endocr Metab Disord*. 2013;14(2):143-157.

26. Ueland GA, Mathie P, Oksnes M, et al. The short cosyntropin stimulation testing in participants with adrenal sufficiency.

27. El-Farhan N, Pickett A, Ducroq D, et al. Method-specific serum cortisol responses to the adrenocorticotrophin test: comparison of gas chromatography-mass spectrometry and five automated immunoassays. *Clin Endocrinol (Oxf)*. 2013;78(5):673-680.

28. Burt MG, Mangelsdorf BL, Rogers A, et al. Free and total cortisol levels via radioimmunoassay vs liquid chromatography mass spectrophotometry in healthy control subjects and patients with adrenal incidentalomas. *Lab Med*. 2018;49(3):259-267.
31. RRID:AB_2783634. Roche Cat# 03255751 190. https://scicrunch.org/resolver/AB_2783634
32. Greene LW, Geer EB, Page-Wilson G, Findling JW, Raff H. Assay-specific spurious ACTH results lead to misdiagnosis, unnecessary testing, and surgical misadventure-a case series. J Endocr Soc. 2019;3(4):763-772.
33. Raff H, Findling JW, Wong J. Short loop adrenocorticotropic (ACTH) feedback after ACTH-(1-24) injection in man is an artifact of the immunoradiometric assay. J Clin Endocrinol Metab. 1989;69(3):678-680.
34. Mayenknecht J, Diederich S, Bähr V, Plöckinger U, Oelkers W. Comparison of low and high dose corticotropin stimulation tests in patients with pituitary disease. J Clin Endocrinol Metab. 1998;83(5):1558-1562.
35. Neary N, Nieman L. Adrenal insufficiency: etiology, diagnosis and treatment. Curr Opin Endocrinol Diabetes Obes. 2010;17(3):217-223.
36. Arlt W. The approach to the adult with newly diagnosed adrenal insufficiency. J Clin Endocrinol Metab. 2009;94(4):1059-1067.
37. Grassi G, Morelli V, Ceriotti F, et al. Minding the gap between cortisol levels measured with second-generation assays and current diagnostic thresholds for the diagnosis of adrenal insufficiency: a single-center experience. Hormones (Athens). 2020;19(3):425-431.
38. Beuschlein F, Fassnacht M, Assié G, et al. Constitutive activation of PKA catalytic subunit in adrenal Cushing’s syndrome. N Engl J Med. 2014;370(11):1019-1028.
39. Munro V, Elnenaei M, Doucette S, Clarke DB, Imran SA. The effect of time of day testing and utility of 30 and 60 minute cortisol values in the 250 mcg ACTH stimulation test. Clin Biochem. 2018;54:37-41.
40. Marik PE, Pastores SM, Annane D, et al.; American College of Critical Care Medicine. Recommendations for the diagnosis and management of corticosteroid insufficiency in critically ill adult patients: consensus statements from an international task force by the American College of Critical Care Medicine. Crit Care Med. 2008;36(6):1937-1949.
41. Manosroi W, Phimphilai M, Khorana J, Arthakomol P. Diagnostic performance of basal cortisol level at 0900-1300h in adrenal insufficiency. PLoS One. 2019;14(11):e0225255.