DOI: 10.4274/jcrpe.galenos.2026.2026-1-17

Original Article

The 40th Minute Cortisol Measurement is the Key Time-Point in the Low-Dose Synacthen Stimulation Test: A Large, Assay-Specific Pediatric Validation Study

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What is already known on this topic?
The LDSST, which is widely used for suspected CAI, has adopted lower peak and basal cortisol cut-offs in mAb immunoassays.

What this study adds?
A large-scale real-world external validation of the previously defined LDSST sampling strategies is provided. The clinical utility of cortisol measurement at the 40th minute and a basal cortisol threshold of 6.5 µg/dL was validated for routine pediatric practice despite real-world variability.

Abstract
Background: Low-dose synacthen stimulation test (LDSST) is widely used to assess central adrenal insufficiency (CAI). With the adoption of monoclonal antibody (mAb) cortisol immunoassays, lower basal and peak cortisol concentration thresholds require external validation under real-world clinical conditions.
Objective: To externally validate previously defined LDSST sampling strategies, basal cortisol thresholds, and gray-zone cut-offs in a large real-world cohort using mAb immunoassays.
Methods: This single-center retrospective study analyzed 646 LDSSTs in patients with suspected CAI, measuring baseline, 40th, and 60th minute cortisol levels after administration of 1 µg of synthetic ACTH. The diagnostic performance of single and combined sampling strategies and previously defined basal cortisol thresholds and grey-zone cut-offs were evaluated across different peak cortisol criteria.
Results: Cortisol measurement at 40th minute provided the most reliable single time-point assessment, with significantly fewer false-negative results than at 60th minute ($p<0.0001$). At the basal cortisol threshold of ≥6.5 µg/dL identified in our previous prospective study, sensitivity decreased from 68% to 57.5% ($p=0.21$) and specificity remained comparable (73.8% vs. 75.1%, $p=0.74$), while negative predictive value declined significantly from 91% to 78.4% ($p=0.02$) and positive predictive value increased to 53% in this retrospective cohort. Validation of basal cortisol gray-zone thresholds confirmed high diagnostic accuracy across different peak cortisol cut-offs.
Conclusions: This study provides robust real-world external validation of LDSST sampling strategies and basal cortisol thresholds. Cortisol measurement at 40th minutes, combined with assay-specific basal cortisol interpretation and a gray-zone framework, offers a practical approach for individualized clinical decision-making in suspected CAI.
Keywords: Adrenocorticotropic hormone, cortisol, children, low-dose synacthen stimulation test, monoclonal antibody immunoassay, central adrenal insufficiency, external validation

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31.01.2026
03.04.2026

Epub: 27.04.2026

Introduction

The low-dose Synacthen stimulation test (LDSST) is considered a safe and widely used diagnostic tool for evaluating patients with suspected central adrenal insufficiency (CAI) (1-3). Compared to the standard-dose Synacthen stimulation test, LDSST has been shown to provide greater sensitivity, as supraphysiological stimulation in standard-dose testing may result in false-negative responses (2-7). The interpretation of LDSST results has evolved with advances in cortisol assay methodologies. The introduction of newer monoclonal antibody (mAb) immunoassays, which offer higher analytical specificity, has led to the adoption of lower peak cortisol cut-off values, most commonly 18 µg/dL (500 nmol/L), with subsequent studies suggesting even lower cut-offs, ranging from 12.6 to 15.7 µg/dL (8-16).
Morning basal serum cortisol concentrations are commonly used as an initial screening tool for the evaluation of adrenal function (17-24). Very low basal cortisol levels are suggestive of adrenal insufficiency, whereas higher concentrations may indicate preserved hypothalamo–pituitary–adrenal (HPA) axis function (4, 11, 25-29). However, a substantial proportion of patients fall into an intermediate range, in which basal cortisol values alone are insufficient for a definitive diagnosis. In everyday clinical practice, particularly in pediatric care, logistical constraints, patient cooperation, and outpatient scheduling often preclude consistent sampling during the early morning hours, thereby limiting the practical utility of basal cortisol measurement. External validation in heterogeneous clinical populations is essential to confirm the generalizability of diagnostic strategies derived from controlled prospective settings (30, 31). Moreover, data on predictive basal cortisol thresholds in children, especially those derived from mAb immunoassays, remain limited.
In our previously published prospective study using mAb cortisol immunoassays, optimal LDSST performance was achieved with cortisol sampling at the 40th minute, alone or in combination with that at the 60th minute. Morning basal serum cortisol concentrations were identified as predictors of LDSST outcomes, particularly in clinical settings where frequent post-stimulation sampling is technically challenging (32). Although these findings were derived under controlled and prospectively defined conditions, their applicability in routine clinical practice and heterogeneous patient populations has not been fully explored.
Accordingly, the present study retrospectively evaluated a large cohort of pediatric patients who underwent LDSST for suspected CAI to externally validate previously defined optimal sampling strategies, basal cortisol cut-offs, and gray-zone thresholds across different peak cortisol criteria using mAb immunoassays. We aimed to evaluate whether the optimal sampling strategy and basal cortisol thresholds

identified in our previous prospective study remained robust and clinically applicable under real-world conditions in a large retrospective pediatric cohort.

Patients and Methods

This single-center retrospective study evaluated all LDSSTs performed in our pediatric endocrinology clinic among patients aged 0–18 years with suspected CAI between November 2016 and February 2022. A total of 646 LDSSTs performed in 537 children (241 females, 44.9%) at different time points were retrospectively analyzed. The indications for LDSST included suspected CAI due to a sellar mass (*n*=28), history of pituitary or cranial surgery or radiotherapy (*n*=121), long-term or high-dose glucocorticoid use (*n*=102), and the presence of other pituitary hormone deficiencies *(n*=286). Owing to the retrospective design and longitudinal follow-up of patients, some individuals underwent repeat LDSSTs. In our cohort, repeat testing was primarily performed in patients with ongoing glucocorticoid exposure, evolving pituitary pathology, or when initial results were borderline or clinically incongruent.

LDSSTs were performed by pediatric endocrine nurses following standard pre-test instructions, including overnight fasting and temporary (24 − 48 h) withholding of steroid therapy. Basal ACTH and cortisol samples were collected between 08:00 and 11:00 a.m. after an intravenous (IV) line was inserted. Plasma cortisol levels were measured 40 and 60 min after an IV bolus of 1 µg tetracosactide (Synacthen®). The samples were analyzed using mAb immunoassays. During the study period, assays from different immunoassay platforms were used, including the Roche® Elecsys Cortisol II assay and Beckman Coulter Access Cortisol assay (run on UniCel DxI analyzers).

A positive LDSST, suggestive of adrenal insufficiency (AI), was defined as a stimulated peak serum cortisol concentration of <18 µg/dL (500 nmol/L). Previously published optimal testing conditions derived from a prospective cohort were applied to a large real-world dataset to assess their consistency and reliability (32). Diagnostic classifications based on a single time-point (0, 40, and 0, 60 min) and combined time-point (0, 40, and 60 min) sampling strategies were compared. In addition, basal cortisol thresholds previously identified in our prospective study to predict peak cortisol cut-off values defined in the literature for mAb immunoassays were assessed for their performance in the current cohort. Specifically, basal cortisol thresholds of 6.5 µg/dL for a peak cortisol cut-off of 18 µg/dL (500 nmol/L), 6.4 µg/dL for a peak cut-off of 15.7 µg/dL (433 nmol/L), and 6.2 µg/dL for a peak cut-off of <12.6 µg/dL (350 nmol/L) were evaluated (8-10, 13). Basal cortisol gray-zone cut-offs previously identified in our prospective study were externally validated in the current cohort. Diagnostic performance metrics, including sensitivity, specificity, positive predictive value, and negative predictive value, were calculated.

Studies were performed with the approval of the Ethics Committee of the Marmara University Faculty of Medicine, Istanbul, Turkey (09.2022.387).

Statistical analysis

Statistical analyses were performed using GraphPad Prism® version 10 (GraphPad Software Inc., San Diego, California, USA). Statistical significance was defined as $p<0.05$. Continuous variables are presented as the mean±SD for normally distributed data. Categorical variables are expressed as counts (percentages). Pairwise comparisons between continuous variables were performed using the Student's t-test, and categorical variables were compared using the chi-square test. Paired categorical outcomes obtained from different sampling time points during the LDSST were compared using McNemar's test.

Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic performance of cortisol levels at different time points during the LDSST. Previously defined basal morning cortisol cut-off values were applied, and their diagnostic performance was assessed by calculating sensitivity, specificity, and the area under the curve (AUC). Positive predictive value (PPV) and negative predictive value (NPV) were calculated for the identified thresholds. At the basal cortisol threshold of 6.5 µg/dL, sensitivity, specificity, PPV, and NPV were calculated for the prospective and retrospective validation cohorts. Between-cohort differences in each metric were compared using tests for equality of proportions (chi-square or Fisher's exact test, as appropriate), with analyses restricted to the relevant denominator for each metric. All tests were two-sided, and a p value <0.05 was considered statistically significant. The association between basal serum cortisol and peak cortisol concentrations during the LDSST was examined using linear regression analysis for descriptive purposes.

Results

The mean age of the patients at the time of testing was 9.2±5.8 years (range: 0.0 – 21.1). Of the 646 LDSSTs performed, 212 (32.8%) failed. Basal and peak serum cortisol levels were significantly different in patients who failed and passed the LDSST ($p<0.0001$). Age at the time of testing, basal cortisol, ACTH, and peak cortisol levels according to the test outcome (passed or failed) are presented in **Table 1**.

Among the patients who passed the LDSST (*n*=434), basal cortisol concentrations ≥18 µg/dL (500 nmol/L) were observed in only 29 patients (6.7%). The majority of the patients (88.6%) had cortisol concentrations ≥18 µg/dL (500 nmol/L) for the first time at the 40th minute, and 4.7% at the 60th minute. Cortisol levels at the 0th, 40th, and 60th minutes are illustrated in **Figure 1.a**. The association between basal cortisol and peak cortisol levels were shown in **Figure 1.b.** Regardless of the LDSST outcome, peak cortisol concentrations occured at the 40th minute in 50.5% of patients (*n*=326) and at the 60th minute in 49.5% (*n*=318). Comparison of the two groups according to age, sex, LDSST outcomes, and clinical indications for testing showed that children who reached peak cortisol at the 60th minute were significantly younger than those peaking at the 40th minute (mean age 6.7±5.5 vs 11.7±4.9 years, $p<0.0001$). Sex distribution and overall test outcomes were similar between groups. However, the distribution of clinical indications differed significantly ($p=0.023$), with long-term/high-dose glucocorticoid exposure being more common among patients reaching peak cortisol at the 60th minute than among those peaking at the 40th minute (30.2% vs 21.5%, $p=0.012$).

In the model of a single time-point cortisol measurement after tetracosactide stimulation, 64.1% (*n*=414) of the tests had an adequate response at the 40th minute and 55.9% (*n*=361) at the 60th minute. When efficiency was calculated based on the overall LDSST results, the most specific time to test for a single cortisol measurement after stimulation was the 40th minute (specificity, 95.6%; AUC:0.989) **(Figure 2.a)**. Cortisol assessment at the 40th minute resulted in significantly fewer false-negative classifications than that at the 60th minute ($\chi^2 = 31.5$, $p <0.0001$). If single sampling had been performed 40 minutes after stimulation, 20 patients (4.6%) with adequate response at the 60th minute would have been misdiagnosed with AI. In addition, comparison between the 0, 40 minute and 0, 40 and 60 minute sampling strategies using McNemar's test demonstrated a significant improvement in diagnostic classification with the inclusion of the 60 minute measurement ($\chi^2 = 18.05$, $p< 0.0001$) **(Figure 2.b)**.

When a basal cortisol threshold of 6.5 µg/dL was applied, 416 children (64.4%) had basal cortisol levels ≥6.5 µg/dL, of whom 326 (78.4%) passed the LDSST, while 90 (21.6%) had a failed test. Among the 230 children with basal cortisol <6.5 µg/dL, 122 (53.1%) failed the LDSST and 108 (46.9%) passed. Using basal cortisol ≥6.5 µg/dL to predict a normal LDSST outcome yielded a sensitivity of 75.1%, specificity of 57.5%, and a negative predictive value of 78.4% for excluding AI. At a basal cortisol threshold of 6.5 µg/dL, diagnostic performance differed between the initial prospective study and the current retrospective validation cohort. The sensitivity decreased from 68% in the prospective cohort to 57.5% in the retrospective validation cohort; however, this difference was not statistically significant ($p=0.21$). Specificity remained comparable between the two cohorts (73.8% vs. 75.1%, $p=0.74$). The positive predictive value increased from 39.1% to 53%, whereas the negative predictive value decreased significantly from 91% to 78.4% ($p=0.02$). Disease prevalence was higher in the retrospective validation cohort than in the prospective cohort (32.8% vs. 19.7%), which was reflected in the observed changes in predictive values. The diagnostic performance of predefined basal cortisol thresholds according to the different peak cortisol cut-offs were shown in **Table 2**.

Validation analyses were performed for the basal cortisol grey zone thresholds previously identified in our prospective study, in which basal cortisol values <2.5 µg/dL demonstrated 100% sensitivity and values >14.6 µg/dL demonstrated 100% specificity for passing the LDSST

using a peak cortisol cut-off of 18 µg/dL (32). Basal cortisol values <2.5 µg/dL were observed in 58 tests, of which 45 failed and 13 passed the LDSST. Basal cortisol values >14.6 µg/dL were observed in 72 tests, of which 68 passed and four failed. The remaining 515 tests had basal cortisol values within the grey zone (2.5 – 14.6 µg/dL), including 352 passed and 163 failed tests. For basal cortisol <2.5 µg/dL, the sensitivity was 97.2% with a positive predictive value of 77.6%. For basal cortisol >14.6 µg/dL, the specificity for excluding adrenal insufficiency was 98.1%, with a negative predictive value of 94.4%. Validation of basal cortisol gray-zone cut-offs according to different peak cortisol cut-offs during the LDSST were shown in **Table 3.**

Discussion:

In this large real-world cohort of pediatric patients evaluated for suspected CAI, we externally validated key findings from our previously published prospective study and demonstrated their applicability in routine clinical practice, in line with recommendations emphasizing the importance of external validation before the clinical implementation of diagnostic tests (30, 33, 34). Our results confirm that cortisol measurement at 40 minutes after LDSST provides the most reliable single time-point assessment, with superior diagnostic performance compared with the 60th minute and significantly fewer false-negative classifications.

The basal cortisol thresholds derived from the prospective cohort also showed consistent diagnostic behavior in the retrospective validation analysis. When the same basal cortisol threshold (≥6.5 µg/dL) was applied, sensitivity was largely preserved, whereas specificity and NPV were reduced, consistent with application of the same threshold under real-world conditions with higher disease prevalence and greater clinical heterogeneity (34-36). Lowering the peak cortisol cut-off was associated with a marked improvement in the NPV of basal cortisol, whereas sensitivity remained relatively stable, consistent with Bayesian principles and the known dependence of predictive values on disease prevalence and spectral effects (36-38). These findings support the utility of basal cortisol as an effective rule-out tool for CAI and reinforce its role in clinical decision-making, particularly in settings where dynamic testing may be delayed, not readily available, or difficult to perform in children due to practical or clinical constraints (17-25, 32, 39, 40).

An additional observation from the subgroup analysis was that patients reaching peak cortisol concentrations at the 60th minute were significantly younger than those reaching peak levels at the 40th minute, regardless of the LDSST outcome. This finding may reflect developmental differences in HPA responsiveness in younger children. Consistent with previous studies showing that younger children more frequently reach peak cortisol at later time points during ACTH stimulation, this pattern may be related to the relative immaturity of the HPA axis, leading to a delayed adrenal response (41-43). Despite this variability in the timing of the maximal cortisol response, the 40th minute remained the most informative single sampling time point in our cohort. However, delayed peak responses may be particularly relevant when interpreting LDSST results in very young children, especially preterm infants, neonates, or those younger than three years of age.

Interestingly, long-term/high-dose glucocorticoid use was also more frequent among patients reaching peak cortisol at the 60th minute. Chronic glucocorticoid exposure may lead to partial suppression of the HPA axis, potentially resulting in a delayed adrenal response to ACTH stimulation.

Overall, this external validation demonstrates that, although the intrinsic diagnostic characteristics of the test are preserved, its rule-out performance reflects the realities of routine clinical practice rather than controlled research conditions (30, 33, 44). Importantly, the underlying diagnostic distribution of suspected CAI was similar between the prospective and retrospective cohorts. Although the age ranges were similar, the retrospective validation cohort had a significantly higher mean age, which may reflect routine pediatric endocrine practice and support the applicability of the proposed diagnostic strategy across a broader pediatric age spectrum. Additionally, variability in test timing, pre-test steroid withdrawal, and execution under everyday care represents the real-world context in which the test is applied, underscoring the value of validating diagnostic thresholds under pragmatic conditions (35, 36, 45).

Our study had limitations including that, this was a single-center study, which may have limited its generalizability. Secondly, although all cortisol measurements were performed using monoclonal antibody–based immunoassays, two different assay platforms (Roche Elecsys Cortisol II and Beckman Coulter Access Cortisol) were used during the study period due to changes in laboratory contracts, which may have introduced some analytical variability. Finally, because of the retrospective design and clinical follow-up, some patients underwent repeated LDSSTs at different time points. However, this reflects routine clinical practice and did not materially alter the overall diagnostic performance of the proposed testing strategy.

The strengths of this study include its large sample size, inclusion of repeated tests reflecting everyday clinical workflows, and consistent use of monoclonal antibody–based immunoassays. In a real-world validation study, pre-analytical and analytical variabilities can not be fully standardized; however, this reflects routine clinical practice and aligns with the primary aim of evaluating test performance under pragmatic conditions.

In conclusion, this study demonstrated the reliability of our initial diagnostic approach by validating its key findings in a large, real-world, retrospective pediatric cohort. In this context, the proposed sampling strategy and cortisol threshold values provide clinicians with a robust framework for interpreting test results in daily practice. By using the optimal conditions defined in the prospective study as a reference, test results can be interpreted on an individual patient basis, allowing informed clinical decision-making, even under the variable conditions of routine care.

Author Declaration

The authors affirm that the manuscript is original, has not been previously published, and is not currently under consideration for publication elsewhere. Each author has contributed significantly to the conception, design, execution, and interpretation of the study and has approved the final version of the manuscript. The authors also agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors meet the ICMJE criteria for authorship.

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Figure Legends

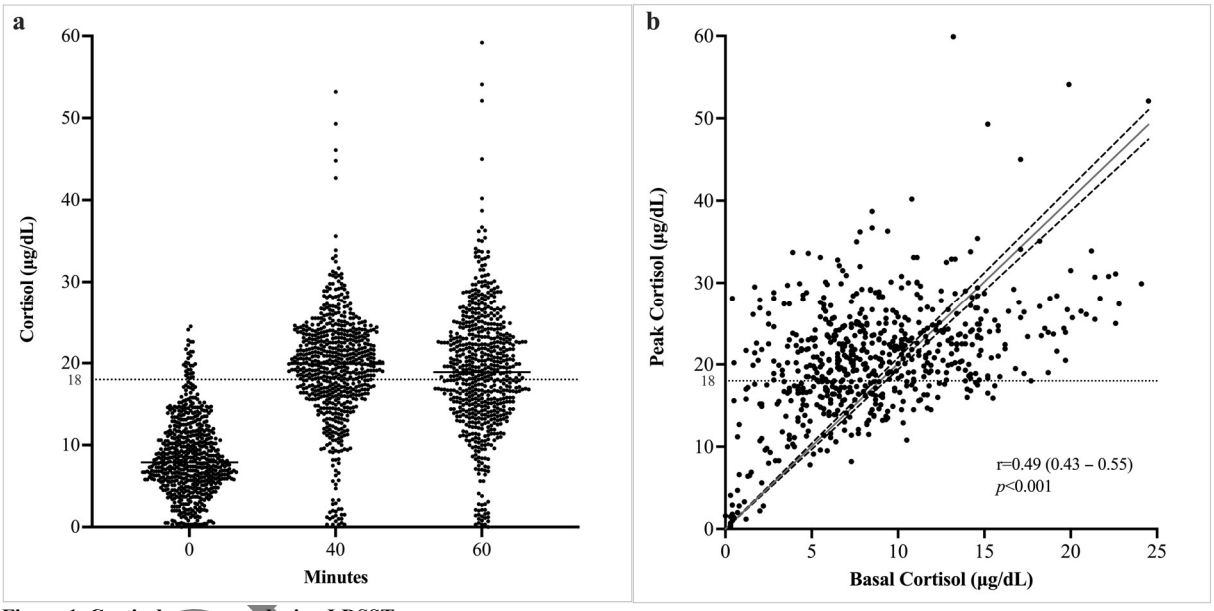

Figure 1. Cortisol responses during LDSST.
(a) Serum cortisol levels at baseline, 40, and 60 minutes after tetracosactide administration.
(b) Association between basal cortisol levels and peak cortisol responses during the LDSST.

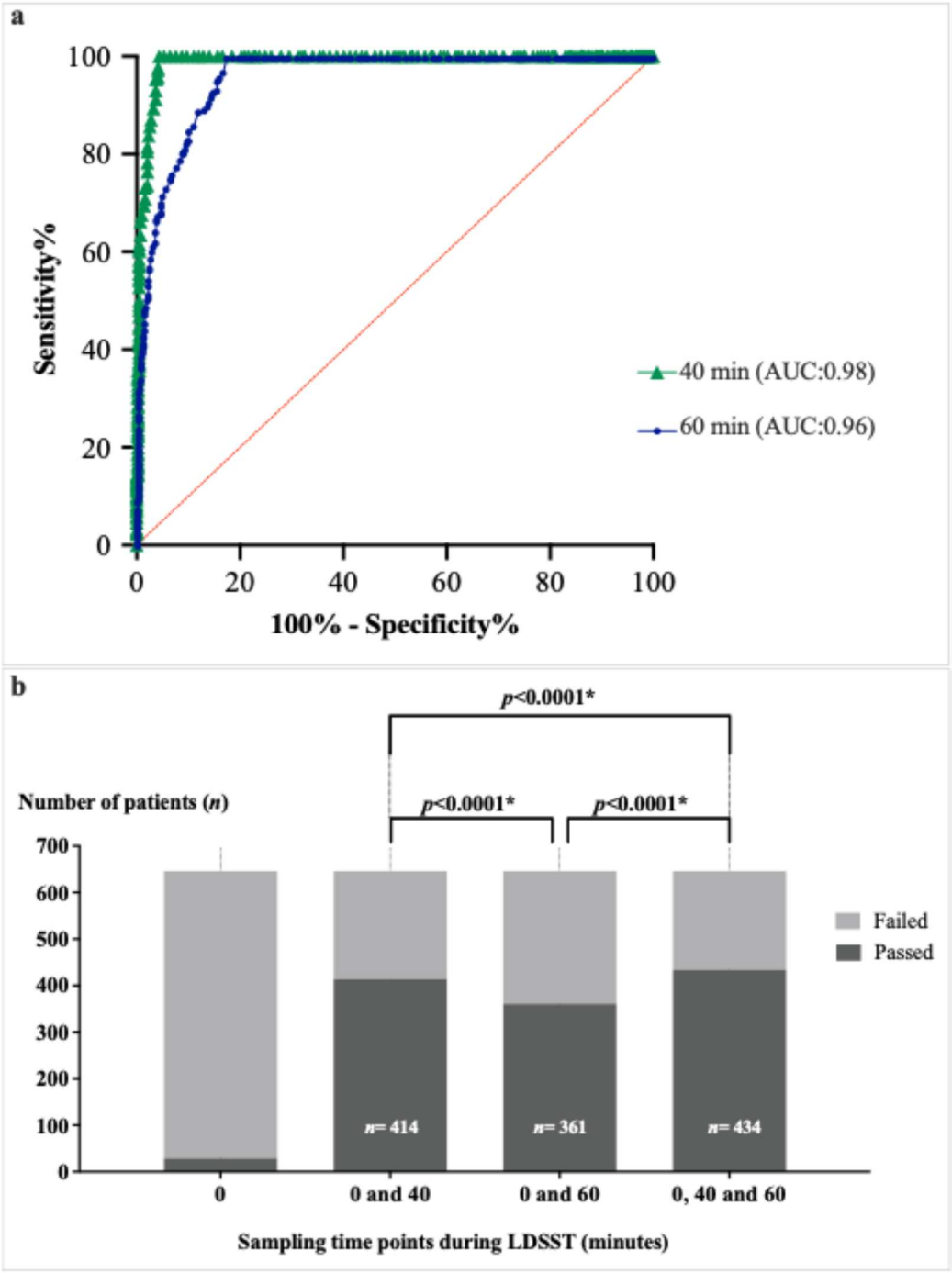

Figure 2. Diagnostic performance of cortisol measurements at different sampling time points during the LDSST.
(a) Models of a single time point cortisol measurement after tetracosactide stimulation.
(b) Stacked bar graph showing patients who passed the LDSST at different sampling time points, with a paired comparison of diagnostic classifications performed using McNemar's test.
*Adrenal insufficiency was defined as a stimulated cortisol level <18 µg/dL (500 nmol/L).
*Failed: Patients whose serum cortisol did not exceed 18 µg/dL (500 nmol/L) during LDSST were labelled as "failed".
*Passed: Patients whose serum cortisol was ≥18 at any time point were labelled as "passed."
*Cortisol (µg/dL) =0.036 nmol/L.

Table 1. Morning basal ACTH and cortisol concentrations of the patients.							
	LDSST Passed (n=434)*			LDSST Failed (n=212)*			*p*
	mean	**median**	**range**	**mean**	**median**	**range**	
Age (years)	8.7±6.0	9.2	0.0 – 20.0	10.4±5.2	11.6	0.0 – 21.1	**0.0003**
Plasma ACTH (pg/mL)	28.0±22.7	21.0	3.0 – 156	28.6±23.3	22.8	4.0 – 134	0.76
Basal Serum Cortisol (mAb) (µg/dL)	9.9±4.7	9.1	0.4 – 24.5	6.0±3.6	5.9	0.0 – 15.6	**<0.0001**
Peak Serum Cortisol (mAb) in LDSST(µg/dL)	24.1±5.1	23.1	18 – 59.9	13.2±4.9	15.1	0.3 – 17.9	**<0.0001**
*Adrenal insufficiency was defined as a stimulated cortisol level <18.0 µg/dL (500 nmol/L). Patients whose serum cortisol did not exceed 18 µg/dL (500 nmol/L) during LDSST were labelled as "failed" and those whose serum cortisol was ≥18 at any time point were labelled as "passed". *Cortisol µg/dL =0.036×nmol/L *LDSST, low dose Synacthen stimulation test							

Table 2. Diagnostic performance of basal cortisol thresholds according to different peak cortisol cut-off values during the LDSST						
Adrenal Insufficiency (peak cortisol concentration)	**Basal Cortisol Threshold**	**Failed tests** *n* (%)	**Sensitivity** (%)	**Specificity** (%)	**NPV** (%)	**PPV** (%)
<18.0 µg/dL (500 nmol/L)	6.5 µg/dL (179 nmol/L)	212 (32.8)	75.1	57.5	78.4	53.0
<15.7 µg/dL (433 nmol/L)	6.4 µg/dL (177 nmol/L)	123 (19.0)	73.0	68.3	90.7	37.3
<12.6 µg/dL (350 nmol/L)	6.2 µg/dL (171 nmol/L)	65 (10.1)	73.5	86.2	97.9	26.7
Basal cortisol thresholds were predefined based on previously published prospective data and existing literature derived from monoclonal antibody immunoassays. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated without derivation of new cut-off values.						

Table 3. Validation of basal cortisol grey-zone cut-offs according to different peak cortisol cut-offs during the LDSST						
Adrenal Insufficiency (peak cortisol concentration under)	Lower basal cortisol cut-off	Specificity (%)	PPV (%)	Upper basal cortisol cut-off	Specificity (%)	NPV (%)
<18.0 µg/dL (500 nmol/L)	2.5 µg/dL (69 nmol/L)	97.2	77.6	14.6 µg/dL (403 nmol/L)	98.1	94.4
<15.7 µg/dL (433 nmol/L)	2.7 µg/dL (75 nmol/L)	96.5	67.2	7.4 µg/dL (204 nmol/L)	76.4	91.6
<12.6 µg/dL (350 nmol/L)	2.4 µg/dL (66 nmol/L)	96.4	61.8	7.4 µg/dL (204 nmol/L)	93.8	98.8
**Grey zone* represents basal cortisol values between the lower and upper thresholds. *All thresholds were predefined based on prior prospective data and literature derived from monoclonal immunoassays. *Diagnostic performance metrics were calculated without derivation of new cut-off values. *PPV, positive predictive value (probability of adrenal insufficiency given a positive test result) *NPV, negative predictive value (probability of a normal result given a negative test result) *LDSST, low dose Synacthen stimulation test						