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Clinical Research Article

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Human Corticotropin-Releasing Hormone Tests: 10 Years of Real-Life Experience in Pituitary and Adrenal Disease

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Abbreviations: ACTH, adrenocorticotropin; AI, adrenal insufficiency; AUC, area under the curve; BIPSS, bilateral inferior petrosal sinus sampling; BMAH, bilateral macronodular adrenal hyperplasia; CRH, corticotropin-releasing hormone; CD, Cushing disease; CS, Cushing syndrome; CV, coefficient of variation; DST, dexamethasone suppression test; EAS, ectopic adrenocorticotropin secretion; $hCRH^{test}$, human corticotropin-releasing hormone test; HPA, hypothalamic-pituitary-adrenal; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LNSC, late-night salivary cortisol; LR, likelihood ratio; MACS, mild autonomous cortisol secretion; PPNAD, primary pigmented nodular adrenocortical disease; SE, sensitivity, SP, specificity; UFC, urinary free cortisol; ULN, upper limit of normality.

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Abstract

Context: The human corticotropin-releasing hormone (CRH) test ($hCRH^{test}$) is used to differentiate Cushing disease (CD) from ectopic adrenocorticotropin (ACTH) secretion (EAS), to assess autonomous cortisol secretion by the adrenal glands, and to characterize pseudo-Cushing syndrome (CS) or adrenal insufficiency (AI).

Main Outcome Measure: The main outcome measure of this study was to assess the diagnostic accuracy of the $hCRH^{test}$.

Methods: We measured ACTH and cortisol levels; collected the peak values ($peak^{ACTH}$ and $peak^{cortisol}$), and calculated the percentage increases ($\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$) after an intravenous bolus of 100 μg hCRH.

Design and Setting: This cross-sectional study of hCRH tests from 2010 to 2019 took place in a referral university hospital center.

Patients: We enrolled 200 patients: 86 CD, 15 EAS, 18 adrenal CS, 25 mild adrenal autonomous cortisol secretion, 31 pseudo-CS, and 25 suspected AI.

Results: The $hCRH^{test}$ was performed mainly for the differential diagnosis of ACTH-dependent CS or adrenal lesions ($P = .048$). $Peak^{ACTH}$ and $peak^{cortisol}$ were higher in CD, and $\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$ were able to differentiate CD from EAS with a sensitivity and specificity greater than 80%. In patients with low (< 10 pg/mL) or indeterminate (10-20 pg/mL) $basal^{ACTH}$ levels, an absent or reduced $peak^{ACTH}$ response was able to differentiate adrenal from ACTH-dependent forms. $Peak^{ACTH}$ and $peak^{cortisol}$ after $hCRH^{test}$ were lower in pseudo-CS than in CD, but $\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$ were similar. The role of $hCRH^{test}$ in patients with AI was limited.

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Conclusions: The $hCRH^{test}$ test is the mainstay of the differential diagnosis of ACTH-dependent CS. It is also useful for pointing to a diagnosis of CD in the event of bilateral adrenal masses, and in patients with low $basal^{ACTH}$.

Key Words: Cushing syndrome, Cushing disease, ACTH, ectopic ACTH secretion, adrenal adenoma

The hypothalamic-pituitary-adrenal (HPA) axis is strictly controlled. Corticotropin (adrenocorticotropin, ACTH) is synthesized and secreted in response to corticotropin-releasing hormone (CRH) and vasopressin, thereby enabling adrenal cortisol synthesis and release. The HPA axis amplifies small neural signals into major changes in circulating steroid levels (1). The human CRH test ($hCRH^{test}$) is commonly used in clinical practice to assess the integrity of the HPA axis both at the pituitary and hypothalamic levels (2-4).

ACTH-dependent Cushing syndrome (CS) accounts for 80% to 85% of endogenous hypercortisolism (4). It is due to an ACTH-secreting pituitary adenoma (Cushing disease, CD) or to ectopic ACTH secretion (EAS) (5). In clinical practice, bilateral inferior petrosal sinus sampling (BIPSS) is the gold standard for identifying the source of ACTH secretion (6), and a pituitary lesion larger than 6 mm is considered sufficient to diagnose CD (4). BIPSS is time-consuming, however, requires a dedicated team, and is not without a risk of adverse events (6). Magnetic resonance imaging is negative or inconclusive in up to 30% of cases (7). Dynamic tests are widely used to differentiate CD from EAS (2), and the $hCRH^{test}$ reaches a high diagnostic accuracy (8), especially when the results are consistent with those of a high-dose dexamethasone suppression test (DST) (9).

Autonomous cortisol secretion by adrenal lesions leads to CRH and ACTH suppression (4). Measuring ACTH requires careful preanalytical sampling and analytical procedures, however. Commercially available ACTH immunoassays are imprecise in the low range, and often unable to identify patients with suppressed or reduced ACTH levels (< 10 pg/mL or 10-20 pg/mL, respectively) (10). In such cases, an $hCRH^{test}$ might unmask the ACTH responsiveness of ACTH-dependent CS (11, 12). In bilateral macronodular adrenal hyperplasia (BMAH), autocrine and paracrine ACTH in steroidogenic cells point to novel regulatory mechanisms contributing to cortisol secretion in case of bilateral adrenal CS (11).

Hypercortisolism is not always sustained by a neoplastic ACTH or cortisol secretion (13). Functional hypercortisolism, called *pseudo-CS*, may also coincide with HPA axis activation. It can be seen in chronic alcoholism, psychiatric conditions, severe illness, anorexia nervosa, pregnancy, and some common endocrine diseases (metabolic syndrome or polycystic ovary syndrome) (13, 14). The differential diagnosis between pseudo-CS and endogenous CS poses a significant clinical challenge. The $hCRH^{test}$, alone (15) or after DST (16), has been proposed as a second-line approach to characterize pseudo-CS.

In patients with central adrenal insufficiency (AI), the role of the $hCRH^{test}$ is still a matter of debate (17, 18). It could be used to distinguish hypothalamic from pituitary disease (19), but its sensitivity is lower than that of the insulin tolerance test (20).

Moving from these considerations, we report here on our experience of the $hCRH^{test}$ at our endocrine clinic for suspected diseases of the HPA axis.

Materials and Methods

Patient selection

Using a dedicated query in the web-based database of Padova University Hospital, we collected all consecutive $hCRH^{tests}$ performed from January 2010 to December 2019 (n = 270). We selected only those tests performed at the outpatient clinic of our endocrine unit by trained and dedicated endocrine nurses (n = 243).

Then the following exclusion criteria were considered:

- insufficient data for a conclusive diagnosis (n = 3) or patients not followed up at our unit (n = 14);
- repeat tests (n = 16), in which case we considered only the first, performed for diagnostic purposes;
- concomitant or recent (within the last 2 months) use of substitutive or synthetic glucocorticoids, estroprogestinic or antiepileptic drugs (n = 10).

This left 200 tests available for the present study.

CS was diagnosed according to the Endocrine Society's guidelines (3), based on at least 2 positive first-line screening tests: increased urinary free cortisol (UFC, mean of 3 collections), impaired late-night salivary cortisol (LNSC, mean of 2 collections), and/or unsuppressed serum cortisol after overnight 1-mg DST (cutoff 50 nmol/L). ACTH-dependent CS was suspected on findings of normal/elevated morning plasma ACTH levels (> 10 pg/mL). CD was confirmed by at least one of the following: BIPSS findings (n = 28), histological examination consistent with corticotroph adenoma (n = 62), and/or biochemical remission 6 months after pituitary surgery (n = 62). EAS was confirmed with

BIPSS or histology (neuroendocrine tumor with positive ACTH immunostaining in surgical specimens) in all cases treated surgically. Adrenal CS was suspected in patients with suppressed (< 10 pg/mL) or indeterminate (10-20 pg/mL) basal ACTH levels (2). We considered monolateral or bilateral adrenal disease based on abdominal imaging (computed tomography in all cases). Primary pigmented nodular adrenocortical disease (PPNAD) was confirmed by *PRKAR1A* gene mutation (2, 3, 21). In accordance with recent guidelines, mild autonomous cortisol secretion (MACS) was diagnosed in patients with adrenal adenomas if their serum cortisol was greater than 138 nmol/L after 1-mg DST, or 50 to 138 nmol/L with positive findings for another HPA axis anomaly (increased UFC, suppressed ACTH, impaired LNSC) (22).

Pseudo-CS was diagnosed in those patients presenting with some clinical signs of temporary or more persistent hypercortisolism, characterized by at least one first-line screening test result. After a meticulous and prolonged follow-up (for at least 6 months), overt CS was ruled out in all cases.

Central AI was suspected if basal morning serum cortisol was less than 83 nmol/L, or if the peak ACTH after the low-dose short synacthen test (1 µg) was less than 500 nmol/L (17) in high-risk patients.

In accordance with STARD (standards for reporting diagnostic accuracy studies) criteria, we considered the final diagnosis (based on the above-mentioned criteria) as the reference standard. Patients were then grouped as ACTH-dependent CS (n = 101, CD = 86 and EAS = 15); adrenal lesions (n = 43, CS = 18 and MACS = 25); pseudo-CS (n = 31); or suspected AI (n = 25). This observational study was conducted in accordance with the STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) guidelines (23).

All patients gave their informed consent. The study complied with the principles of the Declaration of Helsinki. The ethics committee of Padova University Hospital (Comitato Etico per la Sperimentazione Scientifica) approved the study (protocol No. 32944-2020). The clinical data were obtained from the web-based database of Padova University Hospital in the form of electronic case reports or records.

Endocrine assessment

First-line screening tests were performed with commercially available immunoassays or liquid chromatography–tandem mass spectrometry (LC-MS/MS). Briefly, to ensure proper collection, all protocols were described in a written step-by-step form. For UFC, patients were instructed to discard the first morning urine void and collect all urine for the next 24 hours, so that the first morning urine void on the second day was the final collection. Urinary volume was carefully recorded, then a sample was collected and refrigerated until UFC was measured using LC-MS/MS (intra-assay/interassay coefficient of variation [CV] < 6%/< 8%) (24). Salivary cortisol was collected before sleeping (to measure LNSC). Patients were advised to soak the absorbent cotton of a Salivette device (Sarstedt) for 2 to 3 minutes, then keep it refrigerated. To avoid contamination, saliva samples were collected at least 2 hours after taking a meal/drink. Patients were allowed to brush their teeth only after saliva collection. Smoking or eating licorice was forbidden. Salivary cortisol was measured with a radioimmunometric assay (Radim, intra-assay/interassay CV < 3%/< 9% [25]) until 2014, then with an LC-MS/MS method (intra-assay/interassay CV < 6%/< 8% [26]). The 1-mg DST was performed as recommended (3): 1 mg of dexamethasone was ingested between 11 PM and midnight, then a blood sample for measuring serum cortisol was collected the next morning before 9 AM. Serum cortisol was measured as detailed later. A sufficient serum dexamethasone level was achieved in all cases (27).

The $hCRH^{test}$ was performed in the morning before 9 AM, after overnight fasting. An indwelling catheter was inserted in a forearm vein, and the participant remained fasting, supine, and resting throughout the test. An intravenous bolus of 100-µg hCRH was injected over 30 seconds. Blood samples for measuring ACTH and cortisol were collected at the baseline at –15 and 0 minutes (immediately before the hCRH injection), and then 15, 30, 45, 60, 90, and 120 minutes after the injection (28). ACTH and cortisol were measured by immunochemiluminescence assay (Immulite 2000, Siemens Healthcare; detection limits 5 pg/mL and 6 nmol/L, respectively).

Statistical analyses

Proportions and rates were calculated for categorical data; continuous data were reported as means and standard error. Groups were compared by chi-square test for categorical variables and by the *t*-test for quantitative variables.

To measure ACTH and cortisol response after hCRH injection, we recorded the peak values (termed $peak^{ACTH}$ and $peak^{cortisol}$) and calculated the percentage increases ($\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$) from the mean ACTH levels before the injection (at times –15 and 0 minutes, $basal^{ACTH}$ and $basal^{cortisol}$). We computed the area under the curve (AUC) at the different time points with respect to the baseline (according to the trapezoidal formula proposed by Pruessner [29]) for ACTH and cortisol (AUC^{ACTH} and $AUC^{cortisol}$). We performed receiver operating characteristic curve analyses to ascertain the sensitivity (SE), specificity (SP), and 95% CI. We calculated the likelihood ratio (LR) of the tests

because this is independent of disease prevalence: a positive LR (LR^{pos}) and negative LR (LR^{neg}) indicate by how much the probability of HPA-axis–related disease increases or decreases if the test is positive or negative, respectively, with the 95% CI calculated using the method proposed by Simel et al (30).

The SPSS 24 software package for Windows (SPSS, Inc) was used to manage the database and perform the statistical analysis. The significance level was set at P less than .05 for all tests. All data analyzed during this study are included in the data repositories of the University of Padova-Research Data UniPD (31).

Results

Indications for the human corticotropin-releasing hormone test over time

As reported in Supplementary Figure 1 (31), we observed a change in the indications for the $hCRH^{test}$ over time, with fewer cases of pseudo-CS and suspected AI, from 2010 to 2019. On the other hand, there was an increasing use of the $hCRH^{test}$ in patients with adrenal lesions (P = .048), especially after 2014 to 2015. The percentage of $hCRH^{tests}$ performed for the differential diagnosis of ACTH-dependent CS (CD and EAS) remained stable over the years, accounting for approximately half the tests in all (101 out of 200).

Differential diagnosis in adrenocorticotropin-dependent Cushing syndrome

Patients with CD were younger than EAS. On the other hand, EAS presented with increased levels of $basal^{ACTH}$, $basal^{cortisol}$, UFC, and LNSC (as summarized in Table 1).

During the $hCRH^{test}$, $peak^{cortisol}$ was higher in EAS, and ACTH showed a blunted response ($\Delta\%^{ACTH}$ 37% vs 229% in CD), as shown in Fig. 1.

As for the differential diagnosis of ACTH-dependent CS (Table 2), the highest diagnostic accuracy for EAS was achieved by considering $basal^{cortisol}$. A marked increase in ACTH and cortisol ($\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$, respectively) after hCRH injection was indicative of CD. A previous publication (9) concerned 48 of the tests identifying ACTH-dependent CS considered in the present work, which were performed up to 2013 (44 CD and 4 EAS). The other 53 (42 CD and 11 EAS), diagnosed from 2014 to 2019, could

Table 1. Basal and dynamic tests by type of disorder. Data are reported as means and standard errors (in parentheses)

	CD (n = 86)	EAS (n = 15)	Adrenal mass (n = 43)	Pseudo-CS (n = 31)	Suspected AI (n = 25)
Sex, female/male, % female	64/24 (74.4%)	12/3 (80%)	33/10 (76.7%)	19/12 (61.3%)	13/12 (52%)
Age at hCRH test, y	44.9 (1.7)	59.7(4)[a]	58.9 (2)[b,g]	40.3 (3.2)[d]	47.6 (3.2)[d,h]
Age at last visit, y	49.1 (1.9)	62.3 (4.8)[c]	61.6 (2.3)[b,g]	46.1 (4.1)[e]	51.5 (2.9)[d,h]
Weight, kg	75.8 (2.6)	68.7 (5.3)	68.2 (2)[c,g]	90.2 (4.8)[c,e]	73.4 (7.1)
BMI, kg/m^2	27.9 (0.9)	26.7 (2.8)	25.6 (0.68)[c,g]	33.5 (2.3)[c]	23.5 (2.1)[g]
ACTH morning, pg/mL	57.4 (4.6)	161.8 (37.4)[a]	9.2 (1.2)[b,f,g]	30.6 (5.7)[a,d]	18.6 (2.82)[b,d,h]
$Basal^{ACTH}$ (pg/mL) $hCRH^{test}$	59 (6.9)	183 (73.1)[b]	10.3 (2)[b,d,g]	26.5 (4.2)[c,e]	22 (5.6)[a,e,h]
$Basal^{cortisol}$ (nmol/L) $hCRH^{test}$	492 (20.6)	1176.8 (211.8)[b]	396 (23.5)[c,d]	375 (29.1)[c,d]	241 (27.2)[b,f,g,h]
$Peak^{ACTH}$ (pg/mL) $hCRH^{test}$	168 (20.3)	217.6 (78.9)	27.6 (5.3)[b,d,g]	69.2 (12.9)[c,e]	68.9 (16.2)[a,e,h]
$Peak^{cortisol}$ (nmol/L) $hCRH^{test}$	805 (30.8)	1324.9 (231.6)[b]	593 (28.4)[b,d]	549 (31.9)[b,d]	448 (51)[b,f,h]
$\Delta\%^{ACTH}$	2.29 (0.31)	0.37 (0.12)[a]	1.71 (0.41)[e]	2.01 (0.36)[d]	2.82 (0.53)[d]
$\Delta\%^{cortisol}$	0.76 (0.06)	0.28 (0.14)[a]	0.64 (0.1)	0.69 (0.14)[e]	1.08 (0.24[d,h]
AUC^{ACTH}	13 324 (1409)	25 600 (9764)[c]	2189 (312)[b,e,g]	5480 (863)[a,d]	5263 (952)[b,e,h]
$AUC^{cortisol}$	85 669 (3234)	161 307 (27 180)[b]	64 661 (3051)[b,f]	60 200 (3706)[b,d]	46 259 (4438)[b,d,g,h]
Cortisol (nmol/L) post–1-mg DST	346 (43)	1327 (559)[a]	196 (29.5)[c,f,g]	68 (17)[b,d]	NA
UFC, ULN	5.1 (1.39)	24.67 (8.38)[b]	1.68 (0.52)[c,f]	0.95 (0.2)[a,d]	NA
LNSC, ULN	6.76 (0.96)	42.86 (12.71)[b]	2.89 (0.67)[b,f]	1.22 (0.32)[a,d]	NA

Abbreviations: ACTH, adrenocorticotropin; AI, adrenal insufficiency; AUC, area under the curve; CD, Cushing disease; CS, Cushing syndrome; DST, dexamethasone suppression test; EAS, ectopic adrenocorticotropin secretion; hCRH, human corticotropin-releasing hormone; LNSC, late-night salivary cortisol; NA, not available; UFC, urinary free cortisol; ULN, upper limit of normal.

[a] P less than .01 vs CD.
[b] P less than .001 vs CD.
[c] P less than .05 vs CD.
[d] P less than .01 vs EAS.
[e] P less than .05 vs EAS.
[f] P less than .001 vs EAS.
[g] P less than .05 vs pseudo-CS.
[h] P less than .05 vs adrenal mass.

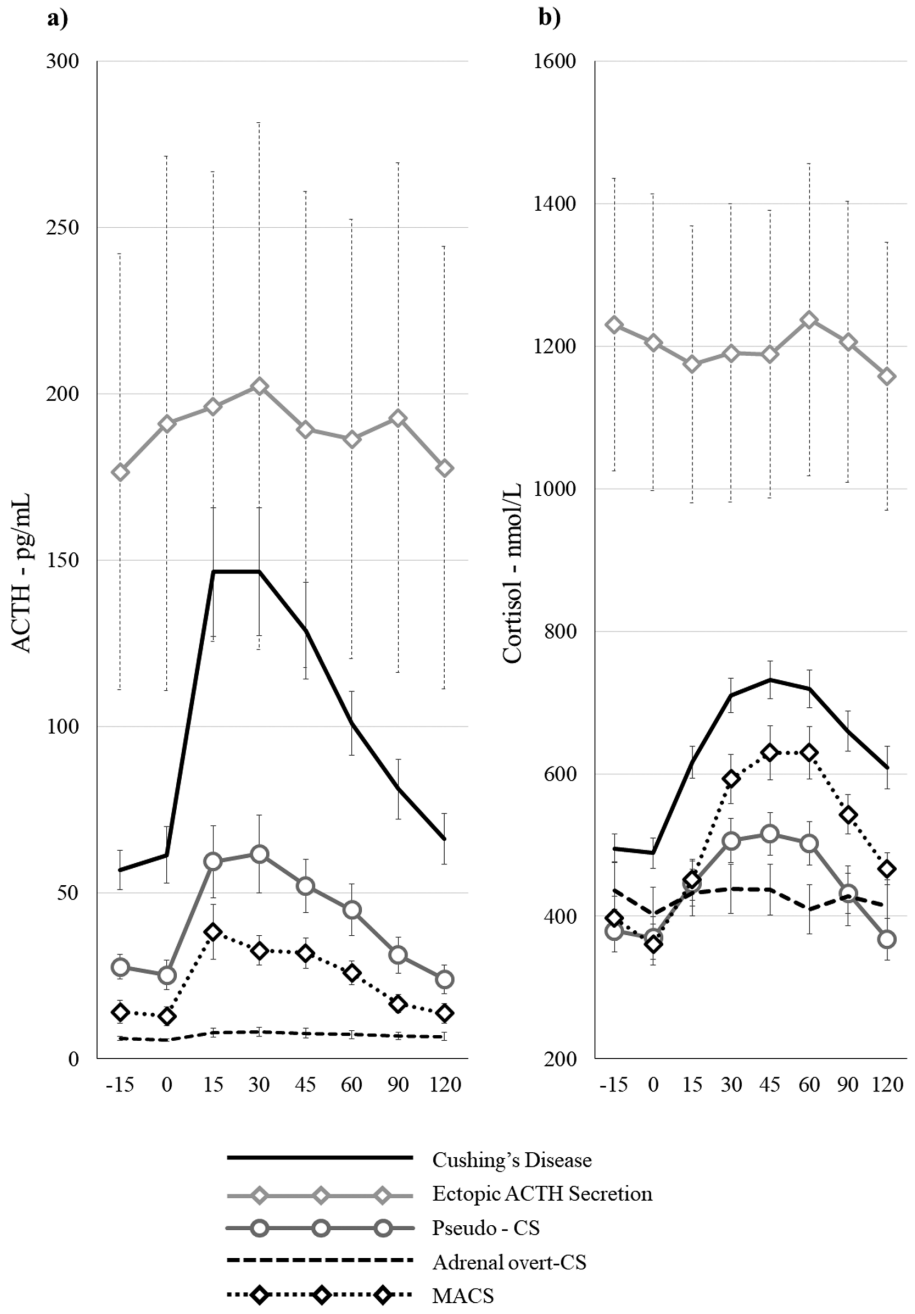

Figure 1. A, Adrenocorticotropin (ACTH) (in picogram per milliliter, pg/mL) and B, cortisol (in nanomole per liter, nmol/L) curve during the human corticotropin-releasing hormone test in the different cohorts considered. Data are given as means and standard errors. CS, Cushing syndrome; MACS, mild autonomous cortisol secretion.

be considered as an independent validation cohort. We calculated the diagnostic accuracy of $\Delta\%^{ACTH}$ taking the year 2013 as a threshold, confirming a similar diagnostic accuracy in $\Delta\%^{ACTH}$ up to 2013 (AUC 0.924, SE 82.2%, SP 100%) and from 2014 onward (AUC 0.894, SE 95.2%, SP 81.8%).

In patients with CD, pituitary magnetic resonance imaging at diagnosis was available for 83 of 86 individuals (97%). Twelve patients had a macroadenoma (> 10 mm in larger diameter), 53 had a microadenoma, and pituitary imaging was negative in 18. Compared with patients harboring a macroadenoma, those with microadenoma or negative imaging had similar basal^{cortisol}, and hCRH-stimulated peak^{ACTH}, and peak^{cortisol}. Only basal^{ACTH} levels were higher in patients with macroadenomas (86 vs 48 pg/mL, $P = .005$).

The human corticotropin-releasing hormone test in patients with Cushing syndrome and low-indeterminate basal adrenocorticotropin levels

In the cohort as a whole, there were 119 patients with overt CS: A total of 101 had ACTH-dependent (86 CD,

Table 2. Diagnostic accuracy of human corticotropin-releasing hormone test in adrenocorticotropin-dependent Cushing syndrome, considering Cushing disease (n = 86) vs ectopic adrenocorticotropin secretion (n = 15). Threshold is indicated by Youden J index

	Level	SE, % (95% CI)	SP, % (95% CI)	LR^{neg}, % (95% CI)	LR^{pos}, % (95% CI)	AUC, % (95% CI)
$Basal^{ACTH}$ (pg/mL) hCRH	58	86.7 (62.1-96.3)	68.6 (58.2-77.4)	0.194 (0.053-0.712)	2.76 (1.906-3.997)	0.799 (0.68-0.919)
$Basal^{cortisol}$ (nmol/L) hCRH	743	93.3 (70.2-98.1)	92.9 (85.4-96.8)	0.072 (0.011-0.477)	13.222 (6.052-28.935)	0.904 (0.715-0.999)
$Peak^{ACTH}$ (pg/mL) hCRH	87	80 (54.8-92.9)	40.7 (30.2-51.3)	0.419 (0.173-1.396)	1.349 (0.992-1.835)	0.539 (0.393-0.685)
$Peak^{cortisol}$ (nmol/L) hCRH	818	86.7 (62.1-96.3)	61.2 (50.5-70.8)	0.218 (0.06-0.801)	2.232 (1.601-3.113)	0.745 (0.604-0.887)
$\Delta\%^{ACTH}$	0.31	90.7 (82.7-95.2)	80 (54.8-92.9)	0.116 (0.057-0.236)	4.535 (1.644-12.506)	0.896 (0.809-0.982)
$\Delta\%^{cortisol}$	0.2	85.9 (76.9-91.9)	80 (54.8-92.9)	0.176 (0.099-0.316)	4.294 (1.555-11.858)	0.871 (0.769-0.973)
AUC^{ACTH}	8880	86.7 (62.1-96.3)	50 (39.6-60.4)	0.2617 (0.072-0.986)	1.733 (1.297-2.316)	0.636 (0.5496-0.776)
$AUC^{cortisol}$	97 942	93.3 (70.2-98.8)	75.6 (65.5-83.4)	0.088 (0.013-0.588)	3.822 (2.573-5.677)	0.843 (0.719-0.968)

Abbreviations: ACTH, adrenocorticotropin; AUC, area under the curve; hCRH, human corticotropin-releasing hormone; LR^{neg}, negative likelihood ratio; LR^{pos}, positive likelihood ratio; SE, sensitivity; SP, specificity.

15 EAS) and 18 had ACTH-independent hypercortisolism. Nineteen patients had low $basal^{ACTH}$ levels (< 10 pg/mL, 16%), including 11 patients with BMAH, 3 with CD, 3 with monolateral adrenal adenoma, and 2 with PPNAD. Thirteen patients had indeterminate $basal^{ACTH}$ levels (10-20 pg/mL, 11%): 10 with CD, 2 with BMAH, and 1 with EAS. In short, 32 patients with overt CS (27%) had low-indeterminate $basal^{ACTH}$ levels, and 14 of them (44%) were cases of ACTH-dependent CS.

Considering patients with low $basal^{ACTH}$ levels and a monolateral adrenal mass, suppressed corticotropin at the baseline did not respond to hCRH ($\Delta\%^{ACTH}$ 0%), and $\Delta\%^{cortisol}$ was less than 10% in all cases. As for BMAH, corticotropin response to hCRH (compared with CD, considering a threshold of $\Delta\%^{ACTH}$ < 31%) was insufficient in 7 cases (ACTH levels remained suppressed throughout the test in 6 of them), partial in 2 ($\Delta\%^{ACTH}$ 38% and 40%, $peak^{ACTH}$ 7 and 9 pg/mL), and high in 2 ($\Delta\%^{ACTH}$ 133% and 140%, $peak^{ACTH}$ 12 and 14 pg/mL). In 3 patients with CD and low $basal^{ACTH}$ levels, baseline corticotropin was not suppressed (being 8 pg/mL in 1, and 9 pg/mL in 2 cases), and showed a strong response ($\Delta\%^{ACTH}$ 450%, 279% and 142%, $peak^{ACTH}$ 44, 36, and 23 pg/mL).

As regards the individuals with indeterminate $basal^{ACTH}$ levels, 2 patients with BMAH showed a corticotropin response after hCRH ($\Delta\%^{ACTH}$ of 90% and 107%), though the $peak^{ACTH}$ achieved was not very high ($peak^{ACTH}$ 19 and 27 pg/mL). The one EAS patient had a cosecreting ACTH pheochromocytoma: $Basal^{ACTH}$ of 19 pg/mL did not increase during the $hCRH^{test}$ ($\Delta\%^{ACTH}$ 12%). Ten patients were cases of CD, and they all responded to hCRH (mean $\Delta\%^{ACTH}$ 276%, range 60%-725%). To conclude, as shown in Fig. 2, a $peak^{ACTH}$ of less than 29 pg/mL showed a high diagnostic accuracy for ACTH-independent CS (SE 96%, SP 100%, AUC 0.998, 95% CI 0.994-1).

In all 6 patients with BMAH and low-indeterminate $basal^{ACTH}$ levels who had a significant ACTH response in the $hCRH^{test}$, other dynamic tests for ACTH-dependent CS (the 8 mg DST and the desmopressin test), and/or dedicated imaging ruled out CD or EAS.

The CV (SD/mean × 100) of the 2 basal levels of ACTH before hCRH injection was similar for patients with low and indeterminate $basal^{ACTH}$ levels (33%-21% and 25%-35%, respectively, at time –15 and 0 minutes).

The human corticotropin-releasing hormone test in adrenal lesions

Patients with adrenal masses were older (except in cases of EAS), and had a lower body mass index than those with overt CS or pseudo-CS. As expected, they had lower $basal^{ACTH}$ and $peak^{ACTH}$ levels than those with CD, EAS, or pseudo-CS. $Basal^{cortisol}$ and $peak^{cortisol}$ levels were also lower than in cases of CD and EAS, and similar to those of patients with pseudo-CS (see Table 1).

Among the 43 patients with adrenal lesions, 15 had monolateral and 28 had bilateral lesions (2 PPNAD and 26 BMAH). In the monolateral forms, 3 were cases of overt CS, and 12 were MACS. Overt cortisol secretion tended to be higher in BMAH, with overt CS diagnosed in 13 cases, and MACS in 13 (Pearson χ^2 P = .058). As shown in Table 3, all the clinical and endocrine parameters considered were similar for patients with monolateral and bilateral adrenal lesions.

The $basal^{ACTH}$ and hCRH-stimulated $peak^{ACTH}$, and the $\Delta\%^{ACTH}$, $peak^{cortisol}$, and $\Delta\%^{cortisol}$ were blunted in cases of overt hypercortisolism, but not in MACS (see Fig. 1). In patients with adrenal masses, the diagnostic accuracy of the $hCRH^{test}$ in distinguishing between overt CS and MACS was satisfactory for $basal^{ACTH}$ (SE 84%, SP 77.8% for overt CS if < 5 pg/mL, AUC 0.839); $peak^{ACTH}$

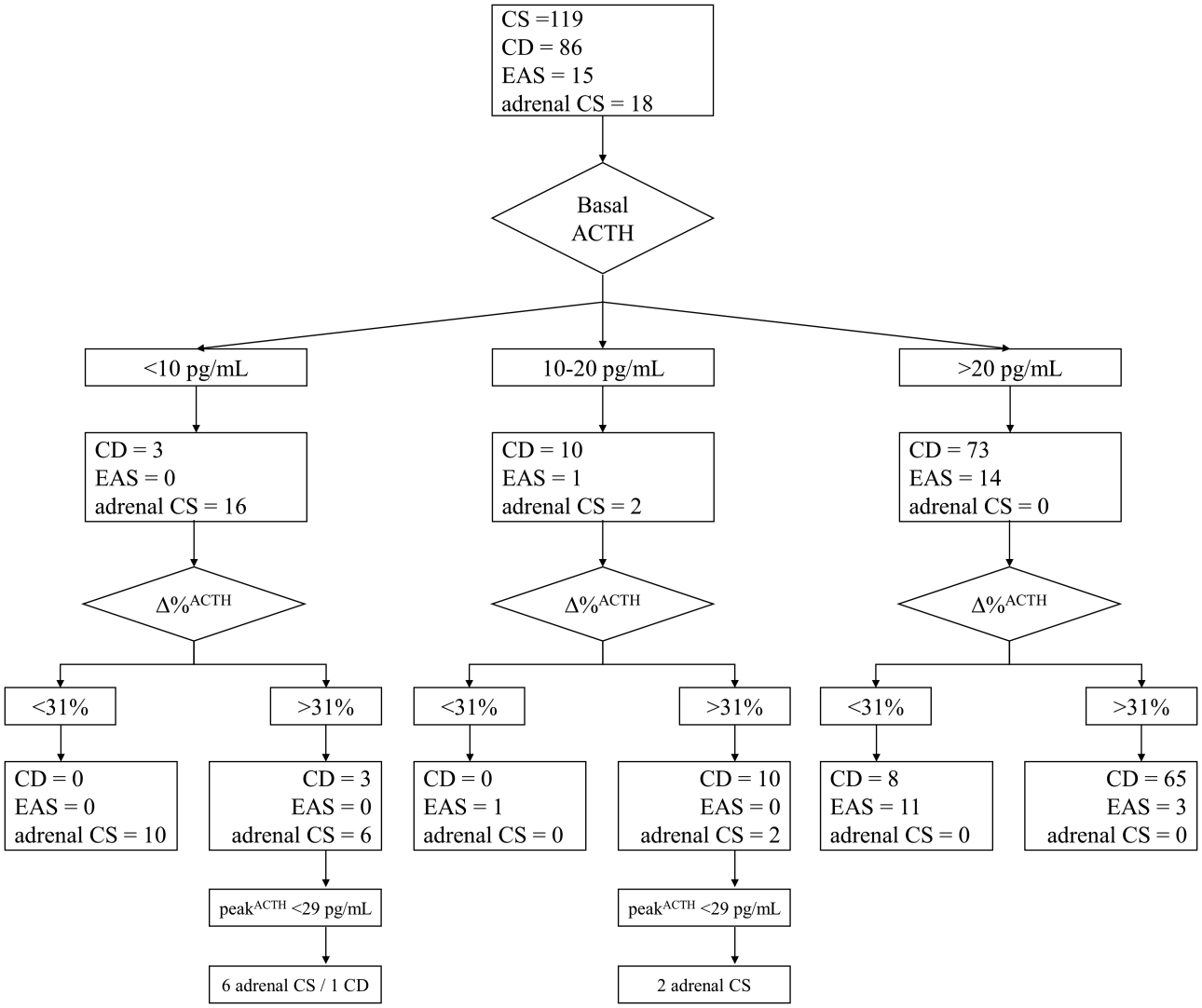

Figure 2. Distribution of patients by thresholds calculated. CD, Cushing disease; CS, Cushing syndrome; EAS: ectopic adrenocorticotropin (ACTH) secretion.

(SE 88%, SP 88.9% for overt CS if < 14 pg/mL, AUC 0.923); and $\Delta\%^{ACTH}$ (SE 84%, SP 83.3% for overt CS if < 95%, AUC 0.893). The $peak^{cortisol}$ (SE 84%, SP 77.8% for overt CS if < 561 nmol/L, AUC 0.818) and $\Delta\%^{cortisol}$ after hCRH (SE 80%, SP 88.9% for overt CS if < 31%, AUC 0.838) were also able to differentiate MACS from overt CS.

As shown in Fig. 3, $\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$ after hCRH injection were reduced in adrenal overt CS, also differentiating between patients with monolateral adenoma and BMAH. The ACTH response to hCRH was maintained, however, not only in patients with MACS, but also in BMAH with overt-CS ($\Delta\%^{ACTH}$ P = .01 in monolateral overt CS vs BMAH with overt CS).

Comparing pseudo-CS with MACS, the $basal^{ACTH}$ was lower in the case of an adrenal mass (13 vs 26 pg/mL, P = .013), whereas $peak^{cortisol}$ was higher in MACS (657 vs 548 nmol/L, P = .011). The other parameters considered did not differ.

The human corticotropin-releasing hormone test in the differential diagnosis of pseudo-Cushing syndrome and central adrenal insufficiency

First-line screening tests for CS were unable to rule out CS reliably in patients with pseudo-CS (n = 31). This was especially true for cortisol after 1-mg DST and LNSC (cortisol was not suppressed to < 50 nmol/L in 44% of cases, and cortisol rhythm was impaired in 48%), whereas UFC was normal in 82% of cases.

$Basal^{ACTH}$ and $basal^{cortisol}$ levels were lower in patients with pseudo-CS than in those with CD, and so were $peak^{ACTH}$ and $peak^{cortisol}$ after hCRH, but $\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$ were similar in the 2 conditions. $Basal^{ACTH}$ and

Table 3. Baseline features, and basal and dynamic tests by type of disorder in patients with monolateral or bilateral (bilateral macronodular adrenal hyperplasia + primary pigmented nodular adrenocortical disease) lesions

	Monolateral n = 15	Bilateral n = 28	Overt-CS n = 18	MACS n = 25
Age at $hCRH^{test}$, y	61.8 (2.7)	57.4 (2.7)	50.9 (3.5)[a]	64.7 (1.5)
Age last visit, y	65 (3)	60.1 (2.9)	53.4 (3.7)[a]	69 (1.4)
Weight, kg	66.7 (4.4)	68.8 (2.2)	67.2 (2.6)	69.4 (3.2)
BMI, kg/m^2	26.3 (1.4)	25.2 (0.8)	25.6 (0.9)	25.6 (1)
ACTH morning, pg/mL	11.3 (2.8)	8.9 (1.3)	6.5 (0.8)[b]	12 (2.1)
$Basal^{ACTH}$ (pg/mL) $hCRH^{test}$	13.6 (5.3)	8.5 (1.1)	5.9 (0.5)[b]	13.4 (3.1)
$Basal^{cortisol}$ (nmol/L) $hCRH^{test}$	419 (32)	383 (32)	419.7 (38.5)	378 (30)
$Peak^{ACTH}$ (pg/mL) $hCRH^{test}$	33 (7.5)	24.4 (7.2)	8.6 (1.4)[a]	41 (8)
$Peak^{cortisol}$ (nmol/L) $hCRH^{test}$	663 (38)	555.6 (40.7)	478.4 (38.3)[a]	675 (37)
$\Delta\%^{ACTH}$	1.89 (0.44)	1.61 (0.58)	0.37 (0.11)[a]	2.68 (0.63)
$\Delta\%^{cortisol}$	0.75 (0.2)	0.58 (0.14)	0.19 (0.08)[a]	0.96 (0.15)
AUC^{ACTH}	2809 (693)	1857 (297)	961 (150)[a]	3074 (453)
$AUC^{cortisol}$	71 652 (3778)	60 965 (4101)	57 240 (4816)	70 005 (3653)
Cortisol (nmol/L) post–1-mg DST	184 (52)	203.3 (34.3)	311.4 (56.4)[a]	132 (22)
UFC, ULN	0.89 (0.22)	2.04 (0.72)	2.83 (1)[b]	0.71 (0.12)
LNSC, ULN	3.29 (1.14)	2.71 (0.83)	4.81 (1.13)[a]	1.27 (0.52)

Abbreviations: ACTH, adrenocorticotropin; AUC, area under the curve; BMI, body mass index; CS, Cushing syndrome; DST, dexamethasone suppression test; hCRH, human corticotropin-releasing hormone; LNSC, late-night salivary cortisol; MACS, mild autonomous cortisol secretion; UFC, urinary free cortisol; ULN, upper limit of normal.

[a]*P* less than .01 vs MACS.

[b]*P* less than .05 vs MACS.

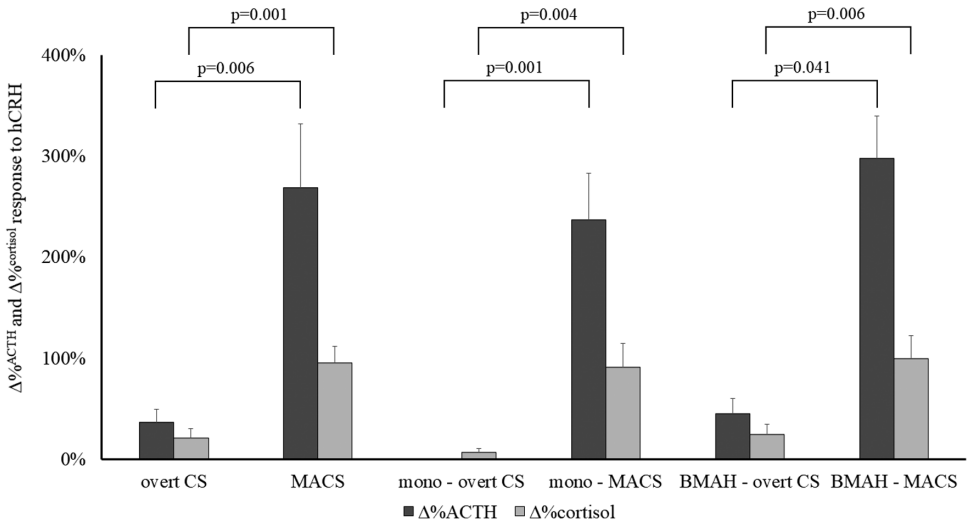

Figure 3. Adrenocorticotropin (ACTH) and cortisol response to human corticotropin-releasing hormone (hCRH) (reported as $\Delta\%^{ACTH}$ and $\Delta\%^{cortisol}$) in patients with monolateral (n = 15) or bilateral adrenal masses (n = 26). BMAH, bilateral macronodular adrenal hyperplasia; CS, Cushing syndrome; MACS: mild autonomous cortisol secretion.

$peak^{ACTH}$ did not reach a satisfactory diagnostic accuracy for the purpose of distinguishing pseudo-CS from CD (a basal ACTH > 23 pg/mL suggests CD with an SE 82.6% and SP 64.5%, AUC 0.768; $peak^{ACTH}$ > 70 pg/mL indicates CD with an SE 69.8% and SP 77.4%, AUC 0.753). The diagnostic accuracy of a $basal^{cortisol}$ greater than 444 nmol/L was unsatisfactory too (SE 63.5%, SP 77.4%, AUC 0.684), and a $peak^{cortisol}$ greater than 685.5 nmol/L was associated with an SE 69.4% and SP 87.1%, AUC 0.808. On the other hand, an increased AUC^{ACTH} was able to differentiate CD from pseudo-CS (> 6277, SE 69.8%, SP 83.9%, AUC 0.794).

Combining the basal and post-hCRH parameters did not improve diagnostic accuracy in terms of distinguishing CD from pseudo-CS: $basal^{ACTH}$ and $basal^{cortisol}$ (> 23 pg/mL and > 444 nmol/L, respectively) reached an SE of 79.4% and an SP of 66.6%. Likewise, combining $peak^{ACTH}$ and $peak^{cortisol}$ levels (> 70 pg/

mL and > 685.5 nmol/L, respectively) reached an SE of 86.3% and an SP of 63.6%.

The diagnostic accuracy of the $hCRH^{test}$ in the differential diagnosis of central AI is reported in the supplementary data available in the data repositories (31).

Discussion

This work concerns a large cohort of patients who underwent an $hCRH^{test}$ for suspected HPA axis dysfunction. The clinical reasons for performing the test have changed in the last 10 years. Nowadays, the $hCRH^{test}$ is performed mainly for the differential diagnosis of ACTH-dependent CS (49% of tests in 2018-2019) and to identify cortisol secretion in adrenal masses (42% in 2018-2019). In our clinical practice, the test's pivotal role in the differential diagnosis in ACTH-dependent CS forms has remained unchanged over the years, in contrast with the situation described in a large European multicenter study (32). Other indications (pseudo-CS and AI) dropped to 7% and 2%, respectively, probably because the role of the $hCRH^{test}$ in the diagnosis of central AI is still a matter of debate (17), and because other screening tests are more reliable for the diagnosis of pseudo-CS (33).

We collected a large series of patients with overt endogenous CS, 101 of them ACTH dependent and 18 ACTH independent (overall 119 CS: 73% CD, 12% EAS, and 15% adrenal CS). In patients with overt hypercortisolism, dynamic tests are used largely to identify ACTH-dependent CS (2). The $hCRH^{test}$ has been used since 2000, and is highly accurate for diagnostic purposes (8, 15, 28), especially if concordant with the results of a high-dose DST (9). We confirmed that patients with EAS have high cortisol and ACTH levels (24, 34), and lower $peak^{ACTH}$ and $peak^{cortisol}$ than those with CD (15, 28, 35). The diagnostic accuracy of the $hCRH^{test}$ in our study is similar to that previously described in a collaborative work (that also considered ovine CRH) (9). Other unconventional indexes, such as the AUC, did not improve the test's overall diagnostic accuracy, as reported elsewhere (35). Despite the proposed $\Delta\%^{ACTH}$ cutoff (ranging from 14% to 43% in the literature [2, 8, 9, 15, 28, 35]), it is important for each center to develop an in-house threshold that can differentiate CD from EAS. The diagnostic accuracy of ovine CRH seems superior to hCRH, but the former is no longer available in Europe.

As recommended by the Endocrine Society guidelines (21), in cases of severe EAS, physicians should prioritize symptomatic over etiological treatments, and should rule out any obvious signs of aggressive neuroendocrine tumors. Invasive procedures or dynamic tests may therefore be incompatible with some patients' general condition and urgent need for treatment (36). In our study, however, the short duration of the $hCRH^{test}$, associated with its good diagnostic performance (the higher the $\Delta\%^{ACTH}$, the lower the likelihood of a diagnosis of EAS), make it suitable even in cases of severe CS.

High ACTH levels in patients with overt CS suggest an ACTH-dependent CS (2, 3), but low ACTH levels do not always indicate adrenal cortisol secretion alone. Negative feedback regulates the HPA axis: Excessive cortisol secretion leads to CRH and ACTH suppression in pituitary corticotroph cells (4). There are some biases to consider, however, particularly as regards ACTH levels. Measuring ACTH precisely is a challenge, requiring careful morning sampling and accurate conservation/storage/centrifugation at low temperatures. Commercially available ACTH immunoassays may be imprecise in patients with suppressed (< 10 pg/mL) or reduced ACTH levels (10-20 pg/mL) (10). In selected patients with low or reduced ACTH levels, an $hCRH^{test}$ is indicated to check pituitary responsiveness (2, 11). In our whole cohort of 119 patients with overt CS, 19 (16%) had suppressed (≤ 10 pg/mL) basal ACTH levels. There were not only adrenal forms (16 patients, 84%: 11 with BMAH, 3 with monolateral adrenal adenoma, 2 with PPNAD), but also 3 cases of CD, all with confirmed ACTH-positive pituitary adenoma in surgical specimens. Baseline suppressed ACTH levels (< 10 pg/mL) did not respond to hCRH in all patients with monolateral adrenal adenoma or PPNAD, while response to hCRH was absent in 7/11 cases of BMAH, partial in 2, and evident in 2. $Peak^{ACTH}$ was not very high in any of these cases, and $\Delta\%^{ACTH}$ showed a relative increase with respect to the low basal levels, thus confirming an adrenal form of CS, with partially preserved positive feedback. Thirteen patients (10 with CD, 1 with EAS, and 2 with BMAH) had indeterminate basal ACTH levels in the so-called "gray zone" between 10 and 20 pg/mL. The 2 adrenal forms revealed low $basal^{ACTH}$ levels and an evident response to hCRH stimulation, but their $peak^{ACTH}$ was not particularly high, suggesting that the adrenal masses were able to suppress corticotrophs. The only patient with EAS with a $basal^{ACTH}$ of 19 pg/mL and no response to the $hCRH^{test}$ had an ACTH-secreting pheochromocytoma. On the other hand, all 13 patients with CD and low or indeterminate $basal^{ACTH}$ levels (3 and 10 individuals, respectively, with < 10 or 10-20 pg/mL) showed a marked response to hCRH. This is no minor concern because CD often causes enlargement of the adrenals and/or adrenal adenoma (37) (2 out of 13 CD patients in our series presented with adrenal incidentaloma), so differentiating it from a monolateral or bilateral adrenal form or MACS could prove a challenge.

As for overt CS in adrenal lesions, basal and stimulated ACTH levels and Δ% or AUC were lower in the overt form of hypercortisolism than in MACS, proving accurate in diagnosing CS (especially peak^{ACTH}, with an SE of 87.5% and an SP of 88.9%). ACTH still responded to CRH, even in patients with overt CS in BMAH, whereas corticotropin remained unstimulated in monolateral forms of adrenal CS. Cortisol also has a tendency to respond to ACTH after hCRH, even if the adrenal glands are under low-basal cortisol levels, indicating that some response to ACTH may persist in BMAH (11). In our sample, the Δ%^{ACTH} was similar in CD and adrenal masses with subclinical hypercortisolism, making an hCRH^{test} useful for detecting a preserved HPA axis in adrenal lesions with MACS.

Distinguishing between endogenous CS (characterized by a neoplastic ACTH or cortisol secretion) and the so-called pseudo-CS (where upregulation of the HPA axis is secondary to a physiological/nonneoplastic condition) can be difficult even at tertiary centers (13, 14). First-line screening tests, especially 1-mg DST, UFC, and LNSC, can rule out endogenous CS in most patients (33, 38, 39), but are reportedly less specific in some pathological conditions (chronic alcoholism, psychiatric conditions, severe illness, anorexia nervosa) and endocrine diseases (metabolic syndrome, polycystic ovary syndrome) (13, 14, 40). In our series, basal^{ACTH} and peak^{ACTH} levels were lower in pseudo-CS than in CD. Increased ACTH (basal, peak, and AUC) levels were sufficiently accurate in diagnosing CD, but the increase in ACTH and cortisol levels after hCRH was much the same in CD and pseudo-CS. In this scenario, the dexamethasone-suppressed CRH test has been proposed to enhance diagnostic performance, but—despite initial enthusiasm—its high diagnostic accuracy has not been confirmed (14). Subsequent reports described a more limited specificity of the dexamethasone-suppressed CRH test (62.5%) (16) in a similar cohort of patients (23 cases). That said, the latter authors also considered ACTH-independent CS cases, thereby including a bias because there is an activation of the HPA axis in pseudo-CS that is ACTH dependent. In 2009, Arnaldi and colleagues reported that the hCRH^{test} alone could detect cases of CD in a series of patients (26 pseudo-CS) based on the association between the basal/peak^{cortisol} or basal/peak^{ACTH} levels (15). In our cohort, associating basal and post-hCRH parameters was unable to confirm any improvement in the test's diagnostic accuracy in terms of distinguishing patients with CD from cases of pseudo-CS. It is worth noting that Arnaldi et al considered all cases of ACTH-dependent CS in their group of patients, including those with EAS (characterized by high basal ACTH and cortisol levels, and a weak response to hCRH). As controls, they considered patients with pseudo-CS and obese individuals without any other clinical features of hypercortisolism. This approach probably influenced the final diagnostic accuracy of the hCRH^{test}. We believe it is more appropriate to compare only pseudo-CS with CD, as done here, because the clinical consequences (ie, hypertension, hypokalemia, sarcopenia, psychiatric disorders) and overt or rapidly increasing hypercortisolism in EAS are generally evident when compared with individuals who are only obese.

An accurate diagnosis of central AI is of the utmost importance for patients with hypothalamic or pituitary disease. We found that reduced basal cortisol levels and hCRH-stimulated AUC^{cortisol} could arouse suspicion of AI, but their role in excluding individuals with a normal HPA axis was limited. The hCRH^{test} is also unable to differentiate hypothalamic from pituitary causes of AI: Clinical judgment, corticotropin testing, and expertise are mandatory to diagnose central AI (41).

Alongside the strengths of this study (a monocentric series with a prolonged follow-up, and a sample size on a par with others previously published), we are also aware of some limitations. First, there is the lack of healthy participants undergoing hCRH^{test} for control purposes. Second, the study design was not prospective.

To conclude, the hCRH^{test} is the cornerstone of the differential diagnosis in ACTH-dependent forms of CS, and has a pivotal role in discriminating a pituitary or adrenal etiology of CS in patients with adrenal masses and indeterminate ACTH levels. Its value in the diagnosis of AI seems to be limited, and further studies are needed to establish its usefulness in the identification of patients with pseudo-CS.

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Additional Information

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