Human corticotropin releasing hormone test performance in the differential diagnosis between Cushing’s disease and pseudo-Cushing state is enhanced by combined ACTH and cortisol analysis

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Abstract

Objective: Corticotropin releasing hormone (CRH) test does not reliably distinguish Cushing’s disease (CD) from normality or pseudo-Cushing state (PC). We assessed whether this could be achieved with a novel approach while preserving the ability of the test to distinguish CD from ectopic ACTH syndrome (EAS).

Design: Retrospective/prospective study.

Subjects and methods: We studied 51 subjects with CD, 7 with EAS, 26 with PC, and 31 controls (CT). Human CRH (hCRH) test was performed at 0830 h by measuring plasma ACTH and serum cortisol at −15, 0, 15, 30, 45, 60, 90, and 120 min.

Results: The area under the curve–ACTH exhibited a significant negative correlation with baseline serum cortisol in CT and PC, but not in CD or EAS patients. ACTH response to hCRH was blunted in PC compared with CT, whereas peak serum cortisol was higher in PC than in CT subjects. These findings suggested that ACTH-dependent Cushing’s syndrome can be diagnosed by the presence of two hCRH test parameters and excluded if either or both are absent. Application of i) basal serum cortisol > 12 μg/dl and peak plasma ACTH > 54 pg/ml, or ii) peak serum cortisol > 21 μg/dl and peak plasma ACTH > 45 pg/ml, had 91.3% (95% confidence intervals (CI) 81–97.1) and 94.8% (CI 85.6–98.9) sensitivity and 98.2% (CI 90.6–99.9) and 91.2% (CI 80.7–97) specificity respectively, in diagnosing ACTH-dependent Cushing’s syndrome. The >14% serum cortisol increase from mean baseline values to the mean of 15 and 30 min values in patients who were positive for the test completely discriminated between CD and EAS.

Conclusions: Simultaneous plasma ACTH and serum cortisol analysis enables the hCRH test to distinguish CD from PC and from normality, while preserving its ability to discriminate CD from EAS.

Introduction

Distinguishing Cushing’s syndrome (CS) from pseudo-Cushing state (PC) is a major clinical challenge (1, 2). Frequently, overlapping clinical and biochemical findings from first-line studies require additional tests, all of which, however, suffer from varying limitations: the dexamethasone-suppressed corticotropin-releasing hormone (DEX/CRH) stimulation test has shown a variable performance in different studies (3–7); the desmopressin (DDAVP) test is not fully validated for clinical use and its evaluation requires larger series (6); sleeping and awake midnight serum cortisol, despite its ability to distinguish subjects with CS from those with PC (5, 6), is uncomfortable and requires overnight hospitalization as well as 48 h hospitalization before sleeping midnight serum cortisol sampling to avoid false positives (6).

Even late-night salivary cortisol has limitations, related to the assay methodology and to the risk of false positives in depressed individuals, in the obese and in subjects with variable bedtimes (6).

The CRH test was introduced into clinical use more than 20 years ago (8), mainly to differentiate between Cushing’s disease (CD) and ectopic ACTH syndrome (EAS) or adrenal CS (9). A degree of overlap of cortisol and ACTH responses found in CD and PC patients and in healthy subjects, has largely prevented its utilization to discriminate CD from the other two conditions (10, 11).
However, a few years ago, a group of researchers demonstrated that CD patients could be discriminated fairly accurately from healthy subjects using the CRH test, thus partially rehabilitating its use in clinical practice (12).

Given these premises, this study was devised to assess whether application of novel criteria could make the human CRH (hCRH) test effective in distinguishing CD from PC and from control subjects (CT), while preserving its ability to discriminate CD from EAS.

Materials and methods

Subjects

We retrospectively and prospectively studied 115 subjects admitted to our centre: 51 with a first diagnosis of active CD, 7 with EAS, 26 with PC, and 31 CT. The diagnosis of CD and EAS was based on clinical and biochemical data (9) and was confirmed on pituitary surgery or by removal of a tumor producing ectopic ACTH respectively, and/or by postoperative clinical and biochemical resolution of hypercortisolism. Out of the seven EAS patients, four had bronchial carcinoids, one had medullary carcinoma of the thyroid, one had a gastric carcinoid and another had small-cell lung carcinoma. Subjects with PC were admitted for suspected CS that was eventually excluded. The diagnosis of PC (2) was based on: a) clinical and biochemical features consistent with CS; b) identification of a clinical condition known to activate the hypothalamic–pituitary–adrenal (HPA) axis; c) a lack of progression of clinical and biochemical abnormalities after treatment of the associated condition; and d) normal pituitary adenoma morphology and biochemical resolution of hypercortisolism. Out of the seven EAS patients, four had bronchial carcinoids, one had medullary carcinoma of the thyroid, one had a gastric carcinoid and another had small-cell lung carcinoma. Subjects with PC were admitted for suspected CS that was eventually excluded. The diagnosis of PC (2) was based on: a) clinical and biochemical features consistent with CS; b) identification of a clinical condition known to activate the hypothalamic–pituitary–adrenal (HPA) axis; c) a lack of progression of clinical and biochemical abnormalities after treatment of the associated condition; and d) normal pituitary adenoma morphology and biochemical resolution of hypercortisolism. Out of the seven EAS patients, four had bronchial carcinoids, one had medullary carcinoma of the thyroid, one had a gastric carcinoid and another had small-cell lung carcinoma. Subjects with PC were admitted for suspected CS that was eventually excluded. The diagnosis of PC (2) was based on: a) clinical and biochemical features consistent with CS; b) identification of a clinical condition known to activate the hypothalamic–pituitary–adrenal (HPA) axis; c) a lack of progression of clinical and biochemical abnormalities after treatment of the associated condition; and d) normal pituitary adenoma morphology and biochemical resolution of hypercortisolism. Out of the seven EAS patients, four had bronchial carcinoids, one had medullary carcinoma of the thyroid, one had a gastric carcinoid and another had small-cell lung carcinoma. Subjects with PC were admitted for suspected CS that was eventually excluded.

The study was performed according to the Declaration of Helsinki and was approved by the institutional ethics committee. All subjects undergoing testing at our centre are asked to sign an informed consent form at admission. The clinical data were obtained as part of the diagnostic work-up: some of the data of PC and CT subjects were acquired in the framework of a research protocol which entailed an additional consent form.

Study protocol

All subjects underwent the standard procedure for CS diagnosis, which includes determination of: a) serum cortisol circadian rhythm (midnight serum cortisol was measured after 24 h hospitalization); b) 24 h UFC; and c) serum cortisol after OST. The hCRH test was performed after overnight fasting by inserting an indwelling catheter at 0800 h in a forearm vein; the subject remained supine during the whole study period. An i.v. bolus of 100 μg hCRH (Ferring Pharmaceuticals Ltd) was injected over 30 s at 0830 h (0 min). Blood samples were collected 15 min before the test, at 0 min and then at 15, 30, 45, 60, 90, and 120 min. Samples for the other CS diagnostic procedures were collected from an indwelling venous catheter placed at least 1 h earlier.

Peak plasma ACTH and peak serum cortisol were the highest concentrations measured during the test. Basal serum cortisol and basal plasma ACTH were the means of the two baseline values (−15 and 0 min) before hCRH administration. Plasma ACTH and serum cortisol increases were calculated from mean baseline values.

Assays

Chemiluminescent immunometric assays were used to measure plasma ACTH (Immulite, DPC, Los Angeles, CA, USA) and serum cortisol and UFC (Advia Centaur; Bayer Diagnostics, Newbury, UK), the latter after urine extraction with dichloromethane. Method sensitivity was 4.54 pg/ml for plasma ACTH and 0.4 μg/dl for both urinary and serum cortisol; intra-assay and interassay variation coefficients were 3.4 and 4.8% for plasma ACTH and 4.4 and 6.0% for both urinary and serum cortisol respectively. Normal ranges in our laboratory are 0–46 pg/ml for plasma ACTH, 0–150 μg/24 h for UFC, and 5–23 μg/dl for morning serum cortisol (0830 h).

Statistical analysis

Values are expressed as mean ± S.E.M. if normally distributed and as median (total range) if not normally distributed. The prevalence of clinical signs was analyzed by the χ² test or, where appropriate, Fisher’s exact test. The net integrated area under the curve (AUC) for plasma ACTH and serum cortisol responses to hCRH was calculated using the trapezoidal method (16). Shapiro–Wilk’s test was applied to verify the normal distribution of quantitative variables. Comparisons between groups were done with ANOVA followed by Tukey’s test.
by Fisher’s post hoc test for normally distributed values; for not normally distributed values the Kruskal–Wallis test was followed by Mann–Whitney’s U test (if significant differences were detected) with P value correction using the Bonferroni–Holm method (17). Pearson and Spearman correlations were done as appropriate: logarithmic transformation was done for the former to obtain a normal distribution where required. Sensitivity (SE), specificity (SP), positive (LR+) and negative (LR−) likelihood ratios and receiver-operator characteristic (ROC) curves were calculated according to standard statistical methods (18). Diagnostic accuracy (DA) was calculated as the proportion of correctly scored patients out of the total number of patients tested. Ninety-five percent confidence intervals (CI) for SE and SP were calculated with the McNemar test. Significance was set at P < 0.05. Statistical analyses were performed using the SPSS 16 package (SPSS Inc., Chicago, IL, USA).

Results

Patients’ characteristics are shown in Table 1. Median (interquartile range) plasma ACTH and serum cortisol levels after hCRH testing are reported in Fig. 1. The diagnostic performances of some first- and second-line tests (6, 9, 19) are shown in Table 2.

Clinical presentation

CS and PC patients were all similarly affected by hypertension, hirsutism, purple striae, impaired fasting glycaemia/diabetes, dyslipidaemia, oligomenorrhoea, acne, overweight/obesity, and muscle weakness (P > 0.05). Bruising and osteoporosis were significantly more prevalent in CS patients (P < 0.05), whereas psychiatric problems were significantly more prevalent among PC patients (P < 0.05).

Plasma ACTH responses

Peak plasma ACTH was significantly greater in CD patients (141 (38–960) pg/ml) and EAS patients (104 (55–200) pg/ml) than among CT (37 (20–212) pg/ml) and PC subjects (29 (10–75) pg/ml; P < 0.001, PC versus CD; P < 0.001, CT versus CD; P < 0.001, PC versus EAS; P < 0.001, CT versus EAS), and was significantly lower (P = 0.014) in the PC compared with the CT group; values did not differ significantly between CD and EAS. AUC–ACTH was significantly greater (P < 0.001) in CD patients (4170 (−5812.5–436 57.5) pg/ml·120 min) compared with CT (1012.5 (−892.5–8437.5) pg/ml·120 min). PC (626.2 (112.5–1732.5) pg/ml·120 min) and EAS subjects (225 (−255–697.5) pg/ml·120 min), and was significantly greater (P = 0.039) among CT than among PC subjects. The measure was significantly lower in EAS patients than in the other groups (P < 0.001, EAS versus CD; P = 0.014, EAS versus PC; P = 0.003, EAS versus CT).

In CT and PC subjects – but not in CD and EAS patients – AUC–ACTH exhibited a significant negative correlation with baseline serum cortisol (CT: r = −0.56, P < 0.001; PC: r = −0.46, P = 0.016). The diagnostic performances of the peak plasma ACTH and basal plasma ACTH cut-offs with the highest SE and SP in discriminating subjects with and without ACTH-dependent Cushing’s syndrome (ACTH-dependent CS) are reported in Table 3.

The maximum percent increase in plasma ACTH was not effective in diagnosing/excluding ACTH-dependent CS (data not shown), nor in discriminating CD from EAS (data not shown); however, the percentage rise of plasma ACTH from mean baseline values to the mean of 15 and 30 min values (20, 21) yielded the highest SE and SP in discriminating CD from EAS patients, a rise of 9% or more being seen in 44 out of 51 patients with CD but in no patient with EAS.

Serum cortisol responses

Peak serum cortisol was significantly greater in CD (33.3 (22–51) µg/dl) and EAS patients (45 (24–99) µg/dl) than in PC (20.7 (12.5–50) µg/dl) or CT subjects (17 (9.3–33) µg/dl; P < 0.001, CT versus CD; P < 0.001, PC versus CD; P < 0.001, CT versus EAS; P < 0.001, PC versus EAS) and was significantly greater in EAS than in CD patients (P = 0.022, EAS versus CD). The difference between CT and PC subjects was nearly significant (P = 0.060). CD patients had significantly greater AUC-cortisol (1078 ± 50.7 µg/dl·120 min) compared with

Table 1 Characteristics of the study subjects.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Body mass index</th>
<th>Basal plasma ACTH (pg/ml)</th>
<th>Basal serum cortisol (µg/dl)</th>
<th>Midnight serum cortisol (µg/dl)</th>
<th>OST cortisol (µg/dl)</th>
<th>UFC (µg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>(n=51)</td>
<td>(8/43)</td>
<td>38.2±1.2</td>
<td>30 (20–52)</td>
<td>64.5 (17.5–360)</td>
<td>20.6 (10.7–36)</td>
<td>23 (8–94)</td>
</tr>
<tr>
<td>EAS</td>
<td>(n=7)</td>
<td>(3/4)</td>
<td>44.4±1.3</td>
<td>30 (28–37)</td>
<td>95 (40–194)</td>
<td>40 (23–87)</td>
<td>30 (25–48)</td>
</tr>
<tr>
<td>PC</td>
<td>(n=26)</td>
<td>(2/24)</td>
<td>35.3±2.4</td>
<td>32.2 (21.4–49.3)</td>
<td>18.7 (9–45.5)</td>
<td>15.5 (7.3–35)</td>
<td>4.2 (0.9–10)</td>
</tr>
<tr>
<td>CT</td>
<td>(n=31)</td>
<td>(8/23)</td>
<td>35.6±2.4</td>
<td>33 (30–41.4)</td>
<td>16.5 (10–42)</td>
<td>11 (6.2–18)</td>
<td>4 (2.3–8)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. if normally distributed and as median (total range) if not normally distributed. *P < 0.01 versus PC, †P < 0.01 versus CT, §P < 0.001 versus PC, ‡P < 0.001 versus CT and †P < 0.001 versus CD.
CT subjects (443.6 ± 80.6 μg/dl; 120 min; \( P < 0.001, \) CT versus CD), PC patients (318.3 ± 75.6 μg/dl; 120 min; \( P < 0.001, \) PC versus CD), and EAS patients (253.9 ± 57.8 μg/dl; 120 min; \( P < 0.001, \) EAS versus CD). In this case, the values of EAS, CT, and PC subjects were not significantly different.

In CT and PC subjects, basal serum cortisol correlated negatively and significantly with AUC–cortisol (CT, \( r = -0.44, P = 0.013; \) PC, \( r = -0.47, P = 0.015). \) The former correlation was not found in CD and EAS subjects.

The diagnostic performances of the peak serum cortisol and basal serum cortisol cut-offs with the highest SE and SP in discriminating subjects with and without ACTH-dependent CS are reported in Table 3.

The maximum percent rise of serum cortisol did not afford an optimal diagnostic performance in diagnosing/excluding ACTH-dependent CS (data not shown).

The best SE and SP distinguishing CD from EAS were those relating to the maximum percent rise in serum cortisol, which was 14% or higher in all patients with CD, but never reached this value in patients with EAS. Complete discrimination of CD from EAS was also achieved by applying as a cut-off the 14% percent rise in serum cortisol from mean baseline values to the mean of values at 15 and 30 min (12).

**Parameter combinations**

The parameter combinations and the cut-offs applied to enhance the diagnostic performance of the hCRH test are reported in Table 3. The two proposed parameter combinations, each capable independently of diagnosing/excluding ACTH-dependent CS, were applied separately to our subjects. Each combination enabled the diagnosis of ACTH-dependent CS, if subjects were simultaneously positive for both parameters, and its exclusion in the absence of either or both.

Values of basal serum cortisol > 12 μg/dl and peak plasma ACTH > 54 pg/ml (Fig. 2, Table 3) yielded 91.3% SE, 98.2% SP, and 94.7% DA. With this combination, SE exceeded the values obtained with basal plasma ACTH (\( P = 0.016 \)) and basal serum cortisol (\( P = 0.021 \)) used singly with the cut-offs derived from the ROC curves, and was not associated with significantly different SP (\( P > 0.05 \) for both measures). The combination also yielded greater SP than peak plasma ACTH (\( P = 0.002 \)) and peak serum cortisol (\( P = 0.001 \)) used singly with the cut-offs derived from the ROC curves, again without significant differences in SE (\( P > 0.05 \)). The combination also afforded an SE that was greater than the one yielded by OST (cut-off 5 μg/dl, \( P = 0.031 \)), without a

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**Table 2 First- and second-line tests routinely performed to distinguish subjects with and without ACTH-dependent Cushing’s syndrome (CS).**

<table>
<thead>
<tr>
<th></th>
<th>SE (CI) (%)</th>
<th>SP (CI) (%)</th>
<th>LR+</th>
<th>LR−</th>
<th>DA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFC: cut-off &gt; 150(^b)</td>
<td>84.4 (72.5–92.6)</td>
<td>54.3 (40.6–67.6)</td>
<td>1.84</td>
<td>0.28</td>
<td>69.5</td>
</tr>
<tr>
<td>OST serum cortisol: cut-off &gt; 5(^b)</td>
<td>81 (68.5–90.1)</td>
<td>94.7 (85.3–98.9)</td>
<td>15.28</td>
<td>0.20</td>
<td>87.8</td>
</tr>
<tr>
<td>OST serum cortisol: cut-off &gt; 1.8(^b)</td>
<td>93.1 (83.2–98)</td>
<td>68.4 (54.7–80)</td>
<td>2.94</td>
<td>0.10</td>
<td>80.8</td>
</tr>
<tr>
<td>Midnight serum cortisol: cut-off &gt; 7.5(^a)</td>
<td>100 (94.9–100)</td>
<td>85.9 (74.2–93.7)</td>
<td>7.09</td>
<td>0</td>
<td>93</td>
</tr>
</tbody>
</table>

Units: UFC, μg/24 h; serum cortisol, μg/dl.
\(^a\)Upper limit of the normal UFC range in our laboratory.
\(^b\)Cut-off commonly used for the diagnosis/exclusion of CS (see ref. 9).
\(^c\)Cut-off according to Papanicolaou et al. (see ref. 19).
significantly different SP ($P>0.05$), and an SP that exceeded that of UFC (cut-off $150 \mu g/dl$, $P<0.001$), OST (cut-off $1.8 \mu g/dl$, $P<0.001$), and midnight serum cortisol (cut-off $7.5 \mu g/dl$, $P=0.039$), without a significantly different SE ($P>0.05$).

Diagnosis/exclusion of ACTH-dependent CS based on peak serum cortisol $>21 \mu g/dl$ and peak plasma ACTH $>45 \mu g/ml$ yielded 94.8 SE, 91.2 SP, and 93% DA (Table 3). This parameter combination yielded a greater SE than basal plasma ACTH ($P=0.004$) and basal serum cortisol ($P=0.006$) used alone with the cut-offs derived from the ROC curves, and did not entail significant differences in SP ($P>0.05$ for both); SP with this combination exceeded the values obtained with peak plasma ACTH ($P=0.031$) and peak serum cortisol ($P=0.006$) used singly with the cut-offs derived from the ROC curves, without a significantly different SE ($P>0.05$ for both). This parameter combination also afforded a greater SE compared with OST (cut-off $5 \mu g/dl$, $P=0.008$), without a significant difference in SP ($P>0.05$), as well as an SP that exceeded that of UFC (cut-off $150 \mu g/dl$, $P<0.001$) and of OST (cut-off $1.8 \mu g/dl$, $P=0.011$), without significant SE differences ($P>0.05$). However, the SE and SP of this combination were not significantly different from those of midnight serum cortisol (cut-off $7.5 \mu g/dl$, $P>0.05$).

Exclusion of CT data from the statistical analysis showed that the SP of the combination 'basal serum cortisol $>12 \mu g/dl$ and peak plasma ACTH $>54 \mu g/ml$' was 96.1% (CI 80.3–99.9). The combination 'peak serum cortisol $>21 \mu g/dl$ and peak plasma ACTH $>45 \mu g/ml$' yielded the same SP value.

### Discussion

In this study, we assessed whether application of novel criteria would enable the hCRH test to distinguish CD from PC and from CT subjects, while preserving its effectiveness in discriminating CD from EAS.

We propose a new, simple method to interpret the hCRH test results based on a combination of parameters. By this method, subjects are diagnosed with ACTH-dependent CS if they are simultaneously positive for two hCRH test parameters; negativity for either or both excludes ACTH-dependent CS.

We propose two distinct parameter combinations, each capable, independently of diagnosing/excluding ACTH-dependent CS: i) basal serum cortisol $>12 \mu g/dl$ + peak plasma ACTH $>54 \mu g/ml$ and its exclusion on the absence of either or both effectively discriminated patients with ACTH-dependent CS (quadrant A) from subjects without ACTH-dependent CS (quadrants B, C, D), with an SE of 91.3% (CI 81–97.1) and an SP of 98.2% (CI 90.6–99.9).

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**Table 3** Human hCRH test: diagnostic performance of the parameters (peak and basal ACTH and cortisol), alone and combined, in subjects with and without ACTH-dependent Cushing’s syndrome (CS).

<table>
<thead>
<tr>
<th>Parameter combination</th>
<th>SE (CI) (%)</th>
<th>SP (CI) (%)</th>
<th>LR+</th>
<th>LR−</th>
<th>DA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performance of the parameters applied singly</td>
<td></td>
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</tr>
<tr>
<td>Peak plasma ACTH $&gt;50^{a}$</td>
<td>94.8 (85.6–98.9)</td>
<td>80.7 (68–89.9)</td>
<td>4.91</td>
<td>0.06</td>
<td>86.9</td>
</tr>
<tr>
<td>Peak serum cortisol $&gt;23^{a}$</td>
<td>96.5 (88–99.5)</td>
<td>73.6 (60.3–84.4)</td>
<td>3.65</td>
<td>0.04</td>
<td>85.2</td>
</tr>
<tr>
<td>Basal plasma ACTH $&gt;41^{a}$</td>
<td>79.3 (66–88.8)</td>
<td>96.4 (87.8–99.5)</td>
<td>22.02</td>
<td>0.21</td>
<td>87.8</td>
</tr>
<tr>
<td>Basal serum cortisol $&gt;18^{a}$</td>
<td>77.5 (64.7–87.4)</td>
<td>89.4 (78.4–96)</td>
<td>7.31</td>
<td>0.25</td>
<td>83.4</td>
</tr>
<tr>
<td>Performance of the parameter combinations$^{b}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal serum cortisol $&gt;12^{a} +$ Peak plasma ACTH $&gt;54^{c}$</td>
<td>91.3 (81–97.1)</td>
<td>98.2 (90.6–99.9)</td>
<td>50.72</td>
<td>0.08</td>
<td>94.7</td>
</tr>
<tr>
<td>Peak serum cortisol $&gt;21^{a} +$ Peak plasma ACTH $&gt;45^{c}$</td>
<td>94.8 (85.6–98.9)</td>
<td>91.2 (80.7–97)</td>
<td>10.77</td>
<td>0.05</td>
<td>93</td>
</tr>
</tbody>
</table>

**Units:** plasma ACTH, pg/ml; serum cortisol, μg/dl.

$^{a}$Cut-off obtained from the ROC curve affording the highest SE and SP in the present sample.

$^{b}$ACTH-dependent CS diagnosis is based on the simultaneous presence of the two parameters; absence of either or both excludes ACTH-dependent CS.

$^{c}$Cut-off affording the best SE and SP combined with the cut-off of the other parameter.

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**Figure 2** Performance of one of the two hCRH test parameter combinations applied in the study to distinguish between subjects with (ACTH-dependent CS+) and without (ACTH-dependent CS−) ACTH-dependent CS. An ACTH-dependent CS diagnosis based on simultaneous positivity for basal serum cortisol $>12 \mu g/dl$ and peak plasma ACTH $>54 \mu g/ml$, and its exclusion on the absence of either or both effectively discriminated patients with ACTH-dependent CS (quadrants A) from subjects without ACTH-dependent CS (quadrants B, C, D), with an SE of 91.3% (CI 81–97.1) and an SP of 98.2% (CI 90.6–99.9).
The selection of these parameter combinations and the resulting improved diagnostic performance of the hCRH test were not accidental, but rather stemmed from the characteristics of the ACTH and cortisol response in the four groups of subjects (Fig. 1), as well as from the remarkable observations that CT and PC subjects (but not CD or EAS patients) displayed significant negative correlations between basal serum cortisol and AUC–ACTH, and between basal serum cortisol and AUC–cortisol.

Such findings, together with the data listed in Table 1, clearly indicate that the negative feedback is normal in CT subjects with simple obesity, that it is preserved to a fair extent in PC patients, and that it is lost in CD and EAS. This key observation and the resulting correlations explain the high DA obtained with the simultaneous analysis of basal serum cortisol > 12 µg/dl and peak plasma ACTH > 54 pg/ml (Table 3), because CT and PC patients with basal serum cortisol > 12 µg/dl tended to have peak plasma ACTH < 54 pg/ml as a result. By contrast, CT and PC patients with basal serum cortisol < 12 µg/dl usually had peak plasma ACTH > 54 pg/ml. In CD and EAS patients, loss of the cortisol-induced negative feedback resulted in the simultaneous presence of basal serum cortisol > 12 µg/dl and peak plasma ACTH > 54 pg/ml. Thus, a diagnosis of ACTH-dependent CS based on positivity for these two parameters, and its exclusion in subjects who were negative for either or both yielded 91.3% SE and 98.2% SP (Fig. 2, Table 3).

Simultaneous analysis of peak serum cortisol > 21 µg/dl and peak plasma ACTH > 45 pg/ml also achieved a diagnostic performance that was not afforded by their separate use (Table 3). This can be explained by the characteristics of the hCRH response in the four subject groups (Fig. 1), where the simultaneous presence of peak serum cortisol > 21 µg/dl and peak plasma ACTH > 45 pg/ml occurred almost exclusively in CD and EAS patients, whereas CT and PC subjects lacked one or both parameters.

Once ACTH-dependent CS has been diagnosed, EAS and CD are completely distinguished by interpreting the 14% serum cortisol increase from mean baseline values to the mean of 15 and 30 min values after hCRH testing. This has previously been considered as the best diagnostic criterion in distinguishing CD from EAS using the hCRH test, although it did not achieve their complete discrimination in a larger study (12).

EAS patients are therefore effectively identified by two characteristics, already described in the literature, i.e. high plasma ACTH and serum cortisol levels and a lack of response to CRH, even though the rare EAS patients with mild hypercortisolism can theoretically escape detection from the parameter combinations (12, 23–26). The characteristics of the hCRH response and the above correlations, reflecting a preserved physiological negative feedback mechanism in CT and PC subjects and its loss in CS patients, have also partially been described. Indeed, PC patients have already been shown to have a significant negative correlation between basal serum cortisol and AUC–ACTH and a blunted ACTH response and higher peak serum cortisol compared with CT subjects (10, 27, 28). A negative correlation between basal serum cortisol and AUC–ACTH and between basal serum cortisol and maximal serum cortisol response has been described even in normal subjects (29), whereas none have been documented in subjects with CD (10, 29).

The methodological approach used in this investigation is also not wholly novel. In 1987, Gold et al. (30) reviewed their previous work directed at distinguishing CD patients from depressed subjects using CRH (10). They noted that depressed subjects with elevated basal serum cortisol values, which therefore overlapped with those of CD patients, had lower peak plasma ACTH and were thus easier to distinguish from CD patients by this criterion; in contrast, depressed subjects with peak plasma ACTH identical to that of CD patients had lower basal serum cortisol, thus being easier to discriminate from CD patients on this criterion.

Ours is the first study to apply this method of data analysis to CD diagnosis/exclusion. The finding of a very high DA for the hCRH test could rehabilitate a tool that has long been considered inadequate (10, 11). In addition, in these subjects, the hCRH test performed significantly better than the UFC, OST, and midnight serum cortisol tests in diagnosing/excluding ACTH-dependent CS. However, unlike the latter assays, the hCRH test enabled CD patients to be discriminated from PC and EAS patients in our small sample.

Although the method requires confirmation and does suffer from some limitations – in particular, like the DDAVP test (4, 22) and, theoretically, the DEX/CRH test (3–5), it cannot be applied in patients with ACTH-independent CS – we suggest its adoption as a second-level test in subjects with normal/high plasma ACTH, who need a differential diagnosis between CD and PC.

The small size of our sample is clearly the main limitation of this study; in particular, the cut-offs of the parameter combinations are to be taken with caution, due to the broad CI of SE and SP, but also to the fact that the use of interpretive cut-offs based on absolute plasma ACTH and serum cortisol values, rather than on their increment, makes the analysis heavily dependent on assay methodology. In addition, they may not apply in women taking contraceptives, given the ability of the latter medications to increase corticosteroid-binding globulin (31). Moreover, the cut-offs adopted here cannot be extended to ovine CRH, which stimulates greater ACTH and cortisol secretion than the hCRH test, resulting in higher peak values, especially of serum cortisol (32). Additional, exhaustive testing is thus required before they can be introduced in clinical practice, and to establish whether one combination is more effective than the other.
Despite these limitations, the key finding of this study is that interpretation of the hCRH test based on a combination of parameters can become a valuable diagnostic tool capable of providing informative data rapidly in patients with unclear clinical and hormonal profiles.

In conclusion, we documented that the hCRH test with a novel interpretive approach has the potential to discriminate CD from PC and CT subjects while maintaining its ability to differentiate EAS from CD. The rationale of the approach is based on the fact that patients with ACTH-dependent CS are distinguished from PC and CT subjects by a combination of hCRH test parameters, while those who are positive can be divided into CD and EAS through the interpretation of the percent rise in serum cortisol from mean baseline values to the mean of values at 15 and 30 min (cut-off 14%).

The high SE and SP of the parameter combinations in diagnosing/excluding CS hinge on normal cortisol-induced negative feedback mechanisms in CT subjects, their reasonable preservation in PC patients and their loss in CD and EAS subjects.

Declaration of interest
The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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References
16 Pruessner JC, Kirschbaum C, Meinschmidt G & Hellhammer DH. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. Psychoneuroendocrinology 2003 28 916–931.

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