CLINICAL STUDY

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

Giorgio Arnaldi*, Giacomo Tirabassi*, Roberta Papa1, Giorgio Furlani, Laura Trementino, Marina Cardinaletti, Emanuela Faloia and Marco Boscaro

Division of Endocrinology, Polytechnic University of Marche, 60100 Ancona, Italy and 1Social Gerontology Unit, Research Department, INRCA (Italian National Institute on Ageing), 60100 Ancona, Italy

(Correspondence should be addressed to E Faloia; Email: e.faloia@univpm.it)

*(G Arnaldi and G Tirabassi contributed equally to this work)

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 0, 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 and/or normal serum cortisol circadian rhythm, and serum 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–Wilks’ test was applied to verify the normal distribution of quantitative variables. Comparisons between groups were done with ANOVA followed by post hoc analysis (Bonferroni’s test). Since we were comparing more than two groups, we have corrected the significance level (by Bonferroni’s test) to avoid type I error.
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 exact method. SE and SP values were compared using 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

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**Table 1** Characteristics of the study subjects.

<table>
<thead>
<tr>
<th></th>
<th>Sex (M/F)</th>
<th>Age (years)</th>
<th>Body mass index (kg/m²)</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>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>
<td>20 (0.4–45)</td>
<td>283 (87–2000)</td>
</tr>
<tr>
<td>EAS</td>
<td>(n=7)</td>
<td>44.4 ± 3.6</td>
<td>30 (28–37)</td>
<td>95 (40–194)</td>
<td>40 (23–87)</td>
<td>30 (25–48)</td>
<td>45 (27–60)</td>
<td>900 (362–1200)</td>
</tr>
<tr>
<td>PC</td>
<td>(n=26)</td>
<td>35.3 ± 2.4</td>
<td>32.2 (21–49.3)</td>
<td>18.7 (9–45.5)</td>
<td>15.5 (7.3–35)</td>
<td>4.2 (0.9–10)</td>
<td>3.2 (0.2–17)</td>
<td>193.5 (155–362)</td>
</tr>
<tr>
<td>CT</td>
<td>(n=31)</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>
<td>1 (0.2–1.3)</td>
<td>70 (17–99)</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.

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Clinical use of human corticotropin releasing hormone test
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

![Figure 1](https://example.com/fig1.png) **Figure 1** Median (interquartile range) plasma ACTH and serum cortisol response to hCRH in subjects with CD, EAS, PC, and CT.

### Table 2

<table>
<thead>
<tr>
<th>Test</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; 150a</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; 5b</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; 1.8b</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; 7.5c</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.

aUpper limit of the normal UFC range in our laboratory.

bCut-off commonly used for the diagnosis/exclusion of CS (see ref. 9).

cCut-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 μg/24 h, P < 0.001), OST (cut-off 1.8 μg/dl, P < 0.001), and midnight serum cortisol (cut-off 7.5 μg/dl, P = 0.039), without a significantly different SE (P > 0.05).

Diagnosis/exclusion of ACTH-dependent CS based on peak serum cortisol > 21 μg/dl and peak plasma ACTH > 45 pg/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 μg/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 μg/24 h, P < 0.001) and of OST (cut-off 1.8 μg/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 μg/dl, P > 0.05).

Exclusion of CT data from the statistical analysis showed that the SE of the combination ‘basal serum cortisol > 12 μg/dl and peak plasma ACTH > 54 pg/ml’ was 96.1% (CI 80.3–99.9). The combination ‘peak serum cortisol > 21 μg/dl and peak plasma ACTH > 45 pg/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 μg/dl + peak plasma ACTH > 54 pg/ml or ii) peak serum cortisol > 21 μg/dl + peak plasma ACTH > 45 pg/ml (Table 3); these parameter combinations yielded the highest SE and SP. The resulting SE and SP, despite the broad CI, exceeded even those of diagnostic tools such as the DDAVP test and the DEX/CRH test, which are considered as the most appropriate second-line tools to differentiate CD from PC (3–7, 22).
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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Author contribution statement
Giorgio Arnaldi: Study Design, Data Acquisition, Statistical Analysis, Data Interpretation, Manuscript Preparation and Literature Search.
Giacomo Tirabassi: Study Design, Data Acquisition, Statistical Analysis, Data Interpretation, Manuscript Preparation and Literature Search.
Roberta Papa: Statistical Analysis and Manuscript Preparation.
Giorgio Furlani: Data Acquisition and Manuscript Preparation.
Laura Trementino: Data Acquisition and Manuscript Preparation.
Emanuela Falciai: Data Acquisition, Data Interpretation, Literature Search and Manuscript Preparation.
Marco Boscaro: Study Design, Data Acquisition, Data Interpretation and Literature Search.

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