

Specificity of First-Line Tests for the Diagnosis of Cushing's Syndrome: Assessment in a Large Series

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Context: The diagnosis of Cushing's syndrome requires highly sensitive screening tests. Therefore, diagnostic cutoffs have been lowered to maximize sensitivity and identify all patients. However, few studies have investigated the impact of these refinements on the specificity of first-line tests.

Objective: The aim of the study was the assessment of the specificity of three widely used screening tests in a large series of Cushing's syndrome suspects referred to our endocrine service.

Patients: We retrospectively reviewed the results of urinary free cortisol (UFC), 1-mg dexamethasone suppression test [overnight suppression test (OST)], and serum cortisol at midnight in 3461, 357, and 864 patients, respectively, with clinical features suggestive of Cushing's syndrome but in whom this diagnosis was subsequently excluded.

Results: UFC and OST at the 5- μ g/dl cutoff exhibited the highest specificities [91% (95% confidence intervals [CI] 90.2–92.1%) and 97%

(95% CI 96.3–98.5%), respectively]. Conversely, midnight serum cortisol yielded 87% (95% CI 84.3–91.1%) specificity only with the 7.5- μ g/dl cutoff, whereas the 1.8- μ g/dl threshold resulted in an unacceptably high proportion of false positives at only 20% specificity (95% CI 16.0–24.4%). Gender and age may lead to misleading results in all three screening tests.

Conclusions: Specificity of tests for Cushing's syndrome varies considerably, with OST and UFC presenting the best performances, and circadian rhythm appearing heavily impaired by lowering of diagnostic cutoffs. Indeed, the vast majority of individuals in our series presented midnight serum cortisol values greater than 1.8 μ g/dl; thus, caution has to be exercised when this criterion is used to exclude Cushing's syndrome. (*J Clin Endocrinol Metab* 92: 4123–4129, 2007)

DESPITE THE INCREASING experience of clinicians and the improvements in laboratory and imaging techniques, the recognition of endogenous Cushing's syndrome remains a challenge. Nowadays, Cushing's syndrome is often diagnosed before patients develop the full-blown clinical picture and may, therefore, present with only subtle clinical features. In light of this, the physician may be called to exclude Cushing's syndrome in obese, depressed, hypertensive, and diabetic patients, who are increasingly present in the general population. Three tests are currently used to screen for Cushing's syndrome: measurement of free cortisol in 24-h urine [*i.e.* urinary free cortisol (UFC)]; assessment of cortisol circadian rhythm; and cortisol suppressibility by low doses of dexamethasone (1, 2). Given the very low prevalence of this disease, test interpretation is geared toward maximal sensitivity, and, indeed, diagnostic thresholds indicative of Cushing's syndrome have been progressively lowered to avoid false-negative results (3–9). However, few studies have evaluated the impact of these developments on the specificity of screening tests. The aim of the present study is the

assessment of the specificity of three widely used screening tests, *i.e.* UFC, serum cortisol at midnight, and after suppression with 1 mg dexamethasone, in a large series of patients referred as Cushing's syndrome suspects in whom the diagnosis was subsequently excluded. We also examined the effect of gender, age, and body mass index (BMI) on specificity of screening tests.

Patients and Methods

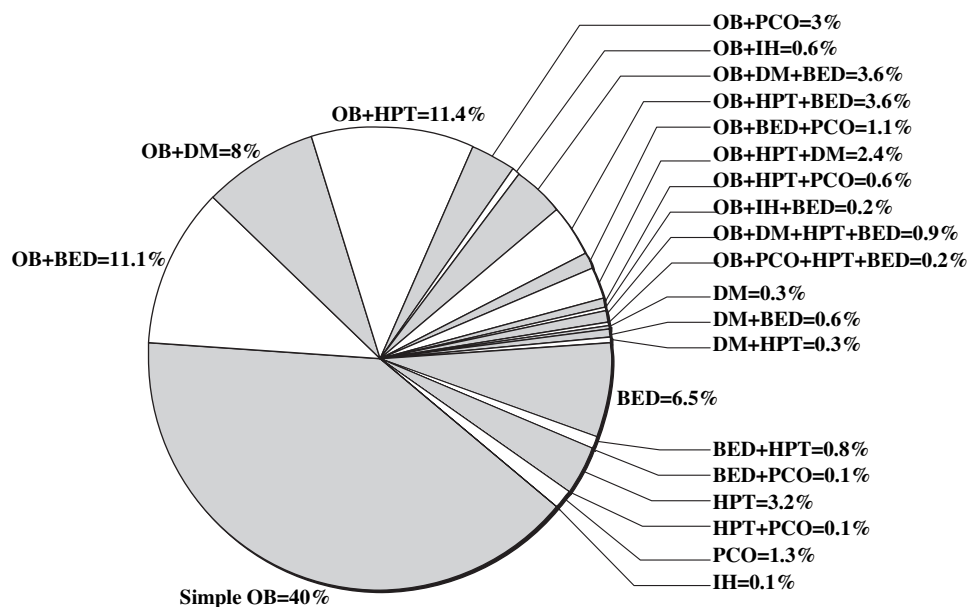
Data from 4126 consecutive patients (3148 women, 978 men, mean age 41.7 ± 0.27 yr, range 13–92) referred to our Institution as Cushing's syndrome suspects from 2001–2005 were analyzed retrospectively. The suspicion had arisen in patients presenting obesity, essential hypertension, impaired glucose tolerance or frank diabetes mellitus, mood disorders, irregular menses, buffalo hump, plethoric appearance and/or hirsutism, and Cushing's syndrome needed to be excluded during the diagnostic workup. Patients referred for adrenal incidentaloma were not included. Cushing's syndrome was diagnosed in 22 patients [17 women, five men; 20 with pituitary ACTH-secreting adenoma, two with adrenal Cushing's syndrome, all with UFC > 80 μ g/24 h, overnight suppression test (OST) > 5 μ g/dl, and cortisol at midnight > 7.5 μ g/dl], and they were excluded from the specificity study. Final diagnosis in 4104 patients was one or more of the following: simple obesity, polycystic ovary syndrome, depression or eating disorders, diabetes mellitus, idiopathic hirsutism, and hypothyroidism (Fig. 1). Data regarding BMI were available in 534 patients [360 obese (BMI > 30 kg/m²) and 174 nonobese (BMI < 30 kg/m²)]. None of the patients was taking drugs known to interfere with pituitary-adrenal axis secretion or cortisol measurement in serum and urine, *e.g.* contraceptives, antidepressants, as such treatments were deferred until the diagnostic workup had been completed

First Published Online August 14, 2007

Abbreviations: BMI, Body mass index; CI, confidence interval(s); NS, nonsignificant; OST, overnight suppression test; UFC, urinary free cortisol.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

FIG. 1. Final diagnosis in the 4104 Cushing's syndrome suspects. Percentages indicate the proportion of patients falling within a given diagnostic group. BED, Depression or eating disorders; DM, diabetes mellitus; HPT, hypothyroidism; IH, idiopathic hirsutism; OB, obesity; PCO, polycystic ovary syndrome.



or had been interrupted at least 3 months previously. The study was approved by our Institution's Ethical Committee.

Study protocol

Testing procedures included:

1. Measurement of UFC and creatinine excretion in 24-h urine collections. Patients collected one to five samples, and we chose to consider the median UFC value if multiple collections were available because this appeared the most reasonable clinical approach.

2. Assessment of cortisol circadian rhythm (*i.e.* venous sampling at 2400 h from an indwelling catheter, the latter in sleeping patients on the second night after admission). Lights off in our ward is at 2200 h, and patients are awakened at 0630 h.

3. Determination of morning serum cortisol (0800 h) after administration of 1 mg dexamethasone the previous midnight (*i.e.* OST).

UFC was measured in 3461 patients, totaling 6606 collections, midnight serum cortisol in 357 patients and OST in 864 patients. UFC and OST were performed on both inpatients (59%) and outpatients (41%), whereas cortisol at midnight was assessed only in inpatients. A total of 235 subjects performed UFC and OST, 335 performed UFC and cortisol circadian rhythm, and 88 patients performed all three tests; no patient was tested with OST and cortisol circadian rhythm only (Fig. 2). All patients presenting supernormal UFC concentrations also had OST and/or cortisol circadian rhythm. Likewise, abnormal OST was complemented by UFC and/or midnight serum cortisol. We did not perform routine adrenal computed tomography scan or scintigraphy in all patients with altered results but maintained a more conservative approach, *i.e.* follow-up, symptomatic treatment. These patients were followed for a median of 2.4 yr (range 8 months to 4 yr), and Cushing's syndrome was excluded after completion of the diagnostic workup and/or normalization upon retesting after weight loss, amelioration of depression and glycemic control and/or absent progression of symptoms (10–12).

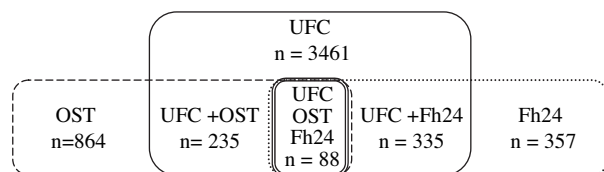


FIG. 2. Diagram showing tests performed in the 4104 Cushing's syndrome suspects. Fh24, Serum cortisol at midnight measurement; OST, cortisol after 1-mg dexamethasone suppression.

Assays

Serum cortisol and UFC were measured by immunometric assay (Byk-Sangtec Diagnostica, Dietzenbach, Germany) and RIA after urine extraction with dichloromethane (Diagnostic Products Corp., Los Angeles, CA), respectively. Analytical sensitivity is 0.21 and 0.20 $\mu\text{g/dl}$ for serum cortisol and UFC, respectively; functional sensitivity of both methods was 0.5 $\mu\text{g/dl}$. Intraassay and interassay coefficients of variation were 3.0 and 4.7% for serum cortisol and 3.5 and 6.2% for UFC, respectively. Normal ranges are 5–25 $\mu\text{g/dl}$ (138–690 nmol/liter) for morning serum cortisol and 10–80 $\mu\text{g/24 h}$ (28–220 nmol/24 h) for UFC. Urine collections in which creatinine concentration was outside the expected range (0.8–2 g/24 h) were excluded from analysis.

Criteria for data interpretation

The following cutoffs to exclude Cushing's syndrome were used: less than 80 $\mu\text{g/24 h}$ (220 nmol/24 h) for UFC; less than 1.8 $\mu\text{g/dl}$ (50 nmol/liter), less than 5 $\mu\text{g/dl}$ (138 nmol/liter), and less than 7.5 $\mu\text{g/dl}$ (207 nmol/liter) for serum cortisol at midnight (13–15); and less than 1.8 $\mu\text{g/dl}$ (50 nmol/liter), and less than 5 $\mu\text{g/dl}$ (138 nmol/liter) for cortisol after OST (16, 17).

Statistical analysis

Statistical analysis was performed using StatView 4.5 (Abacus Concepts, Berkeley, CA) and GB-STAT (Dynamic Microsystems, Silver Spring, MD). Data were examined by the Student's *t* or χ^2 test. Correlations between variables were established by linear regression analysis; logistic regression was used to predict the likelihood of altered hormonal values. For age-specific analysis, frequency distribution across the entire age range was calculated, and patients were subdivided into six groups according to their percentile: up to 10th percentile, 18 yr and younger; 10th to 25th percentile, 19–27 yr; 25th to 50th percentile, 28–41 yr; 50th to 75th percentile, 42–56 yr; 75th to 90th percentile, 57–65 yr; and above 90th percentile, over 65 yr of age. ANOVA was used to analyze the difference in hormonal data across age-percentiles, and χ^2 statistic for $2 \times k$ tables was used to assess differences in specificity per age group and test thresholds (18). Values are given as mean \pm SEM, uncertainty quantified as 95% confidence intervals (CI) (19); *P* values less than 0.05 were considered statistically significant.

Results

UFC

A total of 6606 urine collections was obtained from 3461 patients. A single UFC collection was available in 1846 pa-

tients, two UFC collections in 508 patients, and three or more collections in 1107 patients. UFC concentrations were higher than the reference range in 688 collections, yielding 89.6% (95% CI 88.9–90.3%) specificity. Evaluation per patient, *i.e.* the median UFC value if more than one sample had been collected, revealed comparable specificity (91.2%; 95% CI 90.2–92.1%), with only 305 of 3461 patients presenting supernormal UFC concentrations (Fig. 3). The percentage of supernormal UFC values did not vary in relation with the number of 24-h urine collections [9.9% (95% CI 8.3–11.3%), 8.2% (95% CI 6.5–9.95%), and 6.6% (95% CI 5.8–7.4%) for patients with one, two, or three or more UFC collections, respectively]. Specificity of outpatient collections was barely lower than inpatients [90.3% (95% CI 89.0–91.5%) and 92.5% (95% CI 91.1–93.9%); $P < 0.05$]. Men presented slightly but significantly higher UFC levels (49.7 ± 1.04 vs. 43.5 ± 0.59 $\mu\text{g}/24$ h; $P < 0.001$) and a greater prevalence of false positives than women (10.8 vs. 7.2%, $P < 0.05$). UFC concentrations were weakly and negatively correlated with age ($r = -0.10$; $P < 0.001$), and patients with supernormal UFC values were younger than those with normal values (38.5 ± 0.98 vs. 41.5 ± 0.31 yr; $P < 0.01$). Accordingly, specificity was progressively greater across the six age groups ($\chi^2 = 14.2$; $P < 0.05$; Fig. 6). UFC concentrations presented a weak inverse relationship with BMI ($r = -0.10$; $P < 0.05$), and obese subjects (*i.e.* BMI > 30 kg/m²) presented lower UFC levels compared with nonobese subjects (40.9 ± 1.16 vs. 45.9 ± 2.18 $\mu\text{g}/24$ h; $P < 0.05$), thereby achieving slightly greater specificity compared with nonobese subjects (95.0 vs. 90.2% specificity for BMI > 30 and < 30 kg/m², respectively; $P < 0.05$). At logistic regression analysis, gender and age proved significant predictors of supernormal UFC values ($\beta = 0.286$ with 95% CI 0.005 and 0.529, $P < 0.05$ for gender; $\beta = -0.011$ with 95% CI -0.004 and -0.018 , $P < 0.005$ for age), whereas BMI failed to modify the significance of the regression model [$\beta = -0.0013$ with

Cushing's syndrome suspects (number of subjects)

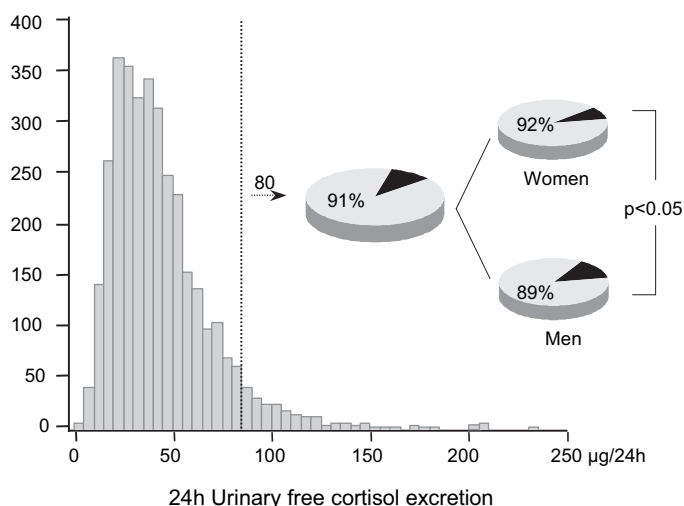


FIG. 3. Twenty-four-hour UFC concentrations in Cushing's syndrome suspects. The median UFC value was considered if more than one collection had been performed. Dashed line indicates the upper limit of the normal range (80 $\mu\text{g}/24$ h). To convert into nmol/24 h, multiply by 2.759. Percentages over gray pie charts indicate specificity over the entire series and among male and female individuals.

95% CI -0.0039 and 0.0013 , nonsignificant (NS)]. Altogether, men were 1.3 times more likely to present abnormal UFC values than women, and the odds of abnormal UFC decreased by 10% every 10 yr in age.

Midnight serum cortisol

Serum cortisol at midnight was measured in 357 patients (286 females, 71 males) and ranged from 0.1–20.8 $\mu\text{g}/\text{dl}$ (Fig. 4). Specificity was very low with the 1.8 $\mu\text{g}/\text{dl}$ cutoff (20.2%; 95% CI 16.0–24.4%) and increased with the higher thresholds [73.9% (95% CI 69.3–78.5%) for 5 $\mu\text{g}/\text{dl}$ and 87.7% (95% CI 84.3–91.1%) for 7.5 $\mu\text{g}/\text{dl}$]. Midnight serum cortisol was positively correlated with age ($r = 0.110$; $P < 0.05$). Accordingly, patients exceeding the 1.8- $\mu\text{g}/\text{dl}$ threshold were older than subjects with less than 1.8 $\mu\text{g}/\text{dl}$ (45.3 ± 0.98 vs. 35.6 ± 1.62 yr; $P < 0.01$). The same was true using the 5- $\mu\text{g}/\text{dl}$ cutoff (46.3 ± 1.75 vs. 42.2 ± 1.00 yr; $P < 0.05$), whereas the difference in age between less than 7.5 $\mu\text{g}/\text{dl}$ and more than 7.5 $\mu\text{g}/\text{dl}$ patients was less pronounced (45.9 ± 2.74 vs. 42.9 ± 0.91 yr; NS). Stratification according to age revealed a progressive increase in the percentage of false positives with the 1.8- $\mu\text{g}/\text{dl}$ threshold ($\chi^2 = 25.6$; $P < 0.01$; Fig. 6), whereas no statistical significance could be detected with the higher cutoffs (both NS). At logistic regression analysis, age proved a significant determinant of cortisol values at midnight greater than 1.8 $\mu\text{g}/\text{dl}$ ($\beta = 0.037$ with 95% CI 0.020 and 0.054; $P < 0.005$), whereas the 5- $\mu\text{g}/\text{dl}$ cutoff was barely significant ($\beta = 0.015$ with 95% CI 0.001 and 0.0298; $P < 0.05$); age failed to predict abnormalities at the 7.5- $\mu\text{g}/\text{dl}$ threshold ($\beta = 0.011$ with 95% CI -0.008 and 0.030; NS). The odds of abnormal cortisol at midnight increased by 9% and by 12% every 10 yr in age for the 1.8 and 5- $\mu\text{g}/\text{dl}$ cutoff, respectively. Conversely, midnight serum cortisol was not affected by gender or BMI (data not shown).

Cushing's syndrome suspects (number of subjects)

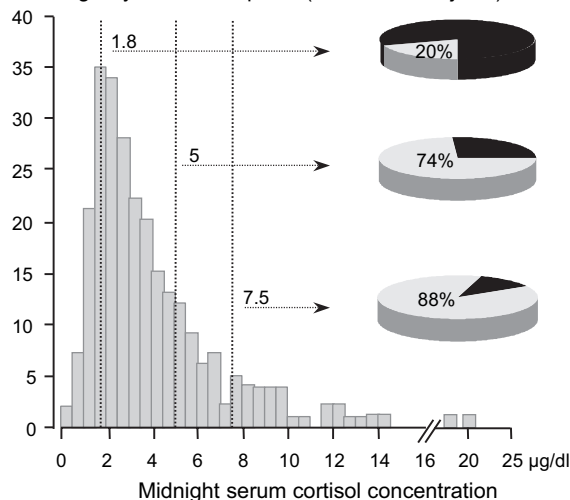


FIG. 4. Serum cortisol concentrations at midnight in Cushing's syndrome suspects. Dashed lines indicate proposed cutoffs, *i.e.* 1.8 $\mu\text{g}/\text{dl}$ (50 nmol/liter), 5 $\mu\text{g}/\text{dl}$ (138 nmol/liter), and 7.5 $\mu\text{g}/\text{dl}$ (207 nmol/liter). To convert into nmol/liter, multiply by 27.59. Percentages over gray pie charts indicate specificity.

OST

Serum cortisol after OST ranged from 0.2–24.5 $\mu\text{g}/\text{dl}$ (Fig. 5). Of 864 patients (661 women, 203 men), 171 subjects failed to suppress cortisol levels less than 1.8 $\mu\text{g}/\text{dl}$, yielding 80.2% (95% CI 77.5–82.9%) specificity. Specificity increased to 97.4% (95% CI 96.3–98.5%) with the 5- $\mu\text{g}/\text{dl}$ cutoff because only 22 patients exceeded this value. Age was again a misleading variable with nonsuppressors (1.8- $\mu\text{g}/\text{dl}$ threshold) appearing older than suppressors (48.3 ± 1.30 vs. 42.5 ± 0.58 yr, for patients with > 1.8 and < 1.8 $\mu\text{g}/\text{dl}$ cortisol after OST, respectively; $P < 0.01$) and, to a lesser extent, with the 5- $\mu\text{g}/\text{dl}$ cutoff (49.4 ± 3.88 vs. 43.5 ± 0.54 yr, for patients with > 5 and < 5 $\mu\text{g}/\text{dl}$ cortisol after OST, respectively; $P = 0.08$). Accordingly, cortisol after OST was positively correlated with age ($r = 0.092$; $P < 0.01$) and, as for midnight serum cortisol, stratification according to age revealed a trend toward age-related increase in false positives ($\chi^2 = 28.0$, $P < 0.01$ for the 1.8- $\mu\text{g}/\text{dl}$ cutoff; $\chi^2 = 5.6$, NS for the 5- $\mu\text{g}/\text{dl}$ threshold; Fig. 6). Age proved a significant predictor of altered OST responses at logistic regression analysis for the 1.8- $\mu\text{g}/\text{dl}$ threshold ($\beta = 0.024$ with 95% CI 0.013 and 0.035; $P < 0.0005$); the coefficient was not significant at the 5- $\mu\text{g}/\text{dl}$ cutoff ($\beta = 0.024$ with 95% CI -0.004 and 0.051; NS). The odds of abnormal cortisol suppression after OST increased by 19% every 10 yr in age. No differences in specificity were observed between inpatients and outpatients, between men and women, and between obese and nonobese subjects (data not shown).

Multiple screening tests

Among patients who performed UFC and OST, specificity of UFC was 69.8% (95% CI 63.9–75.7%) and 65.5% (95% CI 59.4–71.6%) and 93.6% (95% CI 90.5–96.7%) for OST at the 1.8

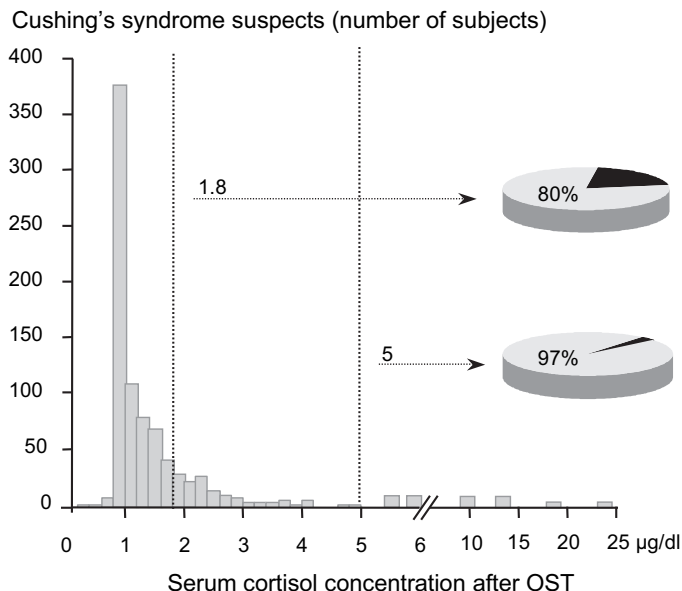


FIG. 5. Serum cortisol concentrations after 1-mg dexamethasone OST in Cushing's syndrome suspects. Dashed lines indicate proposed cutoffs, i.e. 1.8 $\mu\text{g}/\text{dl}$ (50 nmol/liter) and 5 $\mu\text{g}/\text{dl}$ (138 nmol/liter). To convert into nmol/liter, multiply by 27.59. Percentages over gray pie charts indicate specificity.

and 5- $\mu\text{g}/\text{dl}$ thresholds, respectively. Only two patients (0.8%; 95% CI 0–1.9%) presented abnormalities in both tests (UFC > 80 $\mu\text{g}/24$ h, OST > 5 $\mu\text{g}/\text{dl}$). Specificity of UFC was 70.1% (95% CI 65.2–75.0%) among subjects who performed UFC and cortisol at midnight, whereas specificity of the latter was 19.1% (95% CI 14.9–23.3%), 74.3% (95% CI 69.6–79.0%), and 88.0% (95% CI 84.5–91.5%) at the 1.8, 5, and 7.5- $\mu\text{g}/\text{dl}$ thresholds, respectively. Four of 335 (1.2%, 95% CI 0–2.4%) patients who performed UFC and cortisol circadian rhythm presented midnight cortisol higher than 7.5 $\mu\text{g}/\text{dl}$ and supernormal UFC concentrations. In patients submitted to all tests, none presented abnormal responses to all three; specificities were 69.9% (95% CI 60.3–79.5%) for UFC, 67.2% (95% CI 57.2–76.8) and 94.4% (95% CI 89.6–99.2%) for OST at the 1.8 and 5- $\mu\text{g}/\text{dl}$ thresholds, and 15.7% (95% CI 8.1–23.3%), 56.8% (95% CI 46.5–67.1%), and 79.6% (95% CI 81.2–88.0%) for cortisol at midnight at the 1.8, 5, and 7.5- $\mu\text{g}/\text{dl}$ cutoffs. In these patients, cortisol at midnight contributed to two and one false positive, in combination with UFC and OST, respectively. Conversely, no patient presented abnormal UFC and OST and normal circadian rhythm. These data suggest a spectrum of alterations in the hypothalamic-pituitary-adrenal axis in Cushing's syndrome suspects, with some patients presenting greater derangement in cortisol secretion. These patients did not fit in any particular clinical phenotype, possibly a consequence of multiple ailments in the vast majority of Cushing's syndrome suspects in our series.

Comparison of specificity of screening tests

The OST with the 5- $\mu\text{g}/\text{dl}$ cutoff proved superior to all other screening tests (Fig. 7), with UFC proving a close second. Cortisol at midnight using the highest threshold (7.5 $\mu\text{g}/\text{dl}$) presented near to superimposed specificity compared with UFC and was superior to OST with the 1.8- $\mu\text{g}/\text{dl}$ cutoff but was the least specific test at the 1.8- $\mu\text{g}/\text{dl}$ threshold.

Discussion

The choice of optimal laboratory screening procedures for patients in whom the suspicion of Cushing's syndrome has arisen is not firmly established. UFC, midnight serum cortisol, and cortisol suppressibility by low-dose dexamethasone represent three widely used first-line tests for the diagnosis of Cushing's syndrome (1, 2). Over time, the thresholds of these screening procedures have been redefined to maximize sensitivity, aided by the increased specificity of cortisol RIA assays. Indeed, sensitivity is of primary importance in screening tests, in particular for rare diseases such as Cushing's syndrome (16). Some authors (13, 14) suggested that midnight serum cortisol concentrations above 5 or 7.5 $\mu\text{g}/\text{dl}$ should be taken as indicative of Cushing's syndrome, whereas others recommended the lower, 1.8- $\mu\text{g}/\text{dl}$ (50 nmol/liter) cutoff because this appeared the most sensitive (15). The lowering of diagnostic thresholds inevitably carries a price in terms of specificity, but few studies have evaluated the impact of these changes in large populations. Indeed, in most series, the control population against which patients with Cushing's syndrome was compared comprised only small numbers, in some cases even 20–30

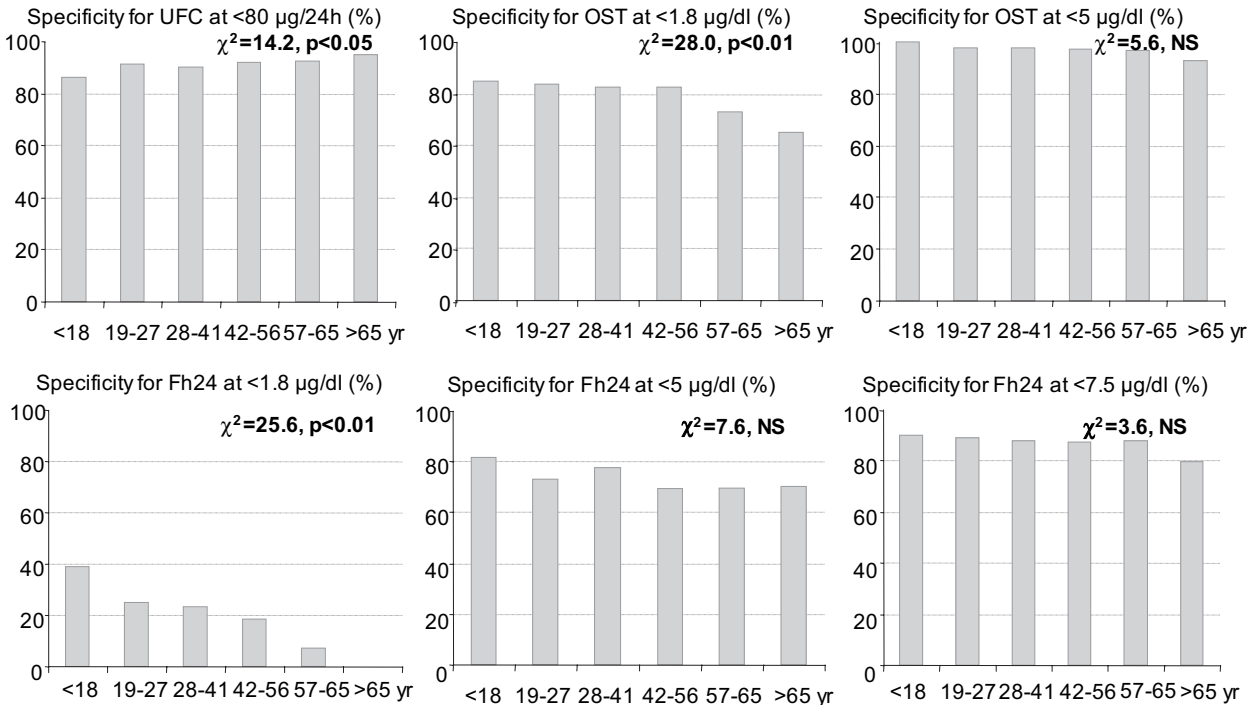
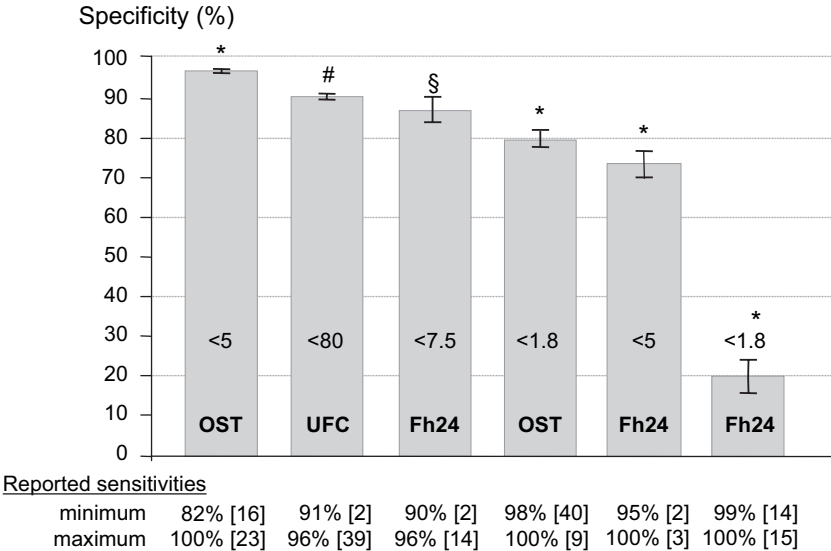


FIG. 6. Specificity of screening tests across age groups. Age percentiles were established according to age distribution and specificity at different cutoffs calculated for each age group. Upper panel, UFC (left graph), cortisol after OST at the 1.8-µg/dl (middle graph) and 5 µg/dl cutoffs (right graph). Lower panel, cortisol at midnight (Fh24) at the 1.8-µg/dl (left graph), 5 µg/dl (middle graph), and 7.5-µg/dl cutoffs (right graph). Significance across age groups is stated for each graph.

subjects (5, 9, 14, 15), thereby allowing only a rough estimate of specificity at best. Larger series (up to 500 controls) date back to the 1980s, when different diagnostic thresholds were used (20–22) and do not provide data applicable to the newest cutoffs. An additional drawback of most studies is that patients with Cushing's syndrome were offset against normal subjects rather than patients with clinical suspicion of hypercortisolism, *i.e.* "Cushing's syndrome suspects" (6, 15). Given the aforementioned results, we evaluated the specificity of screening procedures in a large series of those patients who are most likely to be tested, *e.g.* individuals with

truncal obesity, diabetes, hypertension, mood disorders, and irregular menses. Our study revealed that serum cortisol after 1 mg dexamethasone and UFC achieve superior diagnostic performance, whereas cortisol at midnight should be interpreted with caution. In detail, cortisol suppression by 1 mg dexamethasone yielded the highest diagnostic performance with the 5-µg/dl threshold at 97% specificity, whereas the lower 1.8-µg/dl cutoff resulted in less optimal 80% specificity. As for UFC, concentrations outside the normal range were detected in 10% of Cushing's syndrome suspects. Not surprisingly, these specificities are somewhat less than the

FIG. 7. Comparison of specificity of screening tests for Cushing's syndrome. Specificity and 95% CI are depicted for each test and diagnostic cutoff. Minimum and maximum sensitivity reported in the literature are shown below each test and threshold. References are shown in square brackets. *, $P < 0.001$ vs. all other tests. #, $P < 0.001$ vs. all other tests except for cortisol at midnight with the 7.5-µg/dl cutoff. §, $P < 0.001$ vs. all other tests except for UFC. Fh24, Cortisol at midnight.



near to absolute value obtained using normal subjects as controls (9, 20, 21, 23, 24). The use of even lower thresholds has yielded unacceptable specificity, ranging from 18–73% for UFC (3–5, 25) and 41–68% for OST (3, 5). Among recent studies with the most numerous control groups (up to 130 Cushing's syndrome suspects), 100% sensitivity with OST is reported at 1.2 $\mu\text{g}/\text{dl}$ with 41% specificity (3) or at 3.4 $\mu\text{g}/\text{dl}$ with 94% specificity (4). In these same series, 100% sensitivity with UFC was achieved with values above 55 $\mu\text{g}/24\text{ h}$ and 25 $\mu\text{g}/24\text{ h}$, yielding obviously low specificities, *i.e.*, 73 and 50%, respectively (3, 4). Of note, the upper limit of the normal range in the two aforementioned studies was 75 $\mu\text{g}/24\text{ h}$ and 52.6 $\mu\text{g}/24\text{ h}$, the latter a high-performance liquid chromatography assay. The measurement of dexamethasone levels, an indicator of drug absorption, might clarify nonsuppressing pattern but does not appear practical for screening purposes. We recorded superimposed specificity with the increasing number of UFC collections; on the other hand, obtaining more than one UFC avoids the risk of false negatives and is recommended (1). Reference ranges for UFC differ according to assay and laboratories, and the 80 $\mu\text{g}/24\text{ h}$ threshold reported in this study corresponds to the upper limit of the normal range in our assay. OST and UFC together yielded excellent specificity because only 0.8% of subjects presented abnormal responses to both tests.

Unlike the tests mentioned above, midnight serum cortisol yielded less satisfactory results. In fact, whereas less restrictive criteria (5 and 7.5 $\mu\text{g}/\text{dl}$) allowed the exclusion of Cushing's syndrome with good, although not absolute, reliability (73.9 and 87.7%, respectively), the lowest diagnostic cutoff, *i.e.* 1.8 $\mu\text{g}/\text{dl}$, yielded an unacceptably high proportion of false positives (78.8%). Of note, only one fifth of non-Cushing individuals presented midnight cortisol serum concentrations less than 1.8 $\mu\text{g}/\text{dl}$, which might reflect the difficulty in fulfilling the strict requirements for midnight sampling. In the initial study reporting 100% sensitivity and specificity with the 1.8- $\mu\text{g}/\text{dl}$ threshold and using healthy volunteers as controls, patients were sleeping at the time of sampling, and blood was taken from an indwelling venous catheter (15). The certainty of sleep might be difficult to achieve in everyday clinical practice, even if the protocol is adhered to as closely as possible as we endeavored to, and individual sleeping habits might interfere with cortisol nadir at midnight. Over time, none of the three proposed cutoffs maintained the initially reported 100% specificity that had been achieved in series comprising 10-fold more patients with Cushing's syndrome than Cushing's syndrome suspects (14), just opposite the usual clinical setting. Moreover, smaller series also had failed to confirm 100% specificity of either threshold (3, 5, 14, 26). Assessment of cortisol circadian rhythmicity in saliva rather than serum samples has been proposed as a cost-effective alternative (27, 28) but needs to be validated in large-scale studies.

One feature that has not been fully appreciated in previous studies is the influence of age, gender, and weight on the specificity of screening tests. Gender-related differences were observed for UFC, with male Cushing's syndrome suspects presenting higher UFC levels than their female counterparts, in parallel to what was observed in normal subjects (29), hypertensives (30), and patients with Cushing's disease

(31). Because normal ranges for UFC are the same for both sexes, this leads to a greater prevalence of false positives in male than female Cushing's syndrome suspects. Age is also known to affect hypothalamo-pituitary-adrenal axis activity, most notably nocturnal nadir values (32, 33) and sensitivity to glucocorticoid feedback (34, 35). Accordingly, subjects with absent cortisol circadian rhythm or who failed to suppress after dexamethasone were older than those with normal test responses. Conversely, false positives for UFC were less frequent among the older age percentiles. It is worth underlining that the differing ages of patients with abnormal or normal screening tests spanned the third and fourth decade of life, *i.e.* the age range most commonly affected by Cushing's syndrome. Finally, cortisol secretion and obesity are known to be interlinked in a complex relationship (36), and weight excess might influence the specificity of screening tests. All three tests proved reliable across the entire weight range, with UFC even yielding somewhat higher specificity in obese subjects. Indeed, this finding tallies with lower UFC values in obese subjects (37).

Our study focused on the likelihood of not having Cushing's syndrome although some clinical/biochemical features of hypercortisolism are present. We have shown that the percentage of false positives varies according to applied cutoffs, from 80% with the 1.8- $\mu\text{g}/\text{dl}$ threshold for cortisol at midnight to 3% with the 5- $\mu\text{g}/\text{dl}$ cutoff after OST. In these patients, the clinician can either proceed with further, second-line testing, such as the dexamethasone-suppressed CRH test or desmopressin stimulation (12) or adrenal imaging looking for adrenal incidentalomas, a frequent cause of borderline test results (38), or else proceed more cautiously and maintain vigilant follow-up. Further studies will hopefully clarify which approach is best.

In conclusion, the choice between tests and thresholds capable of identifying all patients with Cushing's syndrome without including unaffected subjects requires a careful balancing act. In this large series of Cushing's syndrome suspects, UFC and cortisol suppression after 1 mg dexamethasone achieved satisfactory specificity. Conversely, serum cortisol concentrations at midnight should be interpreted with caution, especially with the 1.8- $\mu\text{g}/\text{dl}$ cutoff. Furthermore, the influence of age and sex on specificity of screening tests, especially for males or middle-aged subjects, should enter the clinical judgment. These findings carry significant clinical implications for exclusion of Cushing's syndrome by screening tests in the increasing population of obese, depressed, hypertensive, and diabetic patients.

Acknowledgments

Received March 15, 2007. Accepted August 6, 2007.

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Disclosure Statement: The authors have nothing to declare.

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