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Factors Influencing the Adrenocorticotropin Test: Role of Contemporary Cortisol Assays, Body Composition, and Oral Contraceptive Agents

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Context: The normal cortisol response to an ACTH test remains inconsistently defined, possibly caused by various subject- and test-condition-related factors.

Objective: Our objective was to evaluate the impact of newer automated immunoassays; gender, age, body composition, and endogenous sex-hormone levels; corticosteroid-binding globulin levels; and test conditions (fasting/nonfasting, rest/intermittent exercise).

Methods: A 250-μg ACTH test (0800–1000 h) was performed in 100 unmedicated subjects, 13 women taking oral contraception (OC), and six men with nephrotic syndrome. Tests were performed fasting supine (n = 119), nonfasting supine (n = 38), and fasting with intermittent exercise (n = 45). Serum cortisol was analyzed by three immunoassays.

Results: Even with a negligible between-assay mean bias, individual samples from unmedicated subjects differed by as much as 110 nmol/liter. The normative 2.5th percentile for total cortisol ranged from 475–523 nmol/liter when analyzed by the three assays. In multivariate analyses, 30-min total cortisol was predicted by baseline cortisol (men plus women) and central adiposity (men) but not by gender, age, and endogenous sex hormones, corticosteroid-binding globulin, fasting/nonfasting, and exercise. Compared with unmedicated subjects, OC women had 2-fold elevated 30-min cortisol ($P < 0.001$) but lowered calculated free cortisol ($P < 0.001$), whereas nephrotic syndrome patients had lowered 30-min cortisol ($P < 0.01$) in two of three assays, but similar calculated free cortisol ($P > 0.1$).

Conclusion: The normal response to an ACTH test is assay specific, even with newer methods, and this also applies to calculated free cortisol. Both total cortisol and calculated free cortisol were severely affected by OC, and the test is therefore only reliable if OC has been discontinued. The ACTH test is, however, robust for most of the other evaluated subject- and test-condition-related factors. **(*J Clin Endocrinol Metab* 92: 1326–1333, 2007)**

THE ACTH TEST is used to assess adrenocortical function and guides the decision for the need of glucocorticoid replacement. The test is often used in clinical practice because of its simplicity and absence of contraindications and unpleasant side effects. It has certain limitations and carries a risk of false-normal results, *e.g.* in the early phase after pituitary surgery as well as in patients with partially degenerated adrenals. This has caused some clinicians to favor central stimulating tests such as the insulin hypoglycemia or the glucagon test. However, irrespective of test preference, common problems exist relating to the chosen cutoff level distinguishing normality from hypocorticism. The cutoff level is likely to be affected by the test in question but also by the cortisol assay used (1) and issues such as medical treatment with estrogens (2, 3) and possibly anticonvulsants (2), hypoproteinemia caused by liver (4) or kidney diseases (5), and position during blood sampling (6). Gender has been shown to be an influencing factor by some (1, 3) but not by others (7), whereas obesity (8) and menstrual cycle (3, 9) do not seem to influence the results.

We aimed to establish normative reference intervals for total cortisol and free cortisol indices during the 250-μg ACTH test with a focus on the influence of gender, age, endogenous sex hormones, and body composition, elevated and lowered corticosteroid-binding globulin (CBG) levels, fasting/nonfasting, and intermittent exercise as measured by three different commonly used automated immunoassays.

Subjects and Methods

Subjects

Pregnant or breastfeeding women and individuals treated with glucocorticoids or spironolactone were excluded from participation. All participants had thyroid function testing. Two persons were excluded due to elevated serum TSH. A total of 50 healthy men and 50 healthy women not taking oral contraceptives (OC) (36 premenopausal, 14 postmenopausal) were included. In addition, 13 premenopausal women all taking estrogen containing OC (three received 20 μg ethinyl estradiol, four 30 μg, and six 35 μg, always in combination with various progestins) and six men with acute nephrotic syndrome with proteinuria greater than 1.6 g/24 h (tested before initiation of glucocorticoid treatment) were included for examination of the influence of altered CBG concentrations. Ethical approval was obtained, and all participants gave written informed consent.

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Abbreviations: BMI, Body mass index; CBG, corticosteroid-binding globulin; CFC, calculated free cortisol; CI, confidence interval; CV, coefficients of variation; FCI, free cortisol index; FIA, fluoroimmunoassay; FM_{abd}, abdominal fat mass; HPA, hypothalamic-pituitary-adrenal; OC, oral contraceptive; TFM, total fat mass.

Methods

Participants rested 15 min before testing after inserting an indwelling catheter in a large forearm vein. An ACTH test was performed between 0800 and 1000 h, after an overnight fast, administering 250 μg iv $ACTH_{1–24}$ (Synacthen; Novartis Healthcare, Copenhagen, Denmark). All participants were tested in a supine position with sampling at baseline and at 30 and 60 min. Plasma and serum samples were stored at −80 C before analysis. All samples from each subject were measured in the same assay series. Premenopausal women not taking OC were tested in their follicular phase and OC women on the d 10–16 of a new sequence of contraceptive pills. Forty-five subjects were retested for studying the influence of intermittent activity including the first 10 women included in the following groups: postmenopausal women, premenopausal women taking or not taking OC, and 15 age-matched men. Participants were instructed to walk a standardized distance of 100 m every 5 min. To study the importance of fasting, 38 persons were retested in a supine nonfasting setting. The sequence of testing was randomized.

Weight, height, and waist and hip circumferences were assessed in all, whereas body composition using dual-energy x-ray absorptiometry was assessed only in individuals tested in the nonfasting setting. Blood was analyzed for cortisol, CBG, estradiol, testosterone, LH, FSH, and SHBG.

Analytical methods

Total cortisol was analyzed by three different assays: 1) electrochemiluminescence immunoassay (Modular Analytics E170; Roche, GmbH, Mannheim, Germany) with intra- and interassay coefficients of variation (CV) of 1.0–1.7 and 1.4–2.8%, respectively; 2) luminoimmunoassay (Immulite 2000, Diagnostics Products Corp., Los Angeles, CA) with intra- and interassay CV of 6–7 and 8–10%, respectively; and 3) fluoroimmunoassay (FIA) (autoDelfia; Perkin-Elmer, Turku, Finland) with intra- and interassay CV of 0.8–1.9 and 2.9–3.6%, respectively. Data relate to the results from the electrochemiluminescence immunoassay if not otherwise stated. Serum CBG was measured by RIA (BioSource, Nivelles, Belgium) with intra- and interassay CV of 4 and 11–13%, respectively. Estradiol was analyzed by RIA (Pantex, Santa Monica, CA) with intra- and interassay CV of 7.5 and 12.3%, respectively, at a concentration of 75 pmol/liter and 4.3 and 12.9%, respectively, at a concentration of 394 pmol/liter. Testosterone was analyzed by RIA (Diagnostic Products) with intra- and interassay CV of 1.3 and 2.8%, respectively, at a concentration of 4.8 U/liter and 1.9 and 4.5%, respectively, at a concentration of 51.4 U/liter. FSH was measured by FIA (Delfia, Wallac, Finland) with intra- and interassay CV of 1.3 and 2.8%, respectively, at a concentration of 4.8 U/liter and 1.9 and 4.5%, respectively, at a concentration of 51.4 U/liter. LH was measured by FIA (h-LH spec; Delfia) with intra- and interassay CV of 1.7 and 4.4%, respectively, at a concentration of 4.4 U/liter and 1.5 and 4.5%, respectively, at a concentration of 25.0 U/liter. SHBG was measured by FIA (Delfia, Finland) with intra- and interassay CV of 3.1 and 4.3%, respectively, at a concentration of 17 nmol/liter and 5.1 and 4.0%, respectively, at a concentration of 96 nmol/liter.

Calculated free cortisol (CFC) was calculated as described by Coolens *et al.* (10): $CFC = \sqrt{[(0.0167 + 0.182(CBG - T))^2 + 0.0122T]} - 0.0167 + 0.182(CBG - T)$ wherein T correspond to total cortisol. Free cortisol index (FCI) was expressed as total cortisol (nmol/liter)/CBG (nmol/liter).

Dual-energy x-ray absorptiometry (model XP-26/XR-46; Norland Medical Systems, Fort Atkinson, WI) was performed as whole-body scans with separate assessment of the three compartments: total fat mass (TFM), total lean tissue mass, and total bone mineral content. Data on the regional distribution of body components were obtained for abdomen and trunk (thorax plus abdomen). The in-house intraoperator variation was 5%.

Statistics

In the healthy unmedicated subjects, total cortisol followed a Gaussian distribution, whereas CFC and FCI were log Gaussian distributed and thus log transformed before analyses. Data are given as mean (2 SD) (CFC after back transformation), except from subgroup-related reference intervals that are given as median (2.5–97.5th percentiles). Because samples from different populations may differ, 95% confidence intervals (CI) for the 2.5th percentile were calculated, and adjusted 2.5th percentiles thereafter defined as follows: 2.5th percentile − 1.95 × SE (11, 12). Between-group comparisons of continuous data were analyzed by ANOVA with *post hoc* Bonferroni correction. Within-subject differences during different test conditions were analyzed by paired *t* test. Cortisol assay comparison was performed calculating the 2.5 and 97.5% limits of agreement. Correlation analyses were used for trends. Uni- and multivariate regression analyses were conducted to analyze the association between 30-min stimulated total cortisol and dependent variables (CBG, baseline cortisol, and body composition measures). In all cases, a difference was considered significant when $P < 0.05$. All statistical analyses were performed by SAS version 9.1.

Results

Total cortisol: between-assay variability

In healthy subjects not taking OC, the mean between-assay bias ratio ranged from 1–4% at baseline and from 1–9% after ACTH stimulation, with 95% CI indicating that we would expect the cortisol results to differ by as much as 27% between assays (Table 1). Method agreement at 30 min is illustrated in Fig. 1. Using the comparison of Modular and Immulite as an example, it illustrates that despite a negligible mean average bias, for a given individual, results could differ by up to 110 nmol/liter with any discrepancy being approximately equally likely in either direction.

With one exception, the assays performed equally in the low as compared with the high range of the measured cor-

TABLE 1. Bias ratios between three different cortisol assays after mutual comparison

Method	Bias ratio				
	HC total	HC low range	HC high range	OC	Nephrotic syndrome
Modular *vs.* Immulite					
0 min	1.03 (0.24)	1.01 (0.20)	1.06 (0.26)[c]	1.22 (0.36)[b]	1.41 (1.08)[b]
30 min	1.01 (0.16)	1.01 (0.20)	1.02 (0.14)	1.19 (0.40)[b]	1.28 (0.68)[b]
Modular *vs.* Delfia					
0 min	1.04 (0.16)	1.01 (0.18)	1.07 (0.14)	1.43 (0.28)[b]	1.32 (0.58)[b]
30 min	1.09 (0.14)[a]	1.08 (0.14)	1.09 (0.14)	1.19 (0.28)[b]	1.40 (0.26)[b]
Immulite *vs.* Delfia					
0 min	1.01 (0.20)	1.00 (0.16)	1.01 (0.24)	1.19 (0.40)[b]	0.97 (0.46)
30 min	1.06 (0.18)[a]	1.06 (0.20)	1.07 (0.16)	1.38 (0.30)[b]	1.12 (0.52)

Data are given as mean (2 SD). HC, Healthy controls.

[a] $P < 0.001$ compared with bias ratio at baseline.

[b] $P < 0.05$ compared with bias ratio of healthy controls.

[c] $P < 0.05$ compared with low range. Low and high range were defined as below or above mean cortisol, measured by Modular (0 and 30 min, respectively).

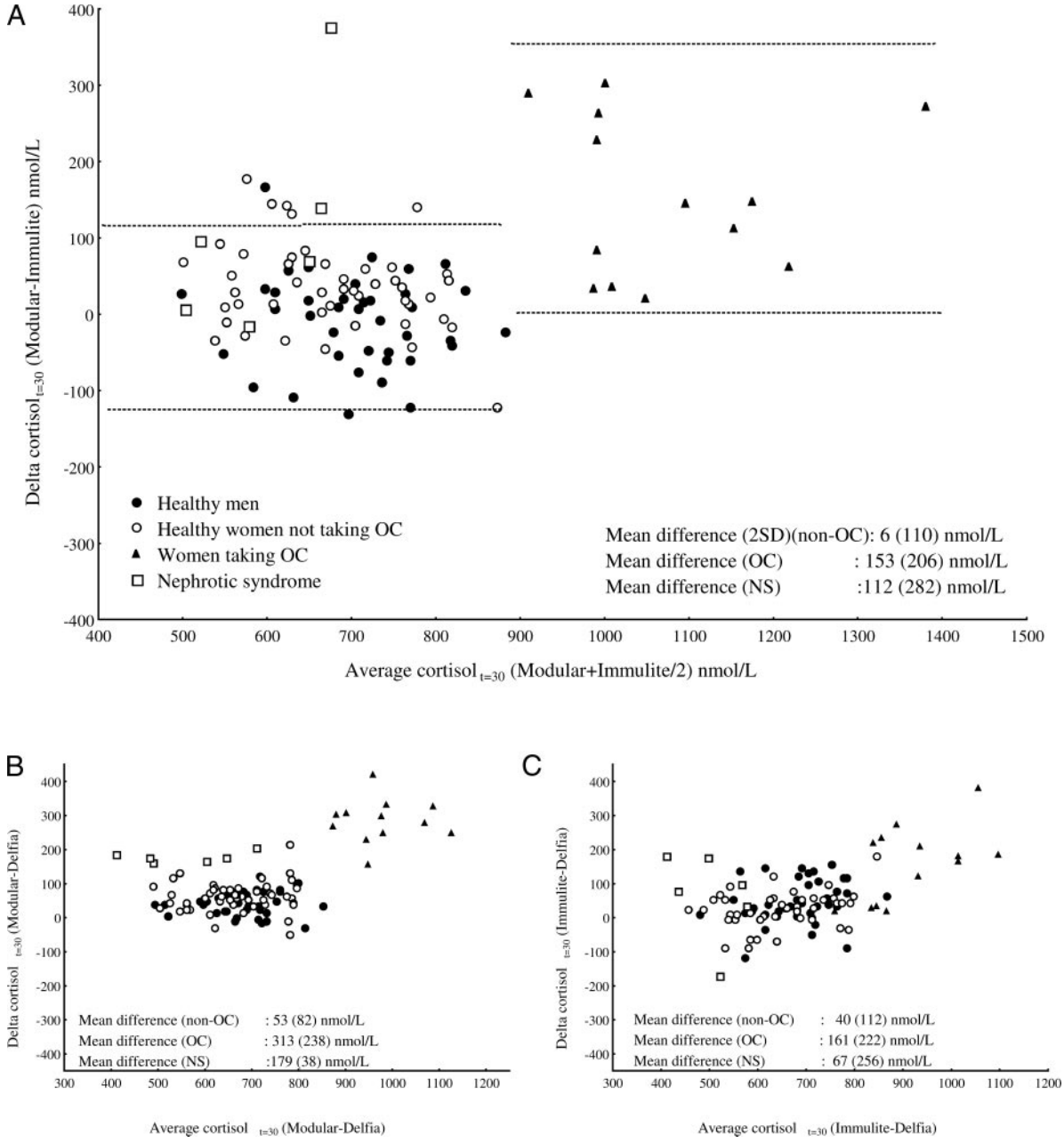

FIG. 1. Bland-Altman plots illustrating the difference between total cortisol concentrations measured by different assays plotted against the average of the assays compared. A, Comparison of Modular and Immulite; B, comparison of Modular and Delfia; C, comparison of Immulite and Delfia. The mean estimates the average bias of one assay relative to the other. The *dotted lines* indicate the 95% limits of agreement in healthy men and women not taking OC and women taking OC, respectively. The limits of agreement estimate how likely the methods are to agree for a given individual. ●, Healthy men; ○, women not taking OC; ▲, women taking OC; □, nephrotic syndrome.

tisol levels (Table 1). On the contrary, the assays performed differently at baseline and after stimulation, with Delfia measuring lower baseline cortisol compared with Modular ($P < 0.001$) as well as lower stimulated cortisol compared with both Modular ($P < 0.001$) and Immulite ($P < 0.001$).

In OC women, higher concentrations were systematically measured with Modular as compared with both Immulite and Delfia as well as with Immulite compared with Delfia (positive mean bias at 30 min: 153, 313, and 161 nmol/liter, respectively). The same tendency was observed in patients with nephrotic syndrome (Table 1 and Fig. 1).

Total cortisol and CFC: normative reference intervals

The distributions and the corresponding adjusted 2.5th percentiles of total and free cortisol measured by the three different assays showed significant differences among assays at baseline as well as after ACTH stimulation in healthy subjects not taking OC, women taking OC, and in patients with nephrotic syndrome (Table 2, only 30-min values shown). Applying commonly used cutoffs of 500 and 550 nmol/liter would have caused false abnormal results in a substantial number of the healthy subjects included (Table 2),

TABLE 2. Normative 95% reference intervals and adjusted 2.5th percentiles for total cortisol and CFC when measured by three different assays

	Total cortisol, 30 min	Adjusted 2.5th percentile, 30 min	False positive (%)		CFC, 30 min	Adjusted 2.5th percentile, 30 min
			500 nmol/liter	550 nmol/liter		
HC						
Modular	691 (523–851)[b]	494	0	6	61.2 (31.8–120.0)[b]	24.7
Immulite	688 (487–841)[b]	449	1	7	58.7 (28.9–103.4)[b]	21.2
Delfia	644 (475–805)	442	7	14	52.0 (27.8–112.6)	21.6
Nephrotic syndrome (n = 6)						
Modular	628 (505–813)		0	17	75.1 (35.7–157.8)	
Immulite	542 (438–615)		50	50	53.5 (17.2–166.6)	
Delfia	465 (319–607)[a]		50	67	42.8 (19.9–91.9)[a]	
OC (n = 13)						
Modular	1125 (1005–1517)		0	0	31.6 (24.9–40.5)	
Immulite	992 (767–1247)[a,b]		0	0	25.1 (16.6–38.0)[a,b]	
Delfia	829 (728–1003)[a]		0	0	19.4 (14.4–23.5)[a]	

Reference intervals are given as median (2.5–97.5th percentiles). CFC values were back transformed after initial log transformation. The adjusted 2.5th percentiles were defined as: 2.5th percentile − 1.96 × SE. False-positive rates are given as the percentage of all subjects with a stimulated cortisol value below 500 or 550 nmol/liter, respectively. HC, Healthy controls.

[a] $P < 0.05$ compared with Modular.

[b] $P < 0.05$ compared with Delfia.

half of whom had between-assay discrepancies of more than 100 nmol/liter. Two healthy subjects failed a cutoff of 550 nmol/liter in all assays, whereas seven failed to reach a level of 500 nmol/liter in at least one of the assays.

In patients with nephrotic syndrome, 30-min total cortisol correlated positively with albumin (r = 0.75; $P = 0.09$) and CBG (r = 0.76; $P = 0.08$), and the reference interval was, accordingly, highly dependant on the CBG concentration in the included patients. Choosing a cutoff level of 500 nmol/liter in these patients resulted in insufficient tests in 0, 50, and 50% using Modular, Immulite, and Delfia, respectively.

Influence of subject-related factors on 30-min cortisol

Data on subject-related factors only included data from healthy subjects not taking OC and were based on total cortisol measured by Modular. Results are shown in Table 3.

Univariate analyses showed 30-min stimulated total cortisol to be unaffected by gender ($P = 0.8$) and age ($P = 0.1$) but positively related to baseline cortisol ($P < 0.001$) and CBG ($P = 0.01$). The relation to CBG, however, did not reach significance in women as a subgroup.

The influence of body composition was gender specific. In men, univariate analyses showed that 30-min total cortisol increased with increasing waist/hip ratio ($P = 0.007$), TFM ($P = 0.02$), and abdominal fat mass (FM_{abd}) ($P = 0.005$), whereas no significant relation was found to body mass index (BMI) ($P = 0.1$). In women, 30-min total cortisol was unrelated to all the given body composition measures ($P > 0.5$). In both men and women, the change in 30-min cortisol was positively correlated to BMI ($r_{men} = 0.64$; $r_{women} = 0.51$), TFM ($r_{men} = 0.79$; $r_{women} = 0.61$), and FM_{abd} ($r_{men} = 0.80$; $r_{women} = 0.62$).

In men, testosterone tended to be negatively related to 30-min stimulated total cortisol ($r_{cort30} = -0.3$; $P = 0.09$). This

TABLE 3. Results from uni- and multivariate regression analyses on the dependency of subject-related factors on 30-min ACTH-stimulated total cortisol

$Cortisol_{t=30}$	Univariate		Multivariate (men)	
	Men (n = 49[a])	Women (n = 50)	Model A (n = 14)	Model B (n = 49)
Reference concentration (intercept)			360 (114)	−86.5 (167.1)
Age (yr)	1.3 (0.8)	0.73 (1.01)		
$Cortisol_{t=0}$ (nmol/liter)	0.25 (0.10)[b]	0.54 (0.07)[b]	0.59 (0.16)[b]	0.28 (0.11)[b]
CBG (nmol/liter)	0.30 (0.12)[b]	0.16 (0.09)	−0.03 (0.11)	0.22 (0.11)
BMI (kg/m^2)	6.8 (4.5)	1.63 (4.15)		
Waist/hip ratio	677 (235)[b]	134 (294)		
Waist (cm)	4.75 (1.87)[b]	0.72 (1.84)		5.72 (1.57)[b]
FM_{abd} (kg)	28.2 (8.3)[b,c]	8.8 (11.5)[d]	30.1 (6.0)[b]	
TFM (kg)	7.3 (2.6)[b,c]	−0.09 (2.45)[d]		
Testosterone (nmol/liter)	−6.71 (3.86)			
LH (IU/liter)	−9.87 (7.50)			
Estrogen (nmol/liter)		−0.12 (0.11)		
FSH (IU/liter)		0.66 (0.47)		
R^2			0.78	0.46

Results are given as regression coefficients (SE). The regression coefficients estimate changes in mean concentrations. R^2 is the estimated variance explained by the model.

[a] One outlier was excluded.

[b] $P < 0.05$.

[c] n = 14.

[d] n = 25.

association disappeared after adjustment for body composition. Free testosterone index, estrogen, LH, and FSH were all unrelated to 30-min total cortisol (in all $P > 0.1$). All parameters of the gonadal axis were unrelated to 30-min total cortisol ($P > 0.2$) in women.

Multivariate regression analyses were performed in men to estimate the independent role of the parameters from the univariate analyses on the 30-min cortisol level. A model including baseline cortisol, CBG, and FM_{abd} explained 78% of the total variance of 30-min total cortisol, with baseline cortisol and FM_{abd} being the only independent explanatory variables. Replacing FM_{abd} with waist circumference yielded a model explaining 45% of the total variance with baseline cortisol and waist being the only independent explanatory variables. Replacement with BMI yielded a model explaining only 31% of the total variance of 30-min cortisol, and BMI was not an independent explanatory factor ($P = 0.4$).

Influence of fasting and intermittent activity

The 30-min stimulated plasma cortisol was similar in the fasting supine setting compared with the nonfasting supine setting [mean differences (95% CI), 10.4 nmol/liter (−12.8 to 33.6); $P = 0.4$] and in the supine setting compared with intermittent exercise in an upright position [mean differences, 3.5 nmol/liter (−21.7 to 28.7); $P = 0.8$].

Influence of OC and nephrotic syndrome

CBG was unchanged over the sampling period ($P = 0.1$). A 2- to 3-fold elevated CBG level was observed at all time points in OC women ($P < 0.001$), whereas patients with nephrotic syndrome had lower CBG levels ($P = 0.04$) compared with healthy men and women not taking OC. In the total study population, CBG was log related to baseline and 30-min total cortisol (Fig. 2, A and B). Concordantly, significantly higher baseline and stimulated cortisol concentrations were observed in women taking OC compared with the other subgroups (Fig. 3A).

In the total study population, CBG and baseline CFC were uncorrelated, whereas CBG showed an inverse log relation to 30-min CFC (Fig. 2, C and D). Concordantly, CFC (and FCI) was not significantly different comparing the five subgroups at baseline, whereas stimulated CFC (and FCI) levels were

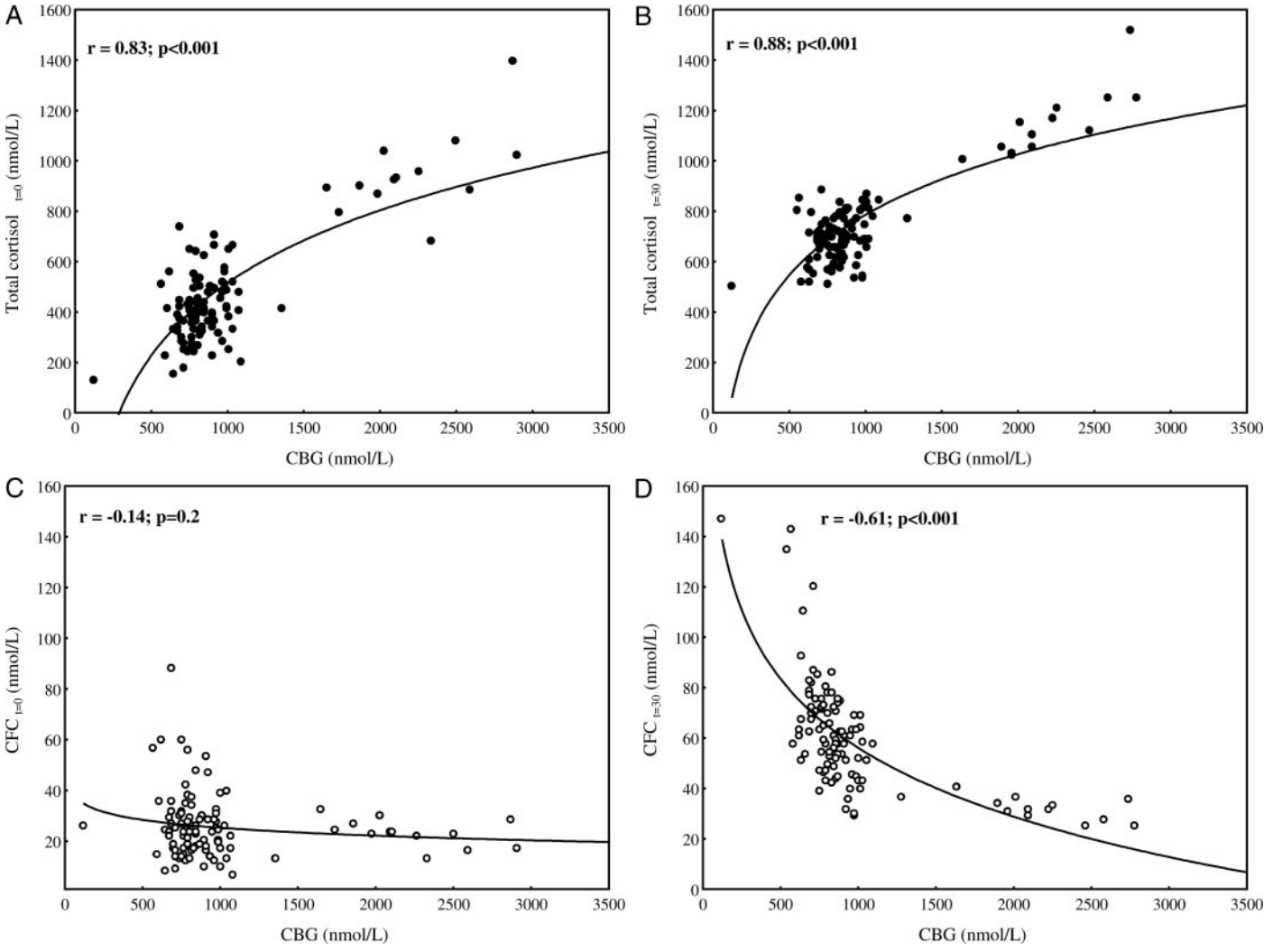

FIG. 2. Correlation between CBG and baseline total cortisol (A), 30-min total cortisol (B), baseline CFC (C), and 30-min CFC (D). Nonlinear fits and related equations are given for each graph. Data are based on total cortisol measured by Modular.

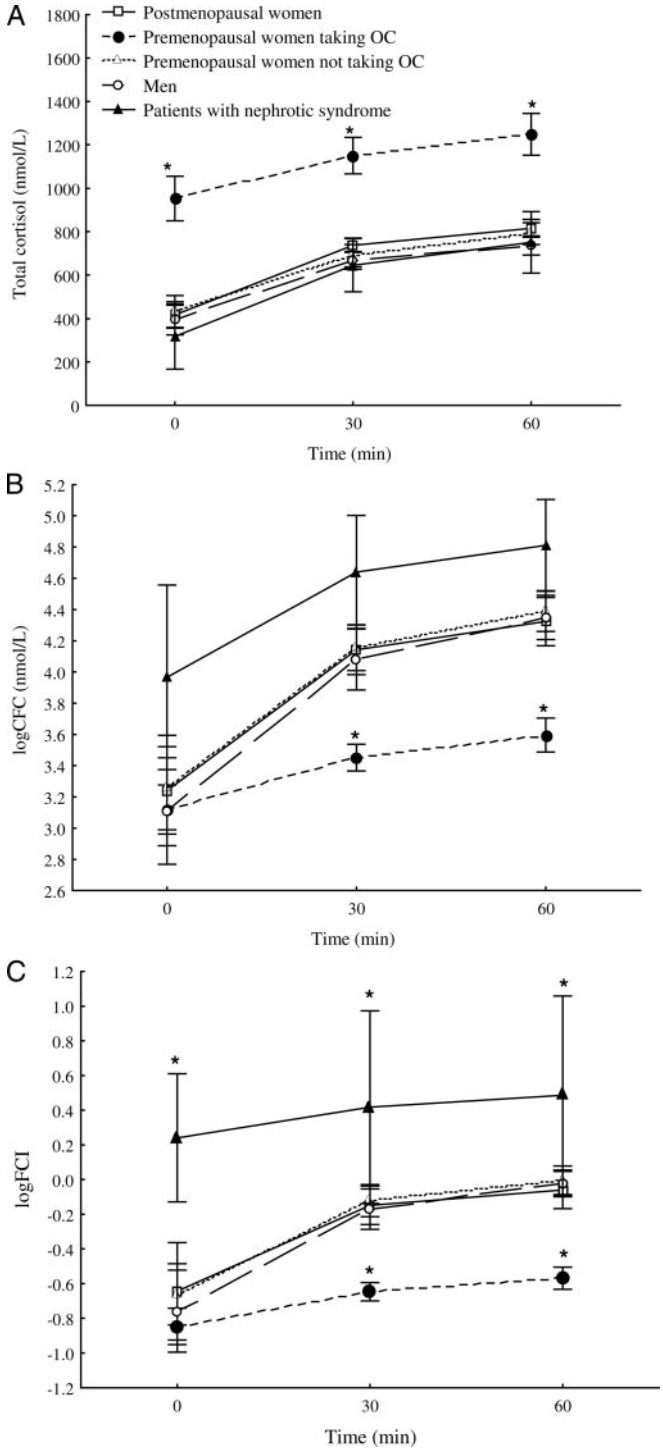

FIG. 3. Total cortisol (A), log-transformed CFC (B), and log-transformed FCI (C) responses to 250 μg ACTH stimulation in women taking OC (●), postmenopausal women (□), premenopausal women not taking OC (△), men (○), and nephrotic syndrome (▲). Data are given as mean, and *whiskers* indicate the 95% CI. *, $P < 0.01$. Data are based on cortisol measured by Modular.

significantly lower in women taking OC compared with the other subgroups (Fig. 3, B and C). Similarly, healthy subjects with CBG above the mean had significantly lower CFC levels compared with those with CBG below the mean ($P < 0.0001$).

Discussion

In a large study of well-defined healthy subjects, we identified the normal response to an ACTH test to be highly assay specific. The use of OC had a tremendous impact, with a substantial risk of misclassification using total cortisol as well as indices of free cortisol. Baseline cortisol and markers of fat mass (men only) were independent positive predictors of the 30-min ACTH-stimulated cortisol concentration, whereas it was robust for the other subject- and test-condition-related factors evaluated.

We presented 2.5th percentiles and adjusted 2.5th percentiles. These should not be confounded with diagnostic cutoffs or decision limits that are normally somewhat higher, and which would require comparison with patients with suspected hypothalamic-pituitary-adrenal (HPA) deficiency by ROC curve analyses. By comparing three currently widely used automated commercial immunoassays, we found the normative reference limits to be assay dependent. All assays performed equally at the low compared with the high range of the cortisol levels, whereas Delfia measured systematically lower stimulated values compared with the other assays. Assay dependency is in accordance with the findings by Clark *et al.* (1), and could to some extent be explained by assay sensitivity and the release of corticosteroids other than cortisol affecting the assays differently. It was also evident that even with a negligible average bias, individual samples may differ by up to 110 nmol/liter when measured by different assays. This may be of clinical relevance, because application of widely used cutoff levels of 500 (13, 14) and 550 nmol/liter (6, 15, 16) would have caused a substantial percentage of healthy individuals, with no clinical or biochemical evidence of HPA deficiency and thus a very low pretest probability of adrenal insufficiency, to be at risk of being misclassified as insufficient by some assays but not by others. The finding of a relatively high proportion of healthy subjects not passing the originally suggested cutoff of 550 nmol/liter was not surprising, because the criterion for a normal cortisol level for the ACTH test has changed over the years, possibly because of reduced interference by noncortisol substances in the newer assays (better detection and separation techniques, *etc.*). In the subgroup of women taking OC and patients with nephrotic syndrome, the interassay agreement was even worse, possibly reflecting a systematic error caused by different effects on the displacement reaction of cortisol from CBG and cross-reactivity. In the nephrotic patients, testing was performed in the acute inflammatory phase with a likely increased excretion of cortisol and other steroid metabolites having a different impact on the evaluated assays. So, as for every hormone, our findings underline the necessity of local assay-specific cortisol cutoff levels and caution interpreting results from patients with altered CBG levels.

We identified baseline cortisol as an independent predictor of 30-min total cortisol in both men and women, whereas markers of fat mass showed sexual dimorphism as predictive only in men. A hypersensitive secretory response of cortisol to a variety of factors is well established in obesity (17–20) This study is, however, the first to show the independent role of central fat accumulation on 30-min ACTH-stimulated total cortisol, indicating that there may be a need of central-fat-

specific cutoff points at least in men. This finding, however, needs to be confirmed.

No gender difference was observed, and we therefore do not find any indication for gender-specific cutoffs. This is in accordance with some (7, 21) but not others (1, 22). Cortisol was normally distributed in our group of healthy subjects not taking OC. This is inconsistent with the findings by, *e.g.* Clark *et al.* (1), which may possibly be explained by their unintentional inclusion of estrogen-treated women. Recent studies have demonstrated an influence of altered estradiol/progesterone (23) and testosterone levels (24) on the HPA axis. In this study, the negative relation between 30-min total cortisol and endogenous testosterone in men was explained by BMI.

Testing in a fasting *vs.* nonfasting state did not influence the 30-min cortisol levels. We did not standardize the time span from food intake, which may account for the discrepancy with previous studies reporting a sustained increase in the cortisol release in relation to food intake (18, 25). From a clinical point of view, these results indicate that it seems unimportant whether the test is performed in a fasting setting. Posture markedly affects the concentration of nonfilterable blood constituents such as CBG (26), and a significant decrease in CBG and total cortisol concentrations has been reported the first 30 min going from a standing to a supine position (6). In this study, CBG concentrations were unchanged during the sampling period. The first samples were standardized to be drawn 15 min after inserting an indwelling catheter, and the most significant decline in the CBG level therefore seems primarily to take place during the first 15 min going from a standing to a supine position. To our knowledge, no study has yet evaluated the difference between cortisol concentrations taken in a supine *vs.* a sitting setting; until then our data indicate that it would be advisable to place the patient in a supine position at least 15 min before sampling. Stimulated cortisol concentrations were unaffected by intermittent light exercise in an upright position, which seems to allow for a toilet visit, for example, during the test.

We found a markedly elevated CBG and baseline and stimulated total cortisol in women taking OC. These are all well-known changes implicating a substantial risk of underestimating hypocortisolism in HPA-deficient women taking OC if OC treatment has not been discontinued before testing. A cortisol increase of more than 200 nmol/liter has been suggested as an alternative definition of a normal response. Because more than half of the OC women in the current study had a response of less than 200 nmol/liter, this alternative remains inappropriate.

Approximately 80% of total cortisol circulates bound to CBG, and alterations in the CBG concentration may therefore cause misleading results if only total hormone concentrations are measured. Free cortisol is considered the bioactive part that unfortunately remains inappropriate for routine analysis. Alternatives for evaluation of the free fraction have been sought, including FCI and CFC, with CFC having the advantage of taking the CBG saturation into account. Baseline values of both indices are highly correlated with free cortisol measured by ultrafiltration (27) or equilibrium dialysis (10), independent of CBG. Accordingly, we found baseline CFC to be within the normal range in OC women. This is in agreement with previous studies, arguing that indices of free cortisol could provide a better discrimination than total cortisol between normality and hypocortisolism in case of CBG alterations (2, 6, 28). Baseline concentrations are, however, rarely used, because most patients have intermediate values and therefore require dynamic testing. We reported that the CFC response declined with increasing CBG, being highly blunted in OC women. The most probable explanation is that the Coolens equation is invalid at very high concentrations during stimulation, in particular in those with altered CBG levels. ACTH-stimulated CFC and serum free cortisol have been reported to be linearly related in subjects with CBG within the normal range (29). ACTH stimulation has, moreover, been shown to cause a higher increase in serum free cortisol in a patient with absent CBG compared with healthy controls (30), and it is thus likely to suspect a blunted increase in free cortisol in subjects with high CBG. Although speculative, this theory is supported by Kirschbaum *et al.* (3), who showed a blunted salivary free cortisol response to ACTH stimulation in women using OC compared with medication-free women and later suggested a compensation at the level of the target tissue by increased glucocorticoid sensitivity (31). However, independent of the physiological explanation, we showed that although baseline CFC is within the normal range in subjects with altered CBG concentrations, the stimulated values are not necessarily so. Thus, relying on a reference interval of stimulated CFC in healthy individuals not taking OC would involve a risk of overestimating hypocortisolism in women taking OC.

In conclusion, method-related differences are highly important and must be accounted for also with newer automated cortisol assays. Local assay-specific cutoff levels for total cortisol and CFC are therefore necessary in the evaluation of the HPA axis. Because the use of OC causes a substantial risk of misclassification using both total cortisol and CFC, test results should be relied upon only if OC has been discontinued. This study is the first to show the independent role of fat mass on 30-min ACTH-stimulated total cortisol in men, indicating a possible need of central-fat-specific cutoffs. The ACTH test, however, seems robust regarding the other subject- and test-condition-related factors evaluated.

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