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Scandinavian Journal of Clinical and Laboratory Investigation

ISSN: 0036-5513 (Print) 1502-7686 (Online) Journal homepage: https://www.tandfonline.com/loi/iclb20

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To cite this article: Kristine Kollerøs Panton, Gustav Mikkelsen, Wenche Øiestad Irgens, Ann Kristin Hovde, Marte Wien Killingmo, Monja Airin Øien, Per Medbøe Thorsby & Arne Åsberg (2019) New reference intervals for cortisol, cortisol binding globulin and free cortisol index in women using ethinyl estradiol, Scandinavian Journal of Clinical and Laboratory Investigation, 79:5, 314-319, DOI: 10.1080/00365513.2019.1622031

To link to this article: https://doi.org/10.1080/00365513.2019.1622031

SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION
2019, VOL. 79, NO. 5, 314–319
https://doi.org/10.1080/00365513.2019.1622031

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ORIGINAL ARTICLE

New reference intervals for cortisol, cortisol binding globulin and free cortisol index in women using ethinyl estradiol

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ABSTRACT

Healthy women using contraceptives containing a low dose of an estrogen may have a higher serum concentration of cortisol (s-cortisol) and cortisol binding globulin (s-CBG) than the commonly used upper reference limits. There are no published reference intervals for s-cortisol, s-CBG, serum free cortisol index (s-FCI) or cortisol in saliva (sa-cortisol) for these women. The aim was to establish the above-mentioned reference intervals and document the differences in s-cortisol and s-CBG in one group of women using and another group not using ethinyl estradiol (EE). In this cross-sectional study, the reference limits presented were given as the 2.5 and 97.5 percentiles of the distribution of reference values in a population of 277 healthy volunteer women, aged 18–45 years. 157 women were not using any type of estrogen, while 120 women were using contraceptives containing a daily dose of 15–35 μg of EE. Serum and salivary cortisol, and serum CBG were measured using standard laboratory methods. S-FCI was calculated as s-cortisol/s-CBG. The reference intervals for s-cortisol in samples collected at 0800–1030 am in women using and not using EE contraception were: 284–994 nmol/L and 159–569 nmol/L respectively, and for s-CBG: 847–3366 nmol/L and 860–1940 nmol/L, respectively. For s-FCI and sa-cortisol, no clinically significant differences were found. Sa-cortisol may be the preferred measurand for evaluation of possible hypercortisolism in women using estrogens, since cortisol in saliva is not influenced by estrogen. If assessing morning s-cortisol and s-CBG in women using EE, we recommend using separate – and not the commonly used – reference intervals.

ARTICLE HISTORY
Received 29 October 2018
Revised 13 May 2019
Accepted 18 May 2019

KEYWORDS
Cortisol; corticosteroid binding globulin; reference values; agents; estrogen contraceptive; ethinyl estradiol; assay; chemiluminescence

Introduction

The obesity and metabolic syndrome epidemics have increased the number of patients screened for suspected hypercortisolism. Although guidelines worldwide recommend quantifying urine cortisol in a sample from a 24-hour urine collection, short dexamethasone suppression test, or evening salivary cortisol (sa-cortisol) as the initial tests, morning serum concentration of cortisol (s-cortisol) is still widely used. Hence, there is a need for reliable cortisol assays and reference intervals for specific populations. Approximately 5% of cortisol is circulating in a soluble, free form, and 90–95% is bound to proteins [1] – 80% to corticosteroid binding globulin (CBG) with high affinity, and <15% to albumin with low affinity. The plasma level of certain hepatic binding proteins, including CBG, increase in women using oral contraceptive pills (OCPs) containing an estrogen, leading to a higher cortisol-binding capacity. As a result, a new equilibrium between the bound and the free fraction of cortisol is found, making the interpretation of s-cortisol challenging. Due to the technical challenges of directly measuring the unbound fraction of cortisol in circulation, most laboratories measure only total s-cortisol. This is true for both immunoassays and chromatographic (LC-MS/MS) technologies. The effect of estrogens on hepatic binding proteins was first documented by Cobey et al. in 1956 [2] and has later been confirmed by multiple studies [3–7]. Because of this well-known effect, some researchers argue for using the change in hepatic binding proteins as a measure of OCP compliance [8]. Estrogens lead to an altered and most likely higher rate of glycosylation, as seen during pregnancy [9], and higher glycosylation of CBG may lead to an increased CBG half-life [10]. According to the free hormone concept [11], only the unbound fraction of cortisol can elicit its glucocorticoid effects on tissues, but in recent years, it has been debated whether the cortisol fraction bound to albumin can be considered free, as it is bound

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Supplemental data for this article can be accessed here.

with such low affinity [12]. There is also some uncertainty around what role internalization of the CBG-cortisol complex plays in the bioavailability of cortisol [13]. Nearly, all the cortisol in saliva exists in its unbound form [14], as the concentration of CBG in saliva is negligible. Salivary cortisol is one of the most commonly used surrogates for measuring plasma free cortisol. Measurement of free cortisol in a sample from a 24-hour urine collection is also used, but this has got serious pre-analytical disadvantages due to complicated sample collection [15]. Different equations have been used in the attempts to find a serum free cortisol index (s-FCI) or a calculated concentration of free cortisol that best correlate with the actual concentration of free cortisol in circulation [16–20]. However, no consensus on how to calculate the free fraction of cortisol has been reached, partly due to the lack of a 'gold standard' that is accurate enough.

It is still debated whether estrogens affect the basal concentration of unbound cortisol and the hypothalamo–pituitary–adrenal (HPA) axis stress response [4,20–22]. Most previous publications report that the concentration of cortisol in saliva is not affected by estrogens in contraception [23–26].

To the best of our knowledge, there are no published data on reference intervals for s-cortisol, s-CBG, s-FCI or sa-cortisol in women using contraceptives containing a low dose of an estrogen (15–35 µg/24 hours). The aim of the present study was to establish such reference intervals in order to avoid excessive investigations and improve diagnosis of cortisol disturbances in women of reproductive age.

Materials and methods

Population

This study was conducted at Trondheim University Hospital and at the Hormone Laboratory at Oslo University Hospital after the study protocol received approval from the Data Protection Official at Trondheim University Hospital. Regional Committees for Medical and Health Research Ethics (REK) North were consulted regarding the ethical permission for this study. The committee had no objections.

296 females – volunteer blood donors, laboratory workers and students – of reproductive age (18–45 years) were recruited for the study. Nineteen of the 296 women (6,4%) were excluded due to various exclusion criteria (Supplement 1).

A total of 277 women met the eligibility criteria and were divided into two groups; 157 women, aged 18–45 (median 31) years, did not use any form of estrogens and were assigned to the non ethinyl estradiol group (NEEG), while 120 women, aged 19–44 (median 23) years, used a combination contraceptive, containing ethinyl estradiol (EE) and a synthetic progestagen, and were assigned to the ethinyl estradiol group (EEG) (Figure 1).

According to the recommendations of the International Federation of Clinical Chemistry (IFCC) on estimation of reference intervals, a minimum of 120 reference individuals is considered to be sufficient to establish a new reference interval [27]. Each study participant completed a study questionnaire, declared herself as healthy, and gave a written, informed consent.

Measurement procedures

Ten milliliter of blood was collected from each study participant between 0800–1030 am, using two 5-ml Vacuette® *Serum Clot Activator* tubes (Greiner Bio-One GmbH, Kremsmünster, Austria). The blood samples were centrifuged immediately and stored at room temperature until s-cortisol was analyzed within 4 hours of collection. In addition, morning (7–9 am) and evening (9 pm to midnight) samples of sa-cortisol were analyzed. The study participants received equipment for collecting saliva at home (2 Salivette® *with citric acid* (Sarstedt AG & Co, Nümbrecht, Germany)), and returned the test material to the laboratory by ordinary mail or by personal delivery within a few days. When received at the laboratory of clinical chemistry, Trondheim University Hospital, the samples were centrifuged and analyzed immediately thereafter. Serum and salivary cortisol was quantified using the Elecsys® Cortisol assay, an electrochemiluminescence immunoassay (ECLIA) on a Modular PE instrument (Roche® Diagnostics GmbH, Mannheim, Germany). The within laboratory coefficient of variation (CV_w) of the s-cortisol method was 6.9% at 113 nmol/L and 4.5% at 571 nmol/L. The sa-cortisol method had a CV_w of 7.9% at 12 nmol/L. The assays used for measurements of cortisol in serum is no longer available but has been replaced by the Elecsys® Cortisol II assay. Before estimating the reference intervals of s-cortisol, the reference values were calculated into Elecsys® Cortisol II values, using the Passing & Bablok regression equation given by the producer in the assays kit insert, $y = 0.758x + 10.1$ nmol/L. This equation was used on all s-cortisol values given in this publication, to make the values and reference intervals applicable to measurements made with cortisol II.

Serum for analyzing CBG was frozen and stored at −80 °C until it was sent to the Hormone Laboratory at Oslo University Hospital for quantification by competitive radioimmunoassay (DIAsource ImmunoAssays SA, Louvain-La-Neuve, Belgium). The CV_w for the s-CBG method was 7% at 820 nmol/L. S-FCI was calculated as s-cortisol (nmol/L) divided by s-CBG (nmol/L).

Statistical methods

Tukey's method, as implemented in MedCalc Statistical Software version 15.2.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2015) was used to check for far-out values. In MedCalc, a far-out value was defined as a value smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range. The reference limits were estimated using the software Stata, version 14, given as the 2.5 and 97.5 percentiles of the distribution of reference values from the two reference populations. The same method was used to estimate the medians of the various measurands. In Stata, the point estimates of the percentiles were calculated

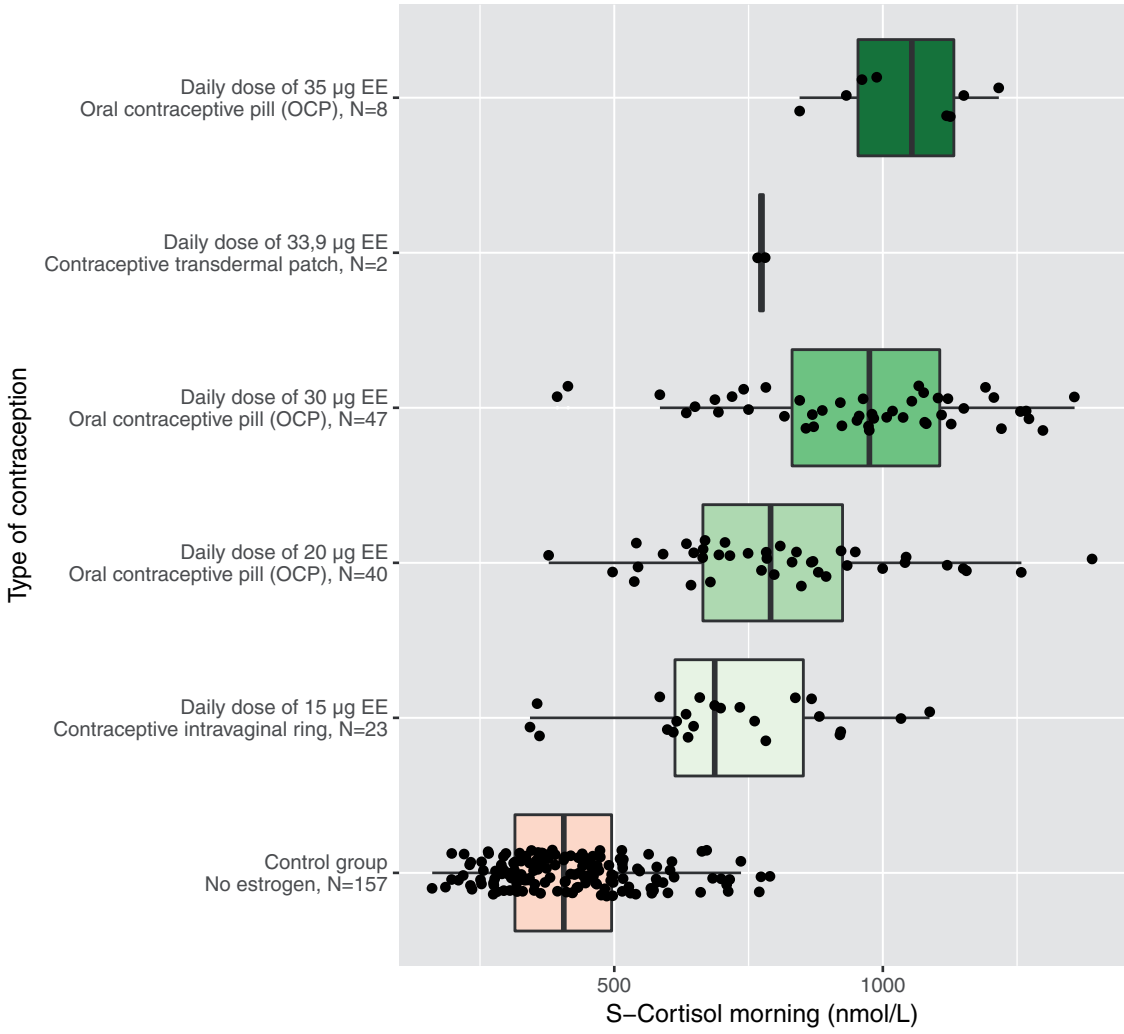

Figure 1. Results shown by subgroup. The group of women using an estrogen containing contraception was divided into subgroups depending on the daily dose of ethinyl estradiol (EE) in the contraception. In the subgroups of women using an oral contraceptive pill or a contraceptive intravaginal ring, the median value of s-cortisol increase with increasing daily dose of EE. N: Number of women in the subgroup; S-Cortisol: serum concentration of cortisol.

according to the CSLI-document C28-A3c [28]. The 'centile' command was used for estimating percentiles and percentile differences along with a bias corrected and accelerated bootstrap technique [29] with 10000 bootstrap samples to estimates the 90% confidence intervals for the 2.5 and 97.5 percentiles. Whether the 2.5, 50 and 97.5 percentiles were different between the two groups of women using and not using an estrogen, was tested by modeling the percentile as a function of group, using quantile regression as implemented in Stata, Version 14. This way, the independent "effect" of using an estrogen was estimated. After a conservative Bonferroni correction, the significance level was 0.003. If the p-value of the group variable was <0.003, the percentile was considered statistically significantly different in the two populations.

Results

The reference interval (median) for morning s-cortisol was 159-569 (318) nmol/L in the NEEG and 284-994 (667) nmol/L in the EEG. The point estimates 2.5, 50 and 97.5 percentiles of morning s-cortisol were 42%, 52% and 43% higher in the EEG than in the NEEG, respectively. For morning s-CBG the reference intervals (medians) were 860-1940 (1101) nmol/L and 847-3366 (2188) nmol/L in the NEEG and EEG, respectively. The results are shown in Table 1. We found a statistically significantly higher value ($p < .001$) in the EEG than in the NEEG for the upper and lower reference limits and median of s-cortisol, and for the median of s-CBG. There was no statistically significant difference between the upper and lower reference limits of s-CBG in the two groups. The 90% CIs for the 2.5 and 97.5 percentiles of s-cortisol were found to be narrower than 0.2 times the width of the reference interval. The 2.5–97.5 percentiles (median) of morning sa-cortisol were 6–26 (15) nmol/L in the NEEG ($n = 121$) and 6–28 (13) nmol/L in the EEG ($n = 101$). The same percentiles for evening sa-cortisol were 3–10 (5) nmol/L in the NEEG ($n = 124$) and 3–9 (5) nmol/L in the EEG ($n = 102$). There was no statistically significant difference between the median values of s-FCI (Table 1), morning or evening sa-cortisol in the two groups of women.

When dividing the EEG into subgroups based on daily dose of EE, a dose-dependent relationship between these subgroups and s-cortisol was found (Figure 1). This

Table 1. Results. Reference intervals given as the 2.5 and 97.5 percentiles of the distribution of reference values, and the corresponding 90% confidence intervals for the reference limits.

	Nonestrogen group (NEEG)			Ethinyl estradiol group (EEG)			
n	Reference interval (median)		*n*	Reference interval (median)		*p*-value for median
	90% CI for 2.5 prc.	90% CI for 97.5 prc.		90% CI for 2.5 prc.	90% CI for 97.5 prc.	
			S-cortisol morning (8–10.30 am)			
157	159–569 (318)		120	284–994 (667)		<0.001
	151–185	543–597		270–311	970–1064	
			S-CBG morning (8–10.30 am)			
156	860–1940 (1101)		114	847–3366 (2188)		<0.001
	833–897	1513–2811		221–1339*	3201–3720*	
			S-FCI morning (8–10.30 am)			
156	0.13–0.50 (0.28)		114	0.16–1.05 (0.29)		0.452
	0.06–0.16	0.47–0.59		0.16–0.17*	0.64–3.39	

The median values and *p*-values from testing whether the medians were statistically significantly different in the two reference groups are also given.
* = Lower (respectively upper) confidence interval limit is held at the minimum (maximum) of sample values.
n: number of reference values; CI: Confidence interval; prc.: percentile; S-cortisol: Serum concentration of cortisol (nmol/L); S-CBG: Serum concentration of CBG (nmol/L); S-FCI: Free cortisol index in serum (s-cortisol/s-CBG).

correlation was also found between the daily dose of EE and s-CBG. The median values of s-cortisol and s-CBG in the EEG subgroups were found to be: 531 and 1759 nmol/L for users of 15 µg/24 hours vaginal ring, 610 and 2093 nmol/L for users of 20 µg/24 hours OCPs, 749 and 2426 nmol/L for users of 30 µg/24 hours OCPs, 809 and 2844 nmol/L for users of 35 µg/24 hours OCPs, and 597 and 2077 nmol/L for users of 33,9 µg/24 hours transdermal patch.

Discussion

New reference intervals of s-cortisol and s-CBG in women using and not using EE were established by the present study. The results confirmed the presence of statistically and clinically significant differences between the reference intervals of s-cortisol of the two groups. A clinically significant difference was here defined as a difference of more than allowed bias based on biological variation: 10% for s-cortisol [30]. There were no clinically significant differences between the NEEG and EEG reference intervals of calculated s-FCI or sa-cortisol. The estrogenic effect on CBG and indirectly on s-cortisol was not found when the s-FCI was calculated. Therefore, we recommend using one common reference interval for s-FCI and sa-cortisol for women 18–45 years, using and not using an estrogen-containing contraception. To our knowledge, no reference interval for s-cortisol or s-CBG has previously been established in a population of women using EE, but the results from the present study support previously published data, regarding the effect of EE on s-CBG and indirectly on s-cortisol [2–7]. The reference intervals (Table 1) will provide useful information for the medical practitioners aiding in explaining unexpected answer of high s-cortisol in women of reproductive age. It can prevent unnecessary, time-consuming, stressful and possibly expensive investigations and blood sampling for the patient. It is important to underscore that this study did not examine the effect of estrogens on s-cortisol and s-CBG in patients suffering from Addison's disease or Cushing syndrome. This issue needs further investigation.

The calculated, morning s-cortisol reference interval for the NEEG, 159–569 nmol/L, correlates well with the reference interval for a population of presumably healthy adults, given by the producer in the kit insert: 133–537 nmol/L. This correlation supports the decision to calculate the original Elecsys® Cortisol I values into Elecsys® Cortisol II values and adds to the validity of the regression equation used, and to the resulting reference interval.

One of the strengths of the study is the size. When collecting material for estimating the reference interval of s-cortisol, the recommendations of the IFCC on estimation of reference intervals were followed [27]. A minimum of 120 reference individuals is considered to be sufficient, and reference limits were calculated based on the distribution of reference values. We used a binomial method for obtaining confidence intervals of the percentiles. This method makes no assumptions about the underlying distribution of the variable. To our knowledge, no other study of this size has been performed on this topic, and the overall size provides important statistical power to the results, with relatively narrow 90% confidence intervals for s-cortisol, meeting the CLSI criteria of being less than 20% of the reference interval [28]. The women in the study represented users of modern types of contraception, various routes of administration, containing daily doses of EE ranging from 15 to 35 µg/24 hours. These different types of contraception are among the most commonly used, and the study provides up to date information on how they affect the circulating s-cortisol and s-CBG.

Previous attempts to calculate a free cortisol index have been made [16–19], using various formulas. Some include only s-CBG and s-cortisol; some are more complex and include several binding proteins (e.g. albumin). Albumin has a several fold lower affinity for cortisol than CBG [12]. Previous studies reported the effect of albumin on the CBG–cortisol interaction to be minimal in healthy individuals [31,32]. Due to these factors, it was decided not to include albumin in the calculations of the s-FCI in the present study. The simplicity of calculating s-FCI as s-cortisol divided by s-CBG would also be an advantage to physicians

Table 2. 'Far-out' values. The 'far-out' values as identified by Tukey's method, with the values of the other relevant parameters.

'Far-out' value	Group	S-CBG	S-cortisol	S-FCI	Sa-cortisol (am)	Sa-cortisol (pm)
High s-CBG	NEEG	4189[a]	230	0.06[b]	21	4
High s-CBG	NEEG	2700[a]	528	0.20	15	3
High s-CBG	NEEG	2411[a]	441	0.18	–	–
Low s-CBG and high s-FCI	EEG (DD 30 µg)	222[b]	753	3.39[a]	11	8*
Low s-CBG and high s-FCI	EEG (DD 30 µg)	221[b]	660	2.99[a]	14	6

[a]Values above the appropriate upper reference limit.
[b]Values below the appropriate lower reference limit.
– Missing value.
* = <50% fall in sa-cortisol from the morning to the evening sample.
NEEG: Non ethinyl estradiol group; EEG: Ethinyl estradiol group; DD: Daily dose of ethinyl estradiol; S-CBG: Serum concentration of CBG (nmol/L); S-FCI: Free cortisol index in serum (s-cortisol/s-CBG); S-cortisol: Serum concentration of cortisol (nmol/L); Sa-cortisol: Salivary concentration of cortisol (nmol/L).

and laboratory workers. CBG has got some diurnal variation, though smaller than cortisol, with the peak early in the afternoon [33]. Due to the diurnal variation of these measurands, it is necessary to standardize the time of sample collection, as the reference interval for s-FCI only will be valid for the time of the day when the samples used as reference values were collected, in this case between 8–10.30 am.

When checking for outliers, using Tukey's method as implemented in MedCalc, three high 'far-out' values of s-CBG were identified in the NEEG: 4189, 2700 and 2411 (Table 2). Two individuals in the EEG, using OCPs containing 30 µg EE, had s-CBG values that were identified as low 'far-out' values: 221 and 222 nmol/L, and s-FCIs identified as high 'far-out' values: 3.392 and 2.986. Possible reasons for these high 'far-out' values are discussed in Supplement 2. If the two s-CBG outliers with low values had been excluded, the lower reference limit would have been 1269 nmol/L in the EEG. This is statistically significantly higher than the low reference limit of 860 nmol/L in the NEEG ($p < .001$).

There were not enough study participants over 30 years of age to evaluate whether there should be separate reference limits for different age groups. We recommend using the same reference limits for women of all ages using EE contraception.

Median age was 23 and 31 years in the EEG and NEEG, respectively. Over the reproductive period of a woman's life, the type of contraception chosen may vary due to several reasons. Stage in life (nulliparity, compatibility with breastfeeding, between pregnancies, finished having children), culture and acceptability of the type of contraception, cost, availability, medical conditions, or family history of thromboembolic event or other cardiovascular illness are among the factors that influence this decision. Surveys in Norway in 2007 [34] and in Great Britain in 2008/2009 [35] show that women in the early reproductive period of their lives more often choose OCPs and that women closer to menopause rather choose intrauterine devices or sterilization as method of contraception. This could explain the difference in median age of the two groups in the present study.

The median values of s-cortisol and s-CBG in the EEG subgroups imply that there is a dose-dependent relationship between daily dose of EE, s-cortisol and s-CBG. This increase in median values of s-cortisol in the subgroups, is visualized in Figure 1. The effect of route of administration can also be studied, even though the subgroups are not large enough to evaluate statistical significance. The results of this study support previous publications claiming that daily dose of EE has a positive correlation with s-CBG [36,37], and that vaginal administration of EE also leads to an increased s-CBG, and indirectly increase the s-cortisol [38], as does OCPs.

Conclusion

When assessing serum cortisol and CBG in women using an estrogen, we recommend using separate – and not the commonly used – reference intervals. There is a statistically and clinically significant difference in s-cortisol between women using and not using EE contraception. This difference is due to the estrogenic influence on CBG. Calculating an s-FCI may help to overcome these differences, but sa-cortisol may be the preferred measurand for evaluating possible hypercortisolism in women using estrogens, since sa-cortisol is not affected by estrogens.

Acknowledgement

We would like to thank the department of Immunology and Transfusion Medicine at St. Olavs Hospital, Trondheim University Hospital, for their cooperation when collecting the material needed for the study. Thanks to the women who donated blood, saliva and a few minutes of their time to this study, and to Dr. Johan Bjerner at Fürst Medical laboratory for his assistance in making the figure.

Disclosure statement

The authors alone are responsible for the content and writing of the article.

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