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## Letter to the Editor

Michel J. Vos\*, Peter H. Bisschop, Martine M. L. Deckers and Erik Endert

# The cortisol-CBG ratio affects cortisol immunoassay bias at elevated CBG concentrations

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To the Editor,

Measurements of baseline, suppressed or stimulated cortisol concentrations are performed in the evaluation of several endocrine disorders such as Cushing's syndrome and adrenal insufficiency. The interpretation of endocrine function test results is often based on historically determined cut-off values without taking differences in analytical performance of immunoassays into account [1]. Therefore, the use of assay dependent cut-off values has been advocated [2]. However, a complicating factor in cortisol immunoassay measurements is the binding of cortisol to cortisol-binding globulin (CBG), which affects the binding of the capturing antibody to cortisol. Therefore, immunoassays utilize a dissociation step to free cortisol from CBG prior to measurement. The displacement agent and concentration used have to be carefully chosen to allow for maximum cortisol displacement without disturbing binding characteristics of the assay antibody [3]. Hence, elevated CBG concentrations which are generally observed during pregnancy and oral contraceptive pill (OCP) usage could theoretically result in incomplete

displacement and falsely lowered total cortisol measurements. Indeed, OCP usage has been associated with a negative cortisol immunoassay bias relative to cortisol LC-MS/MS methods and a recent study has reported that most cortisol immunoassays show decreased recovery of cortisol measured in a pregnant female cohort with elevated CBG concentrations [4, 5]. Both synthetic and endogenous estrogens influence the expression of estrogen-sensitive hepatic proteins including CBG, thyroxin-binding globulin and sex hormone-binding globulin, resulting in higher total hormone concentrations [6–8]. Therefore, women are generally advised to stop OCP usage 4–6 weeks prior to endocrine function tests that use cortisol as a readout to be able to use the generally accepted cut-off values. However, the increase in concentration of binding proteins varies between individuals due to the level of OCP compliance, differences in total estrogen intake during the OCP-cycle and differences in OCP formulations [6, 8]. Nevertheless, in women self-reporting to be non-OCP users, occasionally high baseline cortisol concentrations are observed, suggesting increased CBG concentrations due to high levels of endogenous estrogens.

To substantiate the relationship between cortisol quantification and CBG levels, we studied the role of the cortisol-CBG ratio as a possible determinant of assay related bias. We used serum samples from adrenocorticotrophic hormone (ACTH) stimulation tests (250 µg tetacosactin) which consisted of a baseline sample (t=0 min) and a stimulated sample (t=60 min) from non-OCP and OCP users. Concerning OCP formulations, levonorgestrel- and desogestrel-containing formulations are the most prescribed in the Netherlands (70 and 10% of all OCP users respectively) which contain 20–30 µg ethinyl estradiol [9]. At stable CBG concentrations, the difference in observed immunoassay bias would be mainly attributable to the ACTH-induced production of cortisol and thus the increase in the cortisol-CBG ratio. Cortisol was measured with automated cortisol immunoassays (Siemens Immulite 2000 and Roche Cortisol II) and compared to a routine LC-MS/MS method [10] which is not affected by binding protein concentration due to a protein denaturation step preceding cortisol quantification (LC-MS/MS inter-assay

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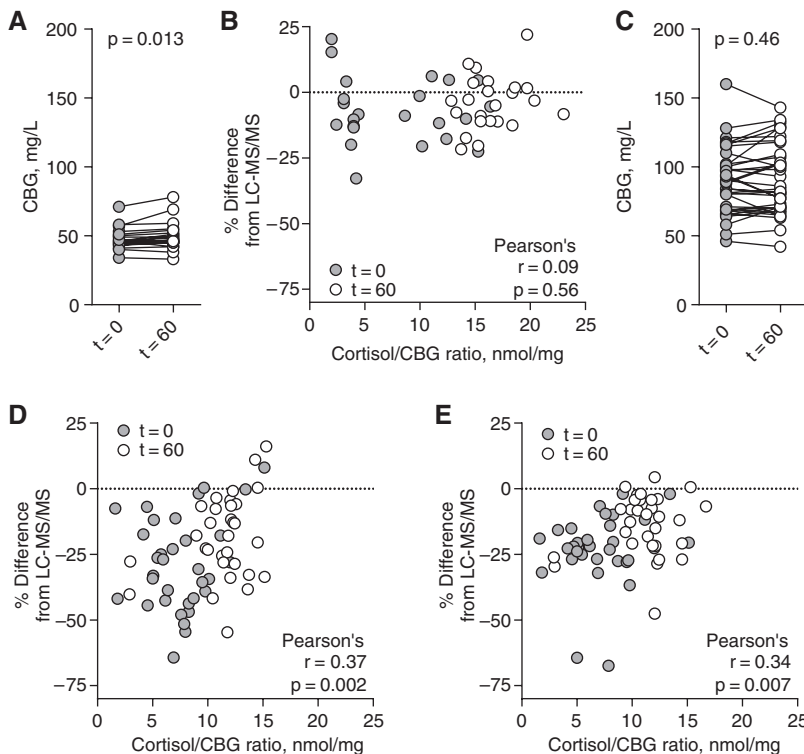
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variation: 7.2% [172 nmol/L]; 13.1% [861 nmol/L]). Samples above the limit of quantification of the immunoassays were omitted from the analysis as such samples require dilution prior to quantification. CBG was quantified using a radio immunoassay (DIAsource) (inter-assay variation: 3.5% [21.1 mg/L]; 9.7% [113 mg/L]).

A control group consisting of 23 females, not using OCP, showed a small increase in CBG concentration during the ACTH-test ( $\Delta 1.9$  mg/L [95%CI 0.45; 3.38],  $p=0.013$ ) (Figure 1A). The mean CBG concentration increased from 47.3 to 49.2 mg/L. At baseline, a significant mean difference between the Immulite cortisol immunoassay and the LC-MS/MS method was observed ( $\Delta 31.8$  nmol/L [95%CI 8.6; 55.1],  $p=0.009$ ). There was no significant difference in cortisol concentration between the two methods in the stimulated samples which showed a large increase from baseline (LC-MS/MS method: mean  $\Delta 430.8$  nmol/L [95%CI 362.8; 498.8],  $p<0.0001$ ). In addition, there was no correlation between the cortisol-CBG ratio (Figure 1B) and the percentage difference in cortisol concentration between the two methods and the observed bias between

time points did not differ significantly (baseline mean bias  $-6.9\%$  [95%CI  $-12.3$ ;  $-1.57$ ] and stimulated mean bias  $-3.9\%$  [95%CI  $-8.4$ ;  $0.54$ ],  $p=0.38$ ).

As expected, the mean CBG concentration in the OCP group ( $n=36$  females) was much higher (89.5 mg/L [95%CI 81.3; 97.6]) compared to the control group ( $p<0.0001$ ). In contrast to the control group, no significant difference in CBG concentration between baseline and stimulated samples was observed (Figure 1C). However, both timed samples showed a significant mean difference between the Immulite cortisol immunoassay and the LC-MS/MS method (baseline  $\Delta 131$  nmol/L [95%CI 93.0; 169.5] and stimulated  $\Delta 142$  nmol/L [95%CI 88.5; 195.3]). Furthermore, the OCP group showed a positive correlation ( $r=0.37$ ) between the cortisol-CBG ratio and the percentage difference in cortisol concentration from the LC-MS/MS method (Figure 1D). A higher cortisol-CBG ratio was associated with less bias from the LC-MS/MS method and stimulated samples overall showed less bias compared to baseline samples (baseline mean bias  $-28.5\%$  [95%CI  $-34.8$ ;  $-22.2$ ] and stimulated mean bias  $-18.4\%$  [95%CI  $-24.3$ ;  $-12.5$ ],  $p=0.02$ ).



**Figure 1:** The effect of elevated CBG concentration on cortisol immunoassay bias.

(A) CBG concentration in the non-OCP group at baseline ( $t=0$  min) and following ACTH-stimulation ( $t=60$  min). (B) The LC-MS/MS cortisol-CBG ratio plotted against the percentage difference between the Siemens Immulite cortisol immunoassay and the routine LC-MS/MS assay for the non-OCP group. (C) CBG concentration in the OCP group at baseline ( $t=0$  min) and following ACTH-stimulation ( $t=60$  min). (D) The LC-MS/MS cortisol-CBG ratio plotted against the percentage difference between the Siemens Immulite cortisol immunoassay and the routine LC-MS/MS assay for the OCP group. (E) The LC-MS/MS cortisol-CBG ratio plotted against the percentage difference between the Roche cortisol immunoassay and the routine LC-MS/MS assay for the OCP group. A  $p$ -value  $<0.05$  is considered significant.

Immunoassays can show variable bias due to assay characteristics and susceptibility to matrix effects [5, 11]. Therefore, we repeated the measurements of OCP samples using a Roche immunoassay. Both baseline and stimulated samples showed a significant mean difference in cortisol concentration compared to the LC-MS/MS method (baseline  $\Delta 105.6$  nmol/L [95%CI 84.0; 127.3] and stimulated  $\Delta 124.9$  nmol/L [95%CI 80.6; 169.2]). Again, a positive correlation ( $r=0.34$ ) between the cortisol-CBG ratio and the percentage difference in cortisol concentration from the LC-MS/MS method was observed (Figure 1E). A higher ratio showed less bias from the LC-MS/MS method and the stimulated samples showed less bias compared to baseline samples (baseline mean bias  $-23.0\%$  [95%CI  $-27.9$ ;  $-18.2$ ] and stimulated mean bias  $-13.5\%$  [95%CI  $-17.7$ ;  $-9.3$ ],  $p=0.004$ ).

In conclusion, we show that elevated CBG levels affect two automated cortisol immunoassays. Changes in the cortisol-CBG ratio affected the amount of bias from a routine cortisol LC-MS/MS method. This suggests that elevated CBG concentrations prevent effective dissociation of bound cortisol in both immunoassays tested here. Adrenal stimulation by exogenous ACTH effectively increases total cortisol concentration and the cortisol-CBG ratio resulting in a lower immunoassay bias. Through an increase in total cortisol concentration, the measurement error derived from non-dissociated cortisol becomes smaller and hence reduces the bias between immunoassay and LC-MS/MS. The decrease in bias observed after stimulation will however affect the calculated value of the incremental cortisol response between the timed samples, leading to possible overestimation of the increment. Thus, correct measurement of cortisol in the presence of an elevated CBG concentration is dependent on the cortisol-CBG ratio. As this ratio is not known a priori and requires measurement of CBG, efficient and correct quantification of cortisol under these circumstances favors the use of a routine cortisol LC-MS/MS method. In addition, we show that the CBG concentration in the OCP group is subject to large variation (Figure 1C) which is probably related to differences in exposure to synthetic estrogens and the use of different OCP formulations. Therefore, we question the feasibility of determining OCP-specific cut-off values for endocrine testing as synthetic estrogen exposure, OCP formulation and the dynamics of estrogen induced CBG expression during an OCP-cycle will influence the results of endocrine function tests.

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