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The Role of Albumin in the Calculation of Free and Bioavailable Testosterone in Women with Hyperandrogenemia

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Abstract. *Background: The aim of this study was to assess the role of albumin in the calculation of free testosterone (cFT) and bioavailable testosterone (cBT). Patients and Methods: Ninety-two healthy women, who presented at our fertility center for evaluation of possible hyperandrogenemia, were included in the study. Total blood testosterone (TT), free testosterone (FT) and sex hormone-binding globulin (SHBG) were measured using commercially available enzyme immunoassays in one serum sample. cFT and cBT were then calculated, taking into account the individual albumin concentration, but without taking account of each individual albumin serum concentration. Results: No differences were observed in the calculated values for FT and BT, with or without taking into account the individual albumin concentration. A correlation between the two was demonstrated. Conclusion: For clinical routine work, it is not necessary to estimate the individual albumin concentration in hyperandrogenic women in order to calculate the FT and BT concentrations from TT and SHBG serum concentrations, when the albumin concentration is in the physiological range of 40-50 g/L.*

Women with polycystic ovary syndrome (PCOS), hyperandrogenic insulin-resistant acanthosis nigricans syndrome, non-classic adrenal hyperplasia, or isolated oligo-ovulation normally tend to show the typical symptoms of a possible hyperandrogenemic disorder and should be evaluated endocrinologically, as 50% of these patients have an androgen excess (AE) (1). However, it is unclear which androgen fraction reflects the clinical situation most accurately and correlates with the symptoms of hyperandrogenemia. Theoretical arguments have been advanced for measuring non-protein-bound or so-called free testosterone (FT) (2).

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Testosterone (T) circulates in the plasma non-specifically bound to albumin, with the majority being specifically bound to sex hormone-binding globulin (SHBG), and a small fraction remaining unbound (FT). Although immunoassay of total blood testosterone (TT) has long been the standard measurement, the TT concentration is not a reliable index of bioavailable testosterone (BT), as it depends on variations in the concentration of the binding proteins. There is good evidence that FT and the hormone fraction non-specifically bound to albumin in plasma, referred to as BT, more accurately reflect the clinical situation than the total hormone levels in plasma (2). Several methods of estimating FT and BT in plasma have been established. The measurement of FT by equilibrium dialysis (the apparent FT concentration, AFTC) (3, 4) and centrifugal ultrafiltration (5, 6) are the reference measurement procedures (RMP) *in vivo*. However, this is a time-consuming and complex manual procedure that is not routinely practical in large laboratories, which rely increasingly on automated multiplex assay platforms.

Alternative methods of estimating FT have been established using additional assay steps, including the FT analog assay. FT can be measured by an analog ligand immunoassay method (aFT); this is the easiest and fastest method, but it shows substantially lower values than those obtained by dialysis (7-9). Other models have been developed to calculate FT (cFT) and BT (cBT) from TT and SHBG, with or without the albumin concentration (2, 10). Another simple calculation model used by many researchers is an indirect parameter of FT known as the free androgen index (FAI), which is obtained as the quotient 100x TT/SHBG (11). The FAI has been found to correlate poorly with laboratory FT measurements and to be highly influenced by low SHBG concentrations, so that ultimately it does not correspond to actual FT measurements (12).

Vermeulen *et al.* (2) reported an excellent evaluation of a calculation method for estimating the FT fraction in serum. The calculation comprises theoretical equilibrium binding equations based on the law of mass action, which can be solved for FT from a second-degree equation in TT and

SHBG. The authors compared the AFTC values, which are generally regarded as the best method for evaluating the FT levels, with the cFT and aFT levels. aFT was not a reliable parameter for the FT fraction; although showing a good correlation with the AFTC values, it represented a variable fraction of only 20-60% of AFTC. In contrast, cFT was a reliable index of the FT fraction; the calculated non-specifically bound T (cBT) reliably reflected non-SHBG T (BT), while immunoassayable SHBG was a reliable measure of SHBG-binding sites (2). In men and postmenopausal women, the albumin concentration did not significantly affect the FT values when there were physiological albumin ranges of 40-50 g/L, as Vermeulen *et al.* had shown (2). Hence, when the albumin concentration is expected to deviate significantly from normal, the actual albumin concentration should be determined and used to calculate the FT and albumin-bound T (2).

The effect of albumin was not assessed in hyperandrogenemic women in their reproductive lifespan (it was only estimated in a small group of twelve women with mild hyperandrogenism in the study by Vermeulen *et al.*) (2). Particularly in women with expected hyperandrogenemia, it is generally recommended to estimate T values as part of the diagnostic procedures for the evaluation of possible AE (1).

The aim of the present study was to show the role of each individual albumin concentration in the estimation of cFT and cBT calculated from serum TT and SHBG in a larger population of hyperandrogenic women.

Patients and Methods

Patient population. The study population consisted of 92 healthy Caucasian women who presented at our Division of Gynecological Endocrinology and Reproductive Medicine center, Germany, between January 2004 and January 2005, for the evaluation of possible hyperandrogenemia. The study was approved by the institutional review board of Erlangen University Hospital. All the patients signed informed consent forms and completed a uniform history form emphasizing menstrual frequency and regularity, hirsutism and acne, gynecological history, history of infertility, medications and family history. All of the women underwent a complete physical examination, including weight and height measurements, and an ultrasound examination of the ovaries. Patients who had received hormonal therapy within 3 months of their initial visit were not included in the study. In all of the patients, serum was obtained and immediately assayed in our laboratory for hormonal parameters and albumin.

Biochemical measurements. All the assays were conducted in our routine diagnostic endocrinology laboratory using established commercial assays, regularly monitored by participation in external quality-control programs.

Total testosterone was measured quantitatively with a solid-phase competitive chemiluminescent enzyme immunoassay (Immulite 2000, Diagnostic Products Corp., Los Angeles, CA, USA). The calibration range of the assay was 0.7 to 55 nmol/L, with an analytical sensitivity of 0.5 nmol/L. The intra-assay coefficient of variation (CV) was 11.7, 10 and 8.3% at the levels of 2.98, 5.27 and 9.71 nmol/L, respectively. The corresponding interassay CVs were 13, 10.3 and 9.1%. The cross-reaction with 5alpha-dihydrotestosterone was 2%.

Free testosterone was measured using the single-tube Coat-A-Count (^{125}I-labelled) Free Testosterone radioimmunoassay (Diagnostic Products Corp.). The calibration range of the assay was 1.9 to 173 pmol/L, with an analytical sensitivity of 0.52 pmol/L. The CVs were 18.3, 8.5 and 8% at the levels of 4.1, 30.8 and 69.3 pmol/L, respectively. The cross-reaction with 5alpha-dihydrotestosterone was 0.041%.

SHBG was quantified using an immunometric assay (Immulite 2000, Diagnostic Products Corp.). The calibration range of the assay was up to 180 nmol/L, with an analytical sensitivity of 0.02 nmol/L. The intra-assay CVs were 2.5, 2.5 and 5.3% at the levels of 1.2, 21 and 80 nmol/L, respectively. The corresponding interassay CVs were 4.2, 5.2 and 6.6%. No cross-reactivity with other compounds was known.

Albumin was regularly measured using routine clinical chemistry methods.

Calculation of free testosterone (cFT) and bioavailable testosterone (cBT). Calculations of cFT and cBT were carried out using the formula available on the web site of the International Society for the Study of the Aging Male (ISSAM) (http://www.issam.ch/freetesto.htm) from TT and SHBG, with calculations either taking into account or not taking into account the albumin concentration measured in the same sample from each woman. When each individual albumin concentration was not taken into account in the calculation of FT and BT, a fixed albumin concentration of 43 g/L was used in the formula. This method was described in detail by Vermeulen *et al.* (2).

Statistical analysis. Comparison of the calculated testosterone values were carried out using ANOVA. A p value of less than 0.05 was considered statistically significant. Correlation of the calculated values obtained by different calculation procedures was done using the method of least square regression. Statistical analysis was performed using the Statistical Package for the Social Sciences, version 10.1 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

During the study period, 92 Caucasian women presented with hirsutism, acne and oligo-ovulation for the evaluation of possible hyperandrogenism. To evaluate for an androgen-secreting neoplasm, our TT cut-off value is above 8 nmol/L, at which point we normally carry out computed tomography of the adrenal gland (1). These high levels of TT were not found in any patient. No ovarian tumors were found on routine ultrasound examinations in the study group. To exclude possible 21-hydroxylase deficiency in 16 women with 17-hydroxyprogesterone levels greater than 6 nmol/L, the 17-hydroxyprogesterone levels stimulated by adrenocorticotropic hormone (ACTH) were measured and found to be normal (1, 14) (data not shown). None of the women had hyperandrogenic insulin-resistant acanthosis nigricans syndrome. The baseline characteristics of the study population are shown in Table I. The albumin serum concentration was in

Table I. *Baseline characteristics of the study population (n=92).*

Age (y)	31.5 (7.9)	18-61
BMI (kg/m^2)	25.8 (6.4)	17.5-45.0
TT (nmol/L)	2.0 (1.1)	0.7-5.2
aFT (pmol/L)	7.2 (4.1)	1.1-20.4
SHBG (nmol/L)	61.5 (39.8)	12.0-180.0
Albumin g/L	46.2 (2.7)	40.1-52.3
cFT nmol/L	0.03 (0.02)	0.003-0.1
cBT nmol/L	0.71 (0.52)	0.087-2.37
cFT(alb) nmol/L	0.03 (0.02)	0.003-0.1
cBT(alb) nmol/L	0.74 (0.54)	0.084-2.38

All values are shown as means, standard deviation and ranges (alb=calculation of testosterone considering the individual albumin concentration in the calculation formula). There were no significant differences between cFT and cFT(alb) (p=0.98) or between cBT and cBT(alb) (p=0.68).
aFT, assayed free testosterone; BMI, body mass index; cBT, calculated bioavailable testosterone; cFT, calculated free testosterone; SHBG, sex hormone-binding globulin; TT, total testosterone.

the range 40.1 to 52.3 g/L. No differences were found between cFT using a fixed albumin concentration of 43 g/L in the formula and cFT(alb) using the individual albumin concentration in the formula (p=0.98). There were also no differences between cBT using a fixed albumin concentration of 43 g/L in the formula and cBT(alb) using the individual albumin concentration in the formula (p=0.68). As expected, the use of a fixed albumin concentration in the calculations did not lead to FT and BT values significantly different from the values on taking each individual albumin concentration into account. There was a close correlation between cFT and cFT(alb), with a correlation coefficient of 0.9969 (Figure 1), as well a close correlation between cBT and cBT(alb), with a correlation coefficient of 0.9961 (Figure 2).

Discussion

This study examined the role of albumin in the estimation of FT and BT in hyperandrogenic women, calculated from serum concentrations of TT and SHBG. The testosterone values (cFT and cBT) were estimated employing the formula described in detail by Vermeulen *et al.* (2), initially using a fixed albumin concentration and then using each individual albumin concentration. There were no significant differences between the use of each individual albumin concentration in the formula in comparison with the results of the calculation when a fixed albumin concentration was used.

Women with an AE-related disorder should be evaluated endocrinologically (1). However, there is no consensus regarding which T fraction should be estimated. In general, women with hirsutism, for example, show physiological increases in circulating androgens. Conversely, however, not all women have AE, and 5-15% show normal androgen levels and are described as having "idiopathic hirsutism" (13). In addition, there is a discrepancy between directly immunoassayable T parameters and the clinical symptoms of hyperandrogenemia in women (8).

There has been growing interest in the estimation of FT concentrations in recent decades, as the free hormone hypothesis postulates that this small free fraction is the most biologically active fraction and has greater accessibility to tissue; it has been suggested that this is the best diagnostic test for evaluating the androgen status of women (2, 12). The approaches can be divided into those using simplified laboratory measurements and those based on calculation models using TT and SHBG concentrations from the same sample (12). FT values, measured by simplified immunoassay methods, did not correlate with the values measured by equilibrium dialysis or centrifugal ultrafiltration, which are the original reference methods for FT measurement *in vivo* (2, 12).

FT values measured using simplified immunoassay methods also did not correlate with the clinical symptoms in hyperandrogenic women (1, 14). However, some assays showed a good correlation with the RMP, as reported by Van Uytfanghe *et al.*; it measured only a fraction of the FT concentrations of 20-30%, but not the correct FT fraction (2, 15). In general, FT analog assays have been reported to be invalid, at least when applied to male samples with correspondingly high serum T concentrations (16-18). However, in children and women as well, in whom very low (0.17 nmol/L) and low (<1.7 nmol/L) T concentrations are expected, none of the immunoassays tested has been found to be sufficiently reliable at low T ranges (19, 20). In contrast to immunoassayed FT, there is good evidence that the calculated FT is a reliable index of the FT fraction in serum (2), although the original validation studies for these calculated FT methods had been based on very few samples (2, 10).

A recent study noted flaws in the applicability of the two calculated FT equations, attributable to the notional SHBG-binding affinity and other assumptions (21). Emadi-Konjin *et al.* stated that the algorithm is not directly transferable, so that, before adopting the calculation, each laboratory should compare it with the locally available measurement method to adjust the calculation and optimize the correlation (21). However, the differences appeared to vary within a very small range and the formula used in the present study was validated over a wide range of concentrations (2).

Vermeulen *et al.* reported an excellent correlation between the calculated FT values and the AFTC values measured by equilibrium dialysis in male out-patients and postmenopausal women (2). The FT values for the postmenopausal women (mean FT 0.011 nmol/L) reported in the study by Vermeulen *et al.* were apparently lower than

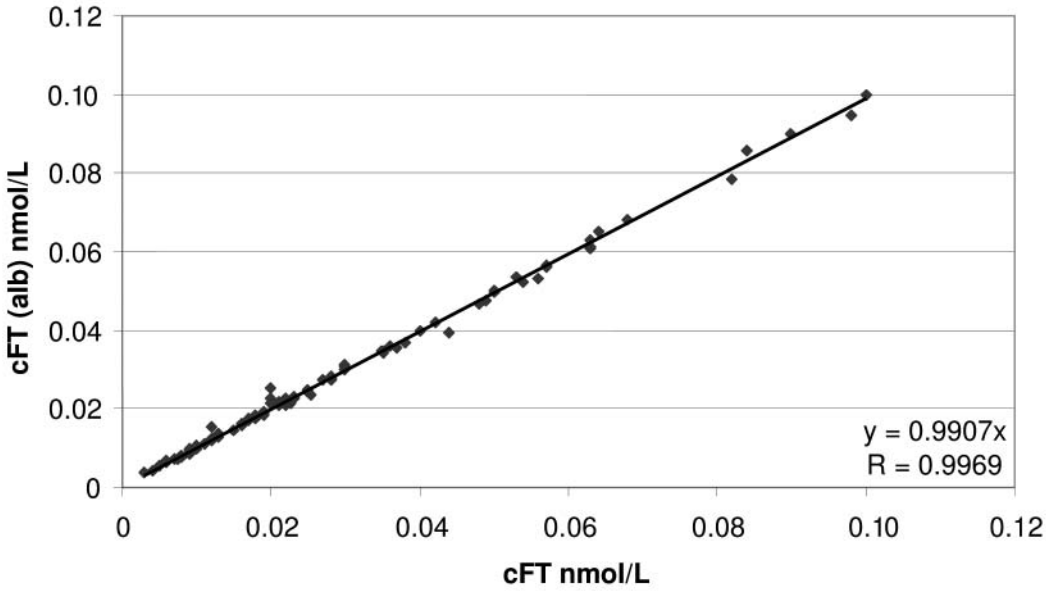

Figure 1. *Correlation of cFT calculated from TT and SHBG, with a fixed albumin concentration of 43 g/L to cFT calculated from TT and SHBG, taking into account the individual albumin concentration, cFT(alb). The calculation of cFT was performed using the method described in detail by Vermeulen et al. (2).*

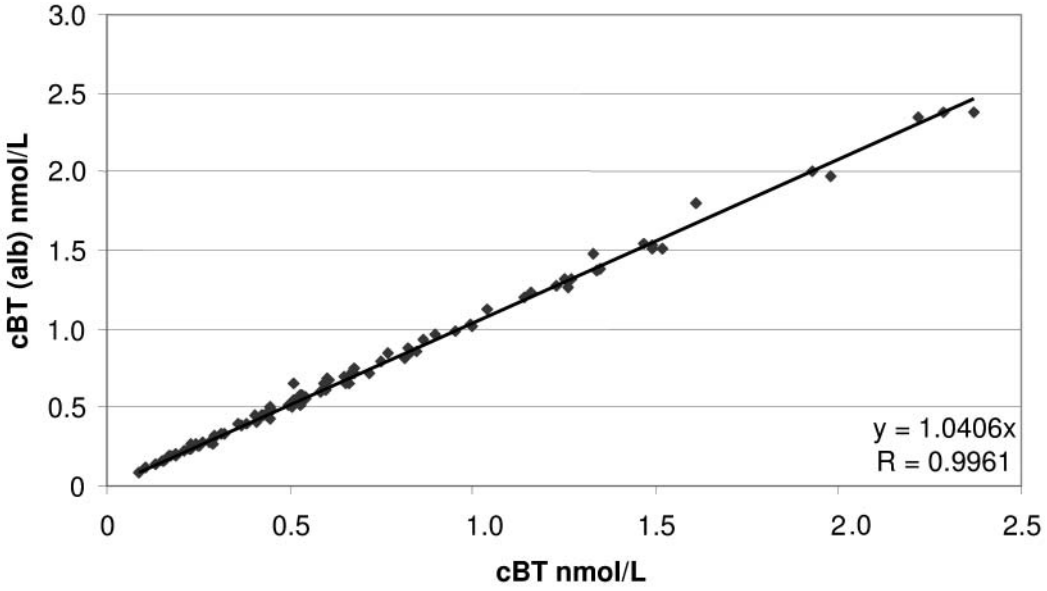

Figure 2. *Correlation of cBT, calculated from TT and SHBG with a fixed albumin concentration of 43 g/L with cBT calculated from TT and SHBG, taking into account the individual albumin concentration, cBT (alb). The calculation of BT was performed using the method described in detail by Vermeulen et al. (2).*

the FT values for the hyperandrogenic women (mean FT 0.03 nmol/L) found in the study population. If the formula developed by Vermeulen *et al.* is practicable in men with high T values (mean FT 0.332 nmol/L) and also in postmenopausal women (mean FT 0.011 nmol/L) with low T values, the formula may also be practicable in hyperandrogenic women. In general, there was a close correlation between the cFT and AFTC values over a wide range of concentrations (2). We, therefore, assume that the formula was also practicable in our study population with a mean FT of 0.03 nmol/L. In agreement with the above-mentioned study, the present results implied that, in

hyperandrogenic women (mean FT 0.03 nmol/L), each individual's albumin concentration does not significantly influence the calculation results of FT from TT and SHBG if the albumin concentration is in the physiological range of 40 to 50 g/L.

In their calculation procedure, Ly and Handelsman also did not recognize the individual albumin concentration mainly responsible for binding the remainder of the blood T. They concluded that their newly-developed formula for calculating blood FT from TT and SHBG in the same sample is a robust, reliable and valid parameter for FT, without taking the albumin concentration into account (12).

Despite the ongoing debate regarding the validity of the different assays for FT measurement (22) and the different calculation formulae that can be used, depending on the levels of serum T in men and women, our results clearly showed that there was no need to take each individual albumin concentration into account in order to calculate FT in hyperandrogenic women if the formula developed by Vermeulen *et al.* is used. In comparison with equilibrium dialyses, the FT value obtained by calculation from serum TT and SHBG, as determined by immunoassay, appears to be a rapid, simple and reliable procedure for estimating FT and practical for clinical routine work.

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