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ORIGINAL ARTICLE

Biological variation of total testosterone, free androgen index and bioavailable testosterone in polycystic ovarian syndrome: implications for identifying hyperandrogenaemia

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Summary

Objective Hyperandrogenaemia is one of the three Rotterdam consensus diagnostic criteria for polycystic ovarian syndrome (PCOS) and may be measured by estimation of total testosterone, free androgen index (FAI) or bioavailable testosterone (BioT). The aim of this study was to compare the biological variability of total testosterone with that of the biological variability of both the FAI and BioT, to determine the least variable measurement for clinical practice.

Design Comparative study.

Patients Blood samples were collected after an overnight fast at 4-day intervals on 10 consecutive occasions from 12 PCOS patients and 11 weight- and age-matched control women.

Measurements Duplicate samples of stored serum were analysed for total testosterone, SHBG and BioT in a single batch.

Results The PCOS group had a significantly higher median BioT, FAI and total testosterone than controls. In both the PCOS and control groups, the intraindividual variance was small and similar for BioT and FAI. There was no significant difference between the within-subject biological coefficient of variation (CV_I) for BioT, FAI and total testosterone. The maximum and minimum critical differences were +58% and –37% for BioT and +70% and –40% for FAI, respectively.

Conclusion FAI appears to be the better diagnostic marker to distinguish hyperandrogenism in patients with PCOS, but once diagnosis has been made, all three methods should be equally good in monitoring further changes in the androgen status.

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Introduction

Polycystic ovarian syndrome (PCOS) is a common disorder of women of reproductive age and is characterized by chronic anovulation and androgen excess. As a consequence, the Rotterdam consensus 2003 has recommended hyperandrogenism as one of the three diagnostic criteria for diagnosis of PCOS.[1] Hyperandrogenism presents as hirsutism, acne or male pattern alopecia; serum concentrations of total testosterone are often at the upper limit of normal or modestly elevated, but free testosterone (direct measurement or using the free androgen index, FAI) is usually elevated.[2] This is recognized as being due to the clearance and bioavailability of testosterone, which is affected by the serum concentration of SHBG; hence, the diagnostic utility of an isolated serum testosterone measurement is questionable and probably adds to the heterogeneity of diagnostic criteria used in PCOS. Bioavailable testosterone (BioT) measurement (which excludes SHBG-bound testosterone) and the FAI, which accounts for the sex hormone-bound fraction of testosterone, are assumed to be inherently superior estimates of hyperandrogenism compared with the measurement of total testosterone concentration alone. While the biological variability of total testosterone has been studied previously[3,4] in normal individuals, no comparative data currently exist on the biological variation of BioT and FAI. These data are crucial for determining the relative suitability and/or superiority of these measures in both the initial and subsequent follow-up of the biochemical assessment of androgen excess in PCOS.

Materials and methods

Subjects were recruited initially for a study to assess the biological variation of insulin resistance in individuals with PCOS.[5] Twelve overweight Caucasian women diagnosed to have PCOS (median age 28 years, range 18–31) and 11 weight-matched Caucasian women (controls) having regular menses (every 28–30 days) and without PCOS (median age 30 years, range 19–33) participated in the study. The diagnosis of PCOS was based on evidence of a history of oligomenorrhoea and either hirsutism or acne, together with hyperandrogenaemia (FAI > 8). FAI was defined as testosterone × 100/SHBG, with both units in nmol/l. Nonclassical 21-hydroxylase deficiency, hyperprolactinaemia and androgen-secreting tumours were excluded by appropriate tests before the diagnosis of PCOS was made.[6] No subject was taking any medication at the start of the study, nor during the preceding 6 months, and there was no concurrent illness. All subjects were on an unrestricted diet and were instructed

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Table 1. Baseline clinical and biochemical features of study subjects

Parameter	PCOS ($n = 12$)	Controls ($n = 11$)	P-value
Age (years)	26·3 ± 4·58	28·4 ± 4·72	ns
BMI (kg/m^2)	33·2 ± 6·32	29·9 ± 3·25	ns
Testosterone (nmol/l)	3·9 (3·1–4·6)	3·2 (2·6–4·0)	< 0·001
SHBG (nmol/l)	22·9 ± 5·06	64·5 ± 7·65	< 0·001
BioT (nmol/l)	1·3 (1·0–1·6)	0·9 (0·7–1·1)	< 0·001
FAI (%)	16·1 (11·6–21·1)	7·4 (5·9–9·4)	0·001
Non-SHBG-bound T (nmol/l)	0·19 (0·13–0·26)	0·07 (0·06–0·11)	0·001

PCOS, polycystic ovarian syndrome; BMI, body mass index; BioT, bioavailable testosterone; FAI, free androgen index; T, testosterone; ns, not significant.
Data presented as mean ± SD or as median (range) due to non-Gaussian distribution.

not to modify their usual eating patterns during the period of sampling. Fasting plasma glucose, age and body mass index (BMI) were obtained. Patients and controls were matched for BMI (Table 1). Fasting venous blood was collected into serum gel tubes (Becton Dickinson, Oxford, UK) at the same time each day (0800–0900 h) on 10 consecutive occasions at 4-day intervals. Samples were separated by centrifugation at 2000 *g* for 15 min at 4 °C, and aliquots of the serum were stored at –20 °C within 1 h of collection. The study was conducted in accordance with the guidelines in the Declaration of Helsinki. All subjects gave their informed written consent prior to entering the study, which had been approved by our local ethics committee. The clinical trial registration number for this study is ISRCTN65353256.

Reagents

Before analysis, all the serum samples were thawed and mixed thoroughly. The duplicate samples (i.e. two per visit) were randomized and then analysed in a single continuous batch using a single batch of reagents. Duplicate aliquots of stored serum were analysed for SHBG, total testosterone (T) and BioT and analysed in singleton. The FAI was obtained as the quotient 100T/SHBG. Serum testosterone was measured on an Architect analyser (Abbott Laboratories, Maidenhead, UK) and SHBG was measured on a DPC Immulite 2000 (Euro/DPC, Llanberis, UK) analyser using the manufacturer's recommended protocol. The intra-assay coefficients of variation (CVs) for total testosterone and SHBG as determined from duplicate study samples were 4·9% and 5·8%, respectively. BioT was measured using minor modifications of the method of Tremblay and Dube[7] and is widely reported as a measure of non-SHBG-bound testosterone.[8–11] In brief, this technique uses ^{3}H-labelled testosterone as a tracer, where SHBG is precipitated from charcoal-stripped serum using saturated ammonium sulfate and the radiolabelled testosterone measured in the supernatant as an estimate of the percentage non-SHBG-bound fraction. The concentration of BioT can then be calculated as this fraction of the total testosterone. Interassay precision was 5·25% at a concentration of 5·0 nmol/l.

Statistical analysis

Statistical analysis was performed using SPSS version 14. Biovariability data were analysed by calculating analytical, within-subject and between-subject variances (SD_A^2, SD_I^2 and SD_G^2, respectively) according to the methods of Fraser and co-workers.[12,13] Using this technique, analytical variance (SD_A^2) was calculated from the difference between duplicate results for each specimen ($SD_A^2 = \Sigma d^2/2N$, where d is the difference between duplicates, and N is the number of paired results). The variance of the first set of duplicate results for each subject on the 10 assessment days was used to calculate the average biological intraindividual variance (SD_I^2) by subtraction of SD_A^2 from the observed dispersion (equal to $SD_I^2 + SD_A^2$). Subtracting $SD_I^2 + SD_A^2$ from the overall variance of the set of first results determined the interindividual variance (SD_G^2). The intraindividual (SD_I) and interindividual (SD_G) variations were estimated as square roots of the respective variance component estimates. The reference change value, or critical difference between two consecutive samples in an individual subject (i.e. the smallest percentage change unlikely to be due to biological variability), was calculated using the formula $2{\cdot}77(CV_I)$, where CV_I is the within-subject biological coefficient of variation.[12] The index of individuality (IoI) was derived from the ratio of intra- and interindividual variation (SD_I/SD_G).[12] When the IoI for a particular test is 0·6 or less, conventional population-based reference intervals are of limited value in the detection of unusual results for a particular individual. When the IoI is 1·4 or more, the variation in an individual will fit the population reference limits more closely, thus being suitable as a screening test.

A power calculation based on our previous biological variability studies[3–5] suggested that a total number of eight patients per arm was sufficient to achieve statistical significance, $P < 0{\cdot}05$.

Results

The baseline clinical and biochemical details of the subjects are shown in Table 1. Figures 1, 2 and 3 show the median and range of BioT, FAI and total testosterone for the individuals in the three groups. The PCOS group had significantly higher median BioT, total T and FAI than the control group [median (range), 1·3 (1·0–1·6) *vs.* 0·9 (0·7–1·1) nmol/l, $P = 0{\cdot}001$; 3·9 (3·1–4·6) *vs.* 3·2 (2·6–4·0) nmol/l, $P = 0{\cdot}001$; and 16·1 (11·6–21·1) *vs.* 7·4 (5·9–9·4), $P = 0{\cdot}001$, respectively]. The distribution of both BioT and FAI in controls and patients with PCOS were non-Gaussian using the Kolmogarov–Smirnov test. Data were therefore log-transformed before calculations were performed. In both the PCOS and control groups the intraindividual variance was small and similar for BioT and FAI (Table 2).

IoI values for FAI, BioT and total testosterone were 0·87, 0·91 and 0·69, respectively, for PCOS and 0·61, 0·71 and 0·43 for controls. There was no significant difference between CV_I of BioT, FAI and total testosterone.

In the group with PCOS, a strong correlation was seen between the BioT values and FAI ($r = 0{\cdot}9$, $P = 0{\cdot}001$). However, this correlation was not observed in the control group ($r = 0{\cdot}2$, $P = 0{\cdot}079$). The minimum and maximum critical difference between two consecutive BioT samples in an individual patient with PCOS was –37% or

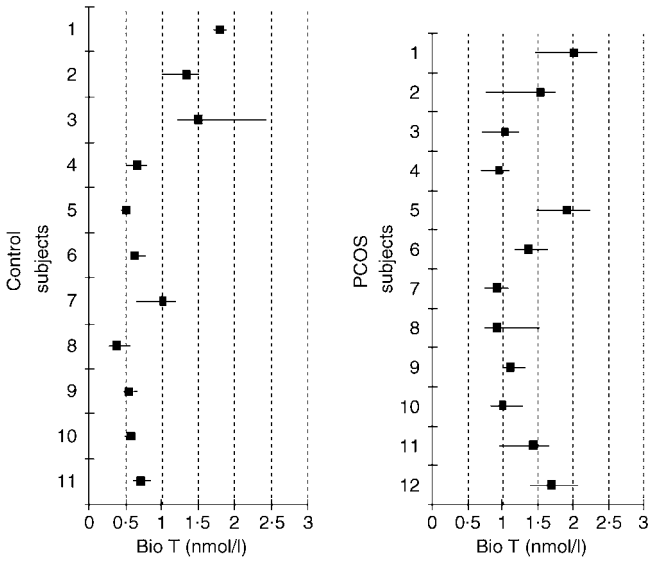

Fig. 1 Median and range of values (unadjusted for analytical variation) for bioavailable testosterone (BioT) in PCOS and controls.

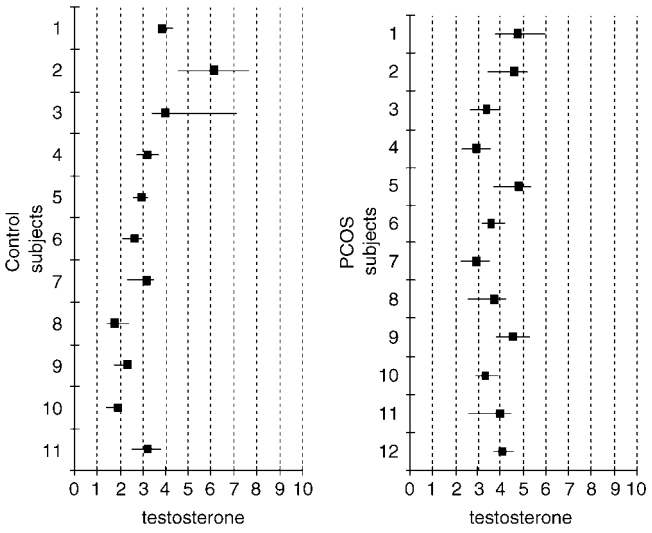

Fig. 3 Median and range of values (unadjusted for analytical variation) for total testosterone in PCOS and controls.

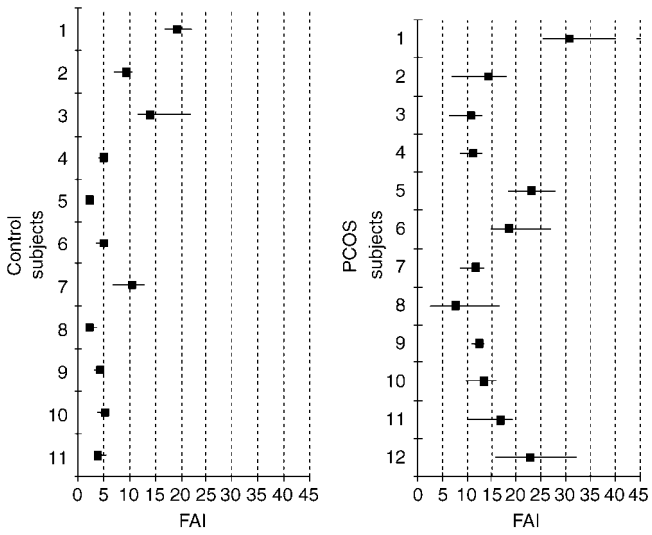

Fig. 2 Median and range of values (unadjusted for analytical variation) for FAI in PCOS and controls.

Table 2. Percentages of contribution from analytical, intra- and interindividual variance to total variance in PCOS and controls

	% analytical variance from total variance	% intraindividual variance from total variance	% interindividual variance from total variance
PCOS			
BioT	2·8	28·8	68·4
FAI	1·8	29·0	69·2
Total T	5·2	30·7	64·1
Non-SHBG-bound T	1·6	21·8	76·6
Controls			
BioT	3·5	5·6	90·8
FAI	1·1	5·4	93·5
Total T	1·5	15·1	83·4
Non-SHBG-bound T	1·0	7·4	91·6

PCOS, polycystic ovarian syndrome; BioT, bioavailable testosterone; FAI, free androgen index; T, testosterone.

+58% of any initial level of BioT. This indicates that a subsequent sample must rise by greater than 58% or fall by more than 37% to be considered significantly different from the first.

In comparison, the critical difference for FAI was –41% and +70%. Total testosterone was normally distributed and the critical difference was ±33%.

Discussion

This is the first study to systematically examine and compare the biological variability of BioT and FAI to total testosterone in PCOS, and demonstrates the relative suitability of each measure in detecting hyperandrogenism in PCOS. Indeed, one of the advantages in specifically assessing the biological variability of these tests is that any differences found between them can be attributed more to the test itself rather than to analytical limitations of the assays involved.

There were high between-subject variations for BioT and FAI in both PCOS and controls, limiting their potential use as a measure to assess the degree of androgen excess present from a single estimate. However, if we consider the range of values in PCOS women, there was an overlap in values between women with PCOS and controls in total testosterone and BioT but no overlaps in FAI, making it a potentially better marker in identifying patients with PCOS than total testosterone alone.

Testosterone circulates in blood bound to both albumin and to the specific binding protein SHBG, with about 1% in women present

in the free, nonprotein state.[14–16] The bioactive component includes the free plus albumin-bound fraction testosterone but not the SHBG-bound testosterone. Therefore, variation in the concentration of SHBG will influence the total concentration of testosterone and thus present difficulty in interpretation of results. SHBG concentration increases during pregnancy, oral contraceptive (oestrogen) usage, hyperthyroidism, administration of antiepileptic drugs and weight loss. However, it decreases with hypothyroidism, obesity or androgen excess.[17] As BioT measures only the bioactive and not the SHBG-bound component and the FAI is a calculated measure of testosterone adjusted for abnormalities in SHBG, they are presumed to be a more reliable index of measurement for hyperandrogenism.

The low IoI value for all three tests in control subjects suggest that none are ideal diagnostic markers for detecting hyperandrogenism in patients with PCOS. This is because a low IoI means that healthy individuals have androgen values that repeatedly remain close to their 'set point' within the population reference range rather than moving within the reference range. As a consequence, it means that individuals who usually have their set point at the lower end of the reference range can develop significant hyperandrogenaemia (in relation to themselves) but be regarded as 'normal' because their result still falls within the population reference range. By contrast, use of a low IoI test to follow up patients already known to have the disease can in fact be of advantage as it means that a relatively small change in their test result will probably be of significance.[18]

In this respect, the IoI and CV_I of total testosterone, BioT and FAI in PCOS patients were relatively similar, suggesting that all three are as useful (or otherwise) in monitoring androgen levels once diagnosis has been made. Both the strong correlation seen between BioT levels and FAI in the group with PCOS and the similar intra/inter-individual variance suggest that when the diagnosis of PCOS has been made, measurement of either BioT or FAI could be used with equal utility when assessing androgen status using a single measure. This correlation was not seen in the control groups, possibly because of the much smaller range of results among the healthy participants compared to those with PCOS. The critical difference value determined in this study means that, for any given individual, when serial measures of BioT are used to assess change in androgen status, a subsequent value must rise by more than 58% or fall by more than 37% to be considered significantly different from the first (i.e. the change is greater than that explained by biological variation alone). The same argument applies for FAI, where the value should be greater than 70% or less than 41%. The Rotterdam criteria suggested the use of hyperandrogenaemia as a means of diagnosing PCOS. However, once the diagnosis is made, monitoring in response to treatment may be useful to assess a treatment effect.

We also calculated the non-SHBG-bound testosterone according to the method of Pearlman and Crepy,[19] which was found to have a good correlation with that of the direct radioimmunoassay of testosterone in ammonium sulfate-precipated plasma.[20] The results are also included in Tables 1 and 2. As expected, the calculated non-SHBG-bound testosterone and its variability was found to be closely correlated to FAI as both represent an indirect measure of free testosterone.

There are limitations to this study. First, this study was executed and analysis performed before solvent extraction of raised testosterone samples was routinely performed by the local laboratory. A recent publication by two of our authors suggested that the Abbott testosterone assay is prone to interference by dehydroepiandrosterone sulfate (DHEAS), which can be removed by a solvent extraction and reanalysis method.[21] This, in turn, could potentially have an influence on our study findings. However, DHEAS interferes not only in the Abbott testosterone assay but also in most other immunoassay methods.[22] Therefore, our results should be comparable to those measured on women in most routine clinical laboratories in the UK, although the proportion of laboratories that would investigate a raised result further by either solvent extraction or referral to another laboratory is unknown. It should be noted, however, that these findings may only be relevant for the defined methodology and caution is necessary for general application. Second, by selecting patients for the study as having PCOS on the basis of a raised FAI, this could itself have introduced a bias in establishing which test was superior at identifying the disease. Nonetheless, any androgen cut-off used for diagnosis, whether it is total testosterone, BioT or FAI, is necessarily arbitrary and our routine use of FAI does not detract from the fact that the overlap between measurements of total testosterone and BioT in healthy and PCOS subjects was far greater than with FAI.

In conclusion, FAI appears to be a better diagnostic marker for hyperandrogenism in patients with PCOS as there is less overlap with normality, but once the diagnosis is made, then all three methods have equal utility for monitoring changes in the androgen status.

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