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ORIGINAL ARTICLE

Establishing the cut off values of androgen markers in the assessment of polycystic ovarian syndrome

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Abstract

Introduction: Hyperandrogenism remains as one of the key features in Polycystic Ovarian Syndrome (PCOS) and can be assessed clinically or determined by biochemical assays. Hirsutism is the most common clinical manifestation of hyperandrogenism. The clinical assessment is subjected to wide variability due to poor interobserver agreement and multiple population factors such as ethnic variation, cosmetic procedures and genetic trait. The difficulty in resolving the androgen excess biochemically is due to a lack of consensus as to which serum androgen should be measured for the diagnosis of PCOS. The aim of the study was to compare and establish the diagnostic cut off value for different androgen biomarker for the diagnosis of PCOS. *Materials and Methods:* A total of 312 patients classified to PCOS (n = 164) and non PCOS (n = 148) cohorts were selected from the Laboratory Information System (LIS) based on serum total testosterone (TT) and sex hormone binding globulin (SHBG) from the period of 1st April 2015 to 31st March 2016. PCOS was diagnosed based on Rotterdam criteria. Clinical hyperandrogenism and ultrasound polycystic ovarian morphology were obtained from the clinical records. The other relevant biochemical results such as serum luteinizing hormone (LH), follicle stimulating hormone (FSH) and albumin were also obtained from LIS. Free androgen index (FAI), calculated free testosterone (cFT) and calculated bioavailable testosterone (cBT) were calculated for these patients. Receiver Operating Characteristic (ROC) curve analysis were performed for serum TT, SHBG, FAI, cFT, cBT and LH: FSH ratio to determine the best marker to diagnose PCOS. *Results:* All the androgen parameters (except SHBG) were significantly higher in PCOS patients than in control ($p<0.0001$). The highest area under curve (AUC) curve was found for cBT followed by cFT and FAI. TT and LH: FSH ratio recorded a lower AUC and the lowest AUC was seen for SHBG. cBT at a cut off value of 0.86 nmol/L had the highest specificity, 83% and positive likelihood ratio (LR) at 3.79. This is followed by FAI at a cut off value of 7.1% with specificity at 82% and cFT at a cut off value of 0.8 pmol/L with specificity at 80%. All three calculated androgen indices (FAI, cFT and cBT) showed good correlation with each other. Furthermore, cFT, FAI and calculated BT were shown to be more specific with higher positive likelihood ratio than measured androgen markers. *Conclusions:* Based on our study, the calculated testosterone indices such as FAI, cBT and cFT are useful markers to distinguish PCOS from non-PCOS. Owing to ease of calculation, FAI can be incorporated in LIS and can be reported with TT and SHBG. This will be helpful for clinician to diagnose hyperandrogenism in PCOS.

Keywords: Androgen excess, diagnostic cut off value, free androgen index, calculated free and bioavailable testosterone, receiver operating characteristic curve

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is one of the most common endocrinopathies in women of reproductive age.[1] The prevalence of PCOS varies depending on which criteria is used to make the diagnosis but is as high as 15% - 20% when the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) criteria are being used.[2] PCOS is a heterogeneous disorder with its aetiology still

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poorly understood. However, there is presence of insulin resistance in most of the cases, with compensatory hyperinsulinemia contributing to hyperandrogenism by stimulation of ovarian androgen secretion and inhibition of SHBG production.[3] Environmental and genetic factors have also been implicated in the development of PCOS.

PCOS was first reported by Stein and Leventhal in 1935. Until now, there has been no consensus on the absolute defining features of the phenotype. Three groups have published diagnostic criteria for PCOS: The National Institutes of Health/National Institute of Child Health and Human Disease (NIH/NICHD); the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM); and the Androgen Excess and PCOS Society (Table 1).[4] While there are certain consistencies between the criteria offered by the different groups, important differences exist. Each issuing group considers PCOS as a diagnosis of exclusion. The NIH/NICHD and the Androgen Excess Society require that patients should have signs or symptoms of hyperandrogenism such as hirsutism, or hyperandrogenaemia, defined as elevated free testosterone, reduced SHBG, elevated free testosterone index, or elevated dehydroepiandrosterone sulphate.[4] However, the ESHRE/ASRM (Rotterdam) criteria allows for the diagnosis of PCOS without the presence hyperandrogenism.

Despite differences in the criteria available, hyperandrogenism remains a consistent key feature in PCOS. Clinical hyperandrogenism is characterised by presence of hirsutism. Hirsutism, considering ethnic differences, is a good marker for hyperandrogenism. It is assessed by modified Ferriman-Gallwey method, but this can be subjected to wide inter – observer variation and does not take into consideration ethnic variations.[5,6]

Defining biochemical hyperandrogenism still remains a major challenge. Testosterone circulating in plasma is almost 97–99% bound to SHBG and albumin. Serum levels of free testosterone are more frequently elevated in women with PCOS. The recommended marker to assess androgen excess is serum free testosterone by equilibrium dialysis.[7] However, only very few laboratories measure free testosterone because the assay is complex, expensive and labour-intensive.[8] Therefore, surrogate markers such as the free androgen index (FAI: the ratio of total testosterone to SHBG multiplied by 100) or calculated free and bioavailable testosterone (cFT and cBT - based on equation by Vermuelen *et al.*[9]), have been used to assess hyperandrogenemia. The calculation of cFT and cBT is based on law of mass action involving total testosterone (TT), SHBG and albumin.

To date, a few studies have been done to determine the appropriate marker to define hyperandrogenism. In our hospital, to assess hyperandrogenism, TT, SHBG and calculated FAI are reported. However, FAI is reported without appropriate diagnostic decision level. Hence, the aim of the present study is to establish diagnostic cut-off level for FAI to be used in the diagnosis of PCOS and to evaluate the usefulness of calculated free and bioavailable testosterone in the diagnosis of PCOS for our local population.

MATERIALS AND METHODS

Subjects

A total of 312 Malaysian females of reproductive age group (ranging from 21 to 46 years old) who were investigated for various gynaecological problems from 1st April 2015 to 31st March 2016 were selected from the Laboratory Instrumentation System (LIS) database. These patients had sought medical treatment for various

TABLE 1: Criteria for diagnosis of polycystic ovarian syndrome[4]

Exclusion of other androgen excess related disorders (congenital adrenal hyperplasia, androgen secreting tumours, Cushing syndrome, thyroid dysfunction and hyperprolactinaemia)		
NIH/NICHD 1992	ESHRE/ASRM (Rotterdam Criteria) 2004	Androgen Excess Society 2006
Includes all of the following	Includes two of the following	Includes all of the following
• Clinical and/or biochemical hyperandrogenism • Menstrual dysfunction	• Clinical and/or biochemical hyperandrogenism • Oligo-ovulation or anovulation • Polycystic ovaries	• Clinical and/or biochemical hyperandrogenism • Ovarian dysfunction and/or polycystic ovaries

symptoms such as menstrual abnormalities, obesity, infertility etc. from the Department of Obstetrics and Gynaecology, University Malaya Medical Centre (UMMC). The study protocol was approved by the Medical Ethics Committee, UMMC.

Data collection

Clinical details and biochemical investigations of each subject were extracted from the clinical notes and LIS database respectively. The study population was categorised to two groups based on the diagnosis into PCOS group (n = 164) and non-PCOS group (n = 148). None of the patients in PCOS and non-PCOS groups were on oral contraceptives.

The diagnosis of PCOS was made according to Rotterdam criteria by the gynaecologist at the time of patient care. History of ovulatory dysfunction and clinical hyperandrogenism (defined by hirsutism, acne and alopecia) were recorded from PCOS patients' clinical notes. Based on ultrasound findings by a sonographer or the radiology department, polycystic ovarian morphology was reported. Complications of PCOS related to insulin resistance such as obesity, impaired oral glucose tolerance and type 2 diabetes mellitus were also noted. The other causes of hyperandrogenism, including Cushing's syndrome, congenital adrenal hyperplasia, and androgen secreting tumours, hyperprolactinemia, and drugs were excluded for the patients who were diagnosed as PCOS. The non-PCOS cases mainly comprised of individuals who were investigated for infertility, endometriosis or fibroids and none of them had any clinical signs and symptoms suggestive of hyperandrogenism.

Biochemical assay

The laboratory investigations such as serum total testosterone (TT), sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicular stimulating hormone (FSH) and serum albumin levels for each patient were extracted from the LIS. Serum TT was measured by competitive direct immunoassay in Advia Centaur® immunoassay platform (Siemens Healthineers, Germany) which has an analytical sensitivity of 0.35 nmol/L. Serum FSH and LH were measured by two site sandwich immunoassay in Advia Centaur® immunoassay platform (Siemens Healthineers, Germany) with analytical sensitivity of 0.3I U/L and 0.07 IU/L, respectively. Serum SHBG was measured by solid phase two site chemiluminescent immunometric assay in Immulite® 2000 system (Siemens Healthineers, Germany) with analytical sensitivity 0.02 nmol/L.

Calculations

Free Androgen Index (FAI) was calculated according to the equation FAI = [(TT/ SHBG) × 100]. Free testosterone (cFT) and bioavailable testosterone (cBT) were calculated using the formula available online at the website http://www.issam.ch/freetesto.htm. LH: FSH ratio was also included as one of the androgen marker.

Statistical analysis

Kolmogorov-Smirnov test was used to determine if the data were Gaussian distributed. Since the data were not normally distributed, they were presented as median and range (lowest - highest range). The Mann-Whitney U test was used for the comparison of PCOS and non-PCOS parameters with p values <0.05 being considered statistically significant. Receiver operating characteristics (ROC) curves and the calculation of area under curve (AUC) for each variable was performed. Sensitivity and specificity for the androgen biomarkers were obtained based on the ROC analysis. A statistical assessment of the difference between the ROC curves for the androgen markers was also performed. Values were reported with 95% confidence intervals. A Spearman correlation analysis was performed to identify the association between the three calculated androgen markers (FAI, cFT, cBT). These statistical analyses were performed using Analyse –it for Microsoft excel (version 2.20) (Analyse-it Software, Ltd. http://www.analyse-it.com/; 2009).

RESULTS

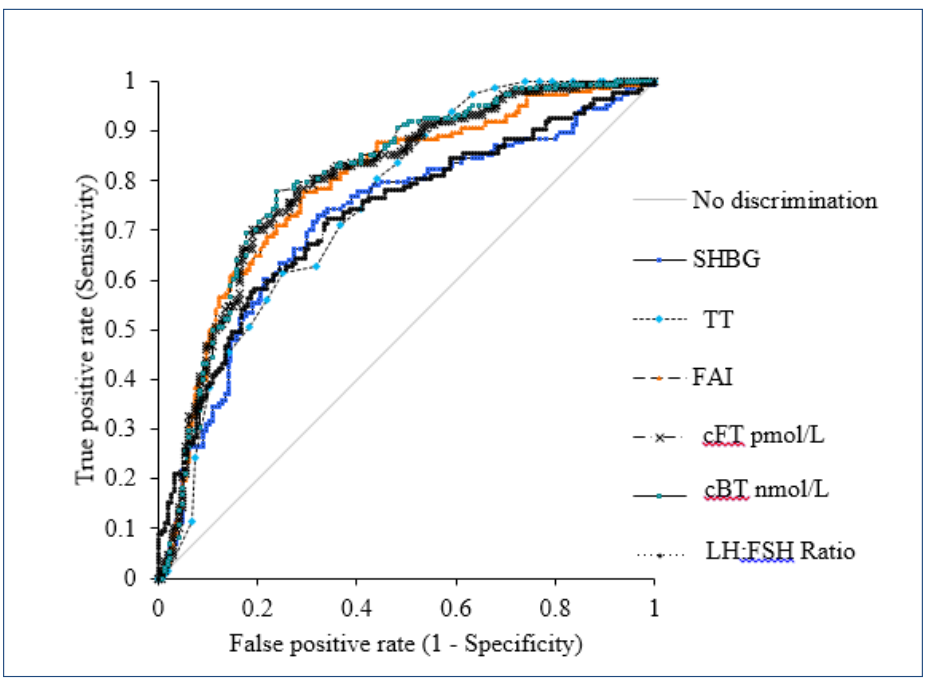
The age and biochemical androgen parameters for the PCOS and non-PCOS cohorts are summarised in Table 2. Significant differences exist between the two groups for the endocrine parameters. As expected, women in the PCOS group showed higher level of TT, FAI, cFT, cBT and LH:FSH ratio and a lower level of SHBG. However, the age distribution between both the groups was not significantly different.

The ROC curves plotted for each of the androgen markers are shown in Fig. 1. The optimum cut-off values determined from the ROC curve analysis, together with diagnostic sensitivity, diagnostic specificity, negative and

TABLE 2: Endocrine variables in PCOS and non-PCOS patients

Variable	Non-PCOS (n = 148)		PCOS (n = 164)		**p Value
	Median	Range	Median	Range	
Age	31	21 – 46	30	21 – 45	0.1838
TT (nmol/L)	1.4	0.6 – 2.6	2.2	0.5 - 5.4	< 0.0001
SHBG (nmol/L)	42.5	6.9 – 140	23.9	8 – 176	< 0.0001
FAI (%)	3.6	0.7 - 33.3	9.2	0.4 - 43.2	< 0.0001
cFT (pmol/L)	23.2	6.2 – 81.5	44.1	3.4 – 166	< 0.0001
cBT (nmol/L)	0.52	0.14 – 1.78	1.06	0.09 - 5.6	< 0.0001
LH:FSH*	0.65	0.1 - 6	1.3	0.29 - 5.12	< 0.0001

*No of samples with LH:FSH differs from other variables above with total (n = 293), non-PCOS (n = 137) and PCOS (n = 156). **p value <0.05 is considered significant.
Polycystic ovarian syndrome (PCOS), total testosterone (TT), sex hormone binding globulin (SHBG), free androgen index (FAI), calculated free testosterone (cFT), calculated bioavailable testosterone (cBT), ratio of luteinising hormone: follicle stimulating hormone (LH: FSH).



Test	Area	95% CI	SE	z value	p value
SHBG	0.72	0.66 to 0.78	0.030	7.51	<0.0001
TT	0.75	0.70 to 0.81	0.027	9.39	<0.0001
FAI	0.79	0.74 to 0.84	0.026	11.13	<0.0001
cFT	0.80	0.75 to 0.85	0.025	11.86	<0.0001
cBT	0.81	0.76 to 0.86	0.025	12.38	<0.0001
LH:FSH	0.73	0.67 to 0.79	0.030	7.72	<0.0001

FIG. 1: Receiver operating characteristic curve for discriminating between different androgen markers in diagnosing polycystic ovarian syndrome. *p value <0.05 is considered significant. (Sex hormone binding globulin (SHBG), total testosterone (TT), free androgen index (FAI), calculated free testosterone (cFT), calculated bioavailable testosterone (cBT), ratio of luteinising hormone: follicle stimulating hormone (LH:FSH), confidence interval (CI), standard error of mean (SE))

positive likelihood ratio (LR) calculated based on optimum cut-off values are presented in Table 3.

cBT had the highest AUC which was 0.81, whereas SHBG had the lowest AUC which was 0.72. Pairwise comparison done for the ROC curves is shown in Table 4. Significant differences are seen between cBT vs CFT, cBT vs FAI, cBT vs TT and cFT vs TT.

The calculated androgen indices which are cBT, cFT and FAI had higher specificity and positive LR. cBT at a cut off value of 0.81 nmol/L had the highest specificity of 83% and positive LR of 3.79. Serum TT with a cut off value of 0.75 nmol/L showed the lowest positive LR of 2.30.

Spearman correlation performed between the three calculated androgen indices is shown in Table 5. All three calculated parameters cBT, cFT and FAI showed good correlation with each other.

DISCUSSION

It has been estimated 80% of women with PCOS have elevated levels of androgens.[10] Even though hyperandrogenism is a key feature of PCOS, there are still controversies surrounding the choice of biomarker to establish the diagnosis hyperandrogenaemia. The paucity of normative data and poor precision and accuracy of commercially available testosterone assays especially in the low range, have made it difficult to establish androgen excess in women.[11] The American Association of Clinical Endocrinologist's (AACE) guidelines for PCOS recommend measurement of free testosterone by equilibrium dialysis for establishing hyperandrogenemia.[7] This method is technically cumbersome, difficult, time-consuming and not automated. Thus, they are not routinely used in most laboratories.[9] When this technique is unavailable, AACE has instead recommended determination of the FAI.[7] Estimation of free testosterone based on other calculated biomarkers such as cFT and cBT have also been used as markers of hyperandrogenaemia. The calculation of FAI, cFT and cBT require the measurement of TT, SHBG and albumin concentrations.

Although there are several assays available for measurement of TT, but there are significant methodological biases between different manufacturers.[12] Therefore, the optimal cut off for FAI to diagnose PCOS has to be based on the specific laboratory method. We undertook this study to establish a diagnostic cut off value for FAI based on the methodology that is used in our laboratory and to evaluate the usefulness of cFT and cBT for the diagnosis of PCOS.

Similar to other studies, we observed higher values for TT, FAI, cBT and cFT and lower values for SHBG in PCOS compared to non PCOS[13,14,15] ROC curve analysis suggests that calculated androgen indices (FAI, cFT and cBT) are superior to the conventional markers (TT, SHBG and LH: FSH ratio) in the diagnosis of PCOS. Spanish and German studies also have noted that calculated indices are useful parameters for the discrimination of PCOS.[13,15] Pasquali R *et al*. have recommended combined use of parameters such as TT, androstenedione and FAI to define hyperandrogenism in 90% of PCOS.[14]

TABLE 3: Analytical statistics for androgen markers based on receiver operating characteristic curve and optimum cut off value

Androgen marker	AUC	Cut off value	Sens. %	Spec. %	Positive LR	Negative LR	PPV	NPV
cBT (nmol/L)	0.81	0.86	64.0	83.1	3.79	0.43	80.8	67.6
FAI (%)	0.79	7.1	62.2	82.4	3.54	0.46	75.6	66.8
cFT (pmol/L)	0.80	35.3	68.9	80.4	3.52	0.39	79.6	70.0
SHBG (nmol/L)	0.72	29	66.5	73.6	2.52	0.46	73.6	66.5
LH: FSH	0.73	1.1	64.7	72.3	2.33	0.49	72.7	64.3
TT(nmol/L)	0.75	2	59.1	74.3	2.30	0.55	71.9	62.1

Area under curve (AUC), sensitivity (Sens.), specificity (Spec.), likelihood ratio (LR), positive predictive value (PPV), negative predictive value (NPV), calculated bioavailable testosterone (cBT), free androgen index (FAI), calculated free testosterone (cFT), sex hormone binding globulin (SHBG), ratio of luteinising hormone: follicle stimulating hormone (LH:FSH), total testosterone (TT).

TABLE 4: Statistical comparison of androgen markers

Comparison	Difference	95% CI	SE	z value	*p value
SHBG v TT	-0.03	-0.10 to 0.04	0.036	-0.90	0.3704
SHBG v FAI	-0.07	-0.10 to -0.04	0.015	-4.66	<0.0001
SHBG v cFT	-0.08	-0.12 to -0.04	0.021	-3.79	0.0002
SHBG v cBT	-0.09	-0.13 to -0.05	0.021	-4.18	<0.0001
SHBG v LH: FSH	-0.01	-0.08 to 0.07	0.038	-0.22	0.8235
TT v FAI	-0.04	-0.08 to 0.01	0.024	-1.46	0.1432
TT v cFT	-0.05	-0.08 to -0.01	0.019	-2.41	0.0161
TT v cBT	-0.05	-0.09 to -0.02	0.019	-2.79	0.0052
TT v LH: FSH	0.02	-0.04 to 0.09	0.032	0.75	0.4508
FAI v cFT	-0.01	-0.02 to 0.00	0.007	-1.43	0.1525
FAI v cBT	-0.02	-0.03 to 0.00	0.008	-2.41	0.0158
FAI v LH: FSH	0.06	-0.01 to 0.12	0.033	1.80	0.0719
cFT v cBT	-0.01	-0.02 to 0.00	0.004	-2.05	0.0405
cFT v LH: FSH	0.07	0.01 to 0.13	0.032	2.19	0.0288
cBT v LH: FSH	0.08	0.02 to 0.14	0.032	2.45	0.0142

*p< 0.05 is considered significant.
Sex hormone binding globulin (SHBG), total testosterone (TT), free androgen index (FAI), calculated free testosterone (cFT), calculated bioavailable testosterone (cBT), ratio of luteinising hormone: follicle stimulating hormone (LH:FSH), confidence interval (CI), standard error of mean (SE).

In our study, FAI at a cut off value of 7.1%, gave a sensitivity and specificity of 62% and 82% respectively. Different cut-off values of 4.97% and 6.1% has been proposed by Hahn *et al*. and Zhou *et al*. respectively.[13,16] The different cut off levels could be attributed to different methodologies for measurement of TT.

In conditions where SHBG levels are low, the FAI calculation may not be reliable.[14] The lower level of SHBG in PCOS is either due to insulin resistance or decreased synthesis of SHBG.[17] Instead of FAI, cFT and cBT may be useful to diagnose hyperandrogenism in conditions with low SHBG.[17] Vermuelen et al. created equation for cBT and cFT.[9] In our study, we found that cBT was a better marker than FAI to diagnose PCOS. At the cut off level of 0.86 nmol/L, the sensitivity and specificity were 64%, and 83% respectively with a positive LR of 3.79.

We noted strong correlation between FAI, cBT and cFT in diagnosing PCOS (Table 5). Hence these three markers could be used with equal efficacy in assessing androgen status. FAI is the most practical marker for routine use owing to its simple formula which will be easy for clinicians to calculate. LIS software requires advanced mathematical functions and equation logic, to incorporate cBT and cFT into LIS.[18] Since our

TABLE 5: Spearman correlation between three calculated androgen parameters (FAI, cFT and cBT)

Spearman correlation	r statistics	95% CI	*p value
FAI vs cFT	0.95	0.93 – 0.96	<0.0001
FAI vs cBT	0.92	0.90 – 0.94	<0.0001
cFT vs cBT	0.96	0.94 – 0.97	<0.0001

*p< 0.05 is considered significant.
Spearman's rank correlation coefficient (r), free androgen index (FAI), calculated free testosterone (cFT), calculated bioavailable testosterone (cBT), confidence interval (CI).

LIS software lacks these functions we could not incorporate cBT and cFT in our reporting.

One of the weaknesses of this study was testosterone being measured by automated immunoassay. The recommended method is by liquid chromatography with tandem mass spectrometry, which is not available in our laboratory. Since this was a retrospective study, there had been some limitations in obtaining thorough clinical information. The non-PCOS population included in this study were not exclusively healthy individuals. However, higher levels for androgen markers were observed in PCOS than non-PCOS.

In conclusion, the calculated testosterone indices such as FAI, cBT and cFT are useful markers to distinguish PCOS from non-PCOS. Based on our study, appropriate cut-off value FAI can be reported with calculated FAI and this will be helpful for clinicians to diagnose hyperandrogenism in PCOS.

Conflict of interest: The authors do not believe that there is a conflict of interest that could potentially be construed to affect the material contained in the manuscript that is being submitted to the journal.

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