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Original Article

Obstetrics and Gynaecology Section

Dihydrotestosterone- A Potential Biomarker of Hyperandrogenaemia in Polycystic Ovary Syndrome: A Case-control Study from North India

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ABSTRACT

Introduction: Polycystic Ovary Syndrome (PCOS) is a complex reproductive disorder characterised by hyperandrogenism, ovulatory dysfunction and polycystic/enlarged ovary. Although clinical and/or biochemical hyperandrogenism is one of the major features of PCOS, biochemical hyperandrogenism in the form of high testosterone and/or Free Androgen Index (FAI) is rarely observed in the Asian Indians.

Aim: To assess various androgens to determine best available biomarker of androgens in PCOS from North India.

Materials and Methods: This case-control study was conducted in the Department of Reproductive Biology, All India Institute of Medical Sciences, New Delhi, India, between January 2016 to December 2019. During this period 137 female with PCOS and 49 female as control were included. Serum total testosterone (T), FAI, Dehydroepiandrosterone Sulphate (DHEAS), androstenedione and Dihydrotestosterone (DHT) were measured besides assessment of hirsutism using the Ferriman-Gallwey (FG) scale. Statistical differences were derived using Mann-Whitney U test, Receiver Operating Characteristic (ROC) curve analysis and Spearman's correlation test.

Results: There were 87 PCOS cases with phenotype A, 25 PCOS cases with phenotype B, 10 PCOS cases with phenotype C and 15 PCOS cases with phenotype D. The mean age was 23.7 years in the PCOS group and 26.2 years in the control group. The mean Body Mass Index (BMI) in the PCOS group was 25.23 kg/m^2 and in the control was 22.6 kg/m^2. FG score of ≥9 was observed in 75.9% PCOS cases. High (mean+2SD) levels of T (≥0.51 ng/mL), FAI (≥2.55), DHEAS (≥309 ug/dL), androstenedione (≥2.2 ng/mL) and DHT (≥462 pg/mL) were observed in 35.29%, 56.25%, 14.18%, 18.62% and 61.38% cases, respectively. Mean DHT value was 584.27 pg/mL in study group whereas in control was 257.15 pg/mL (p-value <0.00001) and area under ROC curve was 0.895. Similarly, area under ROC curve was 0.86, 0.817, 0.721 and 0.63 for FAI, testosterone, DHEAS and androstenedione, respectively. Spearman's correlation test of androgens with BMI, age and FG Score did not find any associations with DHT.

Conclusion: Dihydrotestosterone (DHT) is best available biomarker and can be considered as diagnostic biomarker of hyperandrogenemia in PCOS women from North India.

Keywords: Androgens, Diagnostic criteria, Hirsutism, Phenotypes

INTRODUCTION

The Polycystic Ovary Syndrome (PCOS) is a complex reproductive disorder characterised by clinical hyperandrogenism (hirsutism, acne, excess hair loss, acanthosis nigricans, hoarse voice, etc) and/or biochemical hyperandrogenism {high Testosterone (T) and/or Free Androgen Index (FAI)}, chronic oligo-ovulation or anovulation (oligomenorrhoea or amenorrhoea) and polycystic ovarian morphology (polycystic and/or enlarged ovary). It is the most common endocrinopathy in women of reproductive age with a prevalence of 10-15% [1,2]. PCOS is the leading cause of anovulatory infertility [3]. At present commonly followed PCOS diagnostic criteria world-wide is Rotterdam criteria (2003) but in this study a phenotypic approach to diagnose and classifying PCOS was followed [2,4,5]. This approach classifies PCOS cases into four phenotypes as phenotype A, B, C and D. Although hyperandrogenemia (high total T or high FAI) is one of the major features of PCOS, it is rarely observed in the Asian Indians [6]. To date, no sensitive biomarker of hyperandrogenemia is available in the Asian Indians. Hence, there is a need to search for a better hyperandrogenemia marker, in addition to relook cut-off values for the Asian Indians.

We are working on PCOS for many years and observed mismatch between clinical hyperandrogenism {hirsutism; Ferriman-Gallwey (FG) score ≥9 in about 75% cases} and hyperandrogenemia (high T in 35% cases). At present, testosterone is the most common test in routine clinical practice for the investigation of hyperandrogenemia [7], although it is poorly correlated in Asian Indians. Furthermore, there is no consensus on which androgen to be measured and what analytical technique should be used for quantification [8]. Due to a lack of sensitivity and specificity of older direct immunoassays of testosterone and the very low serum concentrations of testosterone in women. The Endocrine Society recommends avoiding immunoassays and suggesting for more precise mass spectrometry-based assay for testosterone quantifications [9]. Mass spectrometry assay is expensive and requires high end laboratory setup hence difficult to have in most laboratories, particularly in the South Asian countries including India. The new automated architect 2nd generation testosterone assay platform (chemiluminescent microparticle immunoassay) is capable of measuring male samples with comparable accuracy and precision with liquid chromatography-mass spectrometry and with sensitivity of 0.06 ng/mL (0.15 nmol/L). However, assay specificity remains a challenge for measuring testosterone in female. Studies also have shown androgen excess may be missed if only testosterone is measured [10].

Androgens can be secreted from the ovaries and the adrenal glands, but can also be generated from precursors in adipose or peripheral tissues. Serum Dihydrotestosterone (DHT) is almost entirely produced by peripheral conversion of androgens and has a circulating concentration of 3-10% that of testosterone in normal

women. However, in PCOS women excess DHT in circulation may also come from ovary (excess 5α-reductase activity in ovary) [11]. Steroid hormones like androstenedione or testosterone can themselves be further metabolised to other androgens like DHT (more potent androgen). In addition, DHT synthesis may follow the back-door pathway that bypasses testosterone and androstenediol (mainly in adrenal cortex) and depends on androsterone as the predominant backdoor androgen [12] or an alternative route that requires steroid 5α-reductase isoenzyme-1 and bypasses T [13]. Therefore, it is important to investigate PCOS cases for serum DHT also as many could be having atypical congenital adrenal hyperplasia or overactive back door pathway. Hence, the present study was conducted, to investigate DHT with various other androgens for assessment of best available androgen in PCOS patients.

MATERIALS AND METHODS

This case-control study was conducted in the Department of Reproductive Biology, All India Institute of Medical Sciences, New Delhi, India, from January 2016 to December 2019. During this period 263 patients were referred from various parts of North India with provisional diagnosis of PCOS. The study was approved by the Ethics Committee for Postgraduate Research (T-329/23.06.2015, RT-16/22.07.2015) and Institute Ethics Committee (IEC-730/29.12.2017, RP-02/2018). All patients underwent clinical and basic evaluation before included into the study.

Inclusion criteria: PCOS cases were selected for the study after evaluation of reproductive and menstrual history (oligomenorrhoea/amenorrhoea), hirsutism (FG score) [14], testosterone level and ovarian ultrasonography (polycystic and/or enlarged).

Exclusion criteria: Before inclusion as PCOS every case were evaluated for drug induced hyperandrogenism, androgen producing tumors (ovarian neoplasm, adrenal neoplasm, thecoma), hyperprolactinemia, congenital adrenal hyperplasia, Cushing syndrome, hypothyroidism, or premature ovarian failure and excluded from study. In some cases, chromosome analysis was also carried out to exclude rare secondary causes like disorder of sex development/sex reversal.

Sample size calculation: Sample size for the study was computed for comparing testosterone level between PCOS and controls based on the following assumptions: Mean (SD) in PCOS as 1.5 (0.5) nmol/L (0.6 ng/ml) and in controls as 1.0 (0.5) nmol/L (0.4 ng/mL) with 95% confidence level, 90% power and control: PCOS as 1:2 allocation ratio. Study investigators expected more variations in PCOS cases as compared to control female. Therefore, investigators considered 1:2 allocation ratio in sample size calculation. The minimum sample size required was 44 controls and 88 cases. Although study investigators planned for 2:1 ratio but later included all eligible PCOS cases. Investigators enrolled 49 controls and 131 PCOS cases in the study i.e., beyond minimum requirements. The sample size was computed using STATA 15.0 statistical software.

Rotterdam criteria (2003) with modifications in the form of phenotypic classifications by National Institutes of Health (NIH) 2012 criteria. [Table/Fig-1] was followed to assign cases as PCOS [4,5]. PCOS cases were grouped into four as phenotype A (hyperandrogenism, ovulatory dysfunction and polycystic and/or enlarged ovary), phenotype B (hyperandrogenism and ovulatory dysfunction), phenotype C (hyperandrogenism and polycystic and/or enlarged ovary) and phenotype D (ovulatory dysfunction and polycystic and/or enlarged ovary).

A menstrual cycle length of 42 days or more (instead of 35 days) were considered as oligomenorrhoea and 182 days for amenorrhoea. The clinical hyperandrogenism in the form of hirsutism was assessed using FG scale and a score of ≥9 was considered as clinical hyperandrogenism [14]. Polycystic ovarian morphology was considered when targeted ovarian ultrasonography show follicles size of 2-9 mm and count ≥12 in one or both ovaries with ovarian size 10 mL or more (one/both ovaries). All cases were also evaluated for cortisol, Luteinising Hormone (LH), Follicle Stimulating Hormone (FSH), DHEAS, estradiol, progesterone, prolactin and TSH to exclude secondary causes (all were analysed in Abbott Inc. autoanalyser).

Type/Group	*Hyperandrogenism (HA)	**Ovulatory Dysfunction (OD)	***Polycystic Ovary Morphology (PCOM)
Phenotype A	Yes	Yes	Yes
Phenotype B	Yes	Yes	No
Phenotype C	Yes	No	Yes
Phenotype D	No	Yes	Yes

[Table/Fig-1]: Phenotypic classifications of PCOS as per NIH 2012 criteria.
*Hyperandrogenism (clinical and/or biochemical)
Clinical hyperandrogenism (hirsutism): Ferriman-Gallwey score ≥9
Cut-off for this study was ≥9
Biochemical hyperandrogenism: High testosterone (≥0.6 ng/ml)/
Cut-off for this study was ≥0.51 ng/ml
(derived from control mean+2SD)
High FAI (≥4.5)
Cut-off for this study was ≥2.55
(derived from control mean+2SD)
**Ovulatory Dysfunction (oligomenorrhoea/amenorrhoea or oligo-ovulation/anovulation)
Oligomenorrhoea/oligo-ovulation: Menstrual cycle interval >35 days
Cut-off for this study was ≥42 days
as many women have >35 days menstrual cycle
(our experience)
Amenorrhoea/anovulation: No menstruation for >182 days
Cut-off for this study was >182 days
***Polycystic Ovary Morphology
Ovarian follicles of 2-9 mm in size with ≥12 follicles in one or both ovaries and/or Ovarian volume >10 mL in one or both ovary/ovaries on targeted (ovary) ultrasonography using 5 MHz transducer

Testosterone Estimation

Testosterone estimation was carried out using ARCHITECT 2nd Generation Testosterone kit (2P13; ABBL421/R03) and ARCHITECT i2000 System. The ARCHITECT 2nd Generation Testosterone assay releases testosterone from binding proteins and measures total testosterone. The ARCHITECT 2nd Generation Testosterone assay is a delayed one-step immunoassay for the quantitative determination of testosterone in human serum and plasma using chemiluminescent microparticle immunoassay technology. The expected kit ranges for normal females aged 21-49 years was 0.25-2.75 nmol/L (0.072-0.793 ng/mL) with a median value of 0.86 nmol/L or 0.25 ng/mL. This testosterone assay is designed to have a within-laboratory (total) precision of <10% Coefficient of Variation (CV) for samples with testosterone concentrations between 0.5 to 35 nmol/L (0.2 to 14 ng/mL). Sensitivity of kit (limit of quantification) was 0.15 nmol/L (0.06 ng/mL). The 17-hydroxy progesterone (EIA1292) and Sex Hormone Binding Globulin (SHBG) were measured using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit (EIA2996R; DRG International, Inc., USA).

After preliminary evaluation:

- Cases: 137 cases were found as PCOS.
- Control: 49 normal (having normal menstrual cycle and fertility) female in reproductive age as control for the comparison.

Procedure

Blood samples were collected from patients between 2-5 days of menstrual cycle (or following progesterone withdrawal) after obtaining written consent for the study. Blood collected in plain test tube, allowed to coagulate at room temperature for about one hour and then centrifuged at 3000 rpm for 15 minutes, serum (supernatant) collected in serum vial using 1 mL micropipette and either used immediately in Abbott autoanalyser {testosterone (T), Follicle Stimulating Hormone (FSH), Luetinising Hormone (LH), prolactin, Thyroid Stimulating Hormone (TSH), estradiol, progesterone, Dehydroepiandrosterone-sulfate (DHEAS), cortisol} or stored at minus 80ºC for future analysis (DHT, androstenedione, SHBG and 17-hydroxy progesterone). DHT (DBC-Diagnostics Biochem Canada, Inc. Ontario, Canada; Cat No. CAN-DHT-280, version 7) and androstenedione (DRG International, Inc. USA; Cat No. EIA-3265, version 6.1) were estimated using commercial

ELISA kits. The principle of the tests follows the typical competitive binding mechanism. Inter and intra-assay Coefficients of Variability (CV) for DHT were between 3.9-12.1 depending upon value of DHT, higher the values lower the CV. The minimum detection limit (sensitivity) of DHT kit was 6.0 pg/mL. DHT recovery rate of the kit was over 90% and kit to kit variation was <5%. Kit reference range for women in reproductive age group was 24-368 pg/mL. But reference range for North Indian female is unavailable.

STATISTICAL ANALYSIS

Statistical differences between cases and controls were derived using Mann-Whitney U test (two-tailed) method. Predictive values were also evaluated with the use of the Receiver Operating Characteristic (ROC) curve analysis using STATA 15.0 statistical software. Association between androgens and Body Mass Index (BMI) as well as FG score and age was carried out using Spearman's correlation test. The p-value <0.05 was considered as statistically significant.

RESULTS

This study was based on 137 primary PCOS cases and 49 control women. There were 87 PCOS cases with phenotype A, 25 PCOS cases with phenotype B, 10 PCOS cases with phenotype C and 15 PCOS cases with phenotype D. The mean age was 23.7 years in the PCOS group and 26.2 years in the control group [Table/Fig-2]. The age difference was statistically significant (p-value <0.001) in all types of PCOS cases in comparison to controls, although number of cases in phenotype B, C and D were small. The mean Body Mass Index (BMI) in the PCOS group was 25.23 kg/m^2 and in the control was 22.6 kg/m^2. The BMI difference between PCOS (total, phenotype A and B) and control was statistically significant [Table/Fig-2].

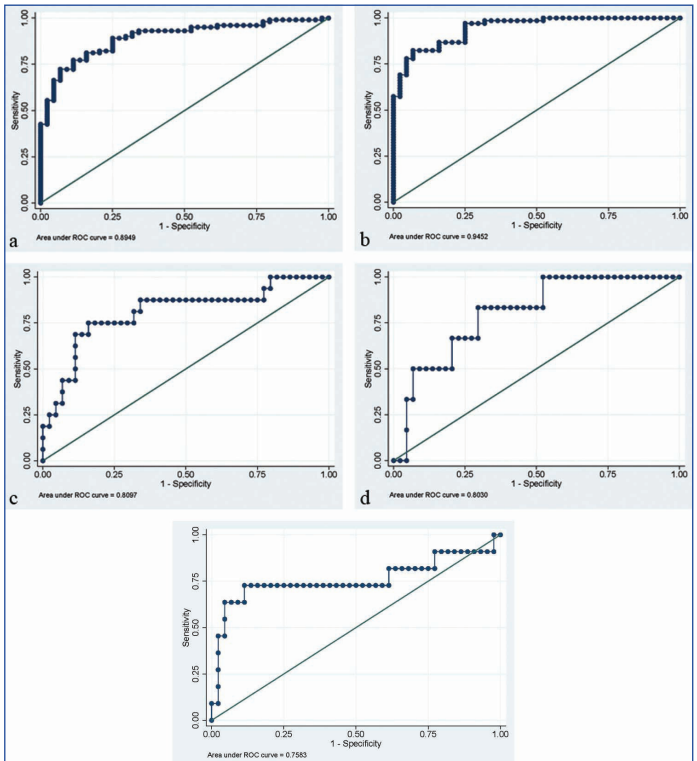
Parameters (cut-off value)[1]	Total (n=137)	Phenotype A (n=87)	Phenotype B (n=25)	Phenotype C (n=10)	Phenotype D (n=15)	Control (n=49)
Age (years) Mean±SD	23.7[c] (4.8)	24.03[c] (4.8)	23.9[c] (4.68)	22.9[c] (3.17)	21.9[c] (5.9)	26.2 (4.4)
BMI (kg/m^2) Mean±SD	25.23[b] (5.11)	25.49[b] (5.07)	25.75[b] (5.88)	24.46[a] (4.33)	22.9[a] (4)	22.6 (3.4)
Ferriman-gallwey score* (≥9)	75.94% (101/133)	77.90% (67/86)	96% (24/25)	100% (10/10)	0% (0/12)	NA
Testosterone (≥0.51 ng/mL)	35.29% (48/136)	41.86% (36/86)	32% (8/25)	40% (4/10)	0% (0/15)	0.262 (0.124)
Free androgen index# (≥2.55)	56.25% (63/112)	72.60% (53/73)	33.33% (6/18)	57.14% (4/7)	0% (0/13)	1.068 (0.74)
Dehydroepiandrosterone sulphate (≥309 µg/dL)	14.18% (19/134)	15.10% (13/86)	20.80% (5/24)	10% (1/10)	0% (0/13)	152.16 (78.45)
Androstenedione (≥2.2 ng/mL)	18.62% (19/102)	27.14% (19/70)	0% (0/15)	0% (0/7)	0% (0/10)	1.07 (0.56)
Dihydrotestosterone (≥462 pg/mL)	61.38% (62/101)	75% (51/68)	25% (4/16)	33.30% (2/6)	45.45% (5/11)	257.15 (102.27)

[Table/Fig-2]: Demographic details and detection rate of various parameters of hyperandrogenism.
[c]p<0.001; [b]p<0.01; [a]p>0.05; (Students t-test was used to compare mean between control and PCOS cases); [1]Cut-off value derived from control mean+2SD (values in control column)
*As per Ferriman-Gallwey 1961 (reference 14); NA (not available): FG score was not recorded in controls as no control women complained for hirsutism; #FAI was calculated according to the equation FAI={(TT/SHBG)×100} i.e., total testosterone (nmol/L or ng/mL) divided by the SHBG (nmol/L or ng/mL), and then multiplying by 100

The clinical hyperandrogenism in the form of hirsutism was assessed using FG scale. FG score of ≥9 was considered as clinical hyperandrogenism [14]. FG score of ≥9 was observed in 75.9% (101/133) cases [Table/Fig-2]. A FG score of ≥9 was observed in 77.9% (67/86) cases with phenotype A, 96% (24/25) cases with phenotype B, 100% (10/10) cases with phenotype C and 0% (0/12) cases with phenotype D. Details of detection rate (>cut-off value, from control mean+2SD) of various parameters of hyperandrogenism (clinical and biochemical) in PCOS cases is represented in [Table/Fig-3]. [Table/Fig-4] shows details of ROC cut-off value with sensitivity/specificity of various androgens in PCOS cases. Best sensitivity and specificity were observed with DHT (77% and 89% at cut-off 382 pg/mL) followed by FAI (71% and 95% at cut-off 1.9) and T (84% and 71% at cut of 0.28 ng/mL). [Table/Fig-5] represents statistical comparisons of androgens between cases and controls. The ROC curve analysis was performed for all androgens [Table/Fig-6]. AUC of ROC >0.9 was observed in DHT and FAI in phenotype A. [Table/Fig-4] shows ROC curve of DHT in PCOS cases, including subtypes. [Table/Fig-7] compares DHT, FG score and age in relation

Parameters	Total (n=137)	Phenotype A (n=87)	Phenotype B (n=25)	Phenotype C (n=10)	Phenotype D (n=15)
Testosterone					
Cut-off (ng/mL)	0.28	0.28	0.28	0.32	0.28
Sensitivity	83.8%	91.8%	72%	70%	53%
Specificity	71.4%	71.4%	71%	72%	71%
Free androgen index					
Cut-off (%)	1.9	1.9	2.2	3.3	1.6
Sensitivity	71%	87.7%	50%	57%	61.5%
Specificity	95.74%	95.74%	95.74%	95.87%	80.85%
Dehydroepiandrosterone sulphate					
Cut-off (µg/dL)	167.5	165.1	163.1	161.5	117.1
Sensitivity	69%	74%	71%	100%	69%
Specificity	71%	71%	67%	65%	41%
Androsterone					
Cut-off (ng/mL)	1.19	1.2	0.75	0.63	0.84
Sensitivity	48%	68.6%	60%	85.7%	70%
Specificity	73%	73.5%	30.6%	22.5%	41%
Dihydrotestosterone					
Cut-off (pg/mL)	382	413	360	279	396
Sensitivity	77%	82%	75%	83%	73%
Specificity	89%	93%	84%	70.5%	89%

[Table/Fig-3]: Details of detection rate (cut-off value with sensitivity and specificity) from ROC of various androgens.
Cut-off value derived from ROC using STATASE 12.1 (StataCorp, Texas, USA)
Sensitivity and specificity in percent
n=number (maximum; varies between parameters)



[Table/Fig-4]: ROC curve for DHT in total (AUC 0.895; a) phenotype A (AUC 0.945; b) phenotype B (AUC 0.81; c) phenotype C (AUC 0.803; d) phenotype D (AUC 0.758; e) PCOS cases.
Y axis denotes sensitivity and X axis denotes specificity with maximum value 1 (equal to 100%) for both the axes.

Parameters	Control	Total	Pheno-type A	Pheno-type B	Pheno-type C	Pheno-type D
Dihydrotestosterone (pg/mL)						
Number	44	101	68	16	6	11
Mean±SD	257.15± 102.27	584.27± 310.45	665.78± 329.87	429.15± 193.5	370.6± 99.2	422.53± 183.8
Z-score*	-	7.54	7.93	3.63	2.3734	2.61965
p-value	-	**<0.00001**	**<0.00001**	**0.00028**	**0.01778**	**0.0088**
Androsterone (ng/mL)						
Number	49	102	70	15	7	10
Mean±SD	1.07± 0.56	1.55± 1.18	1.89± 1.29	0.78± 0.16	0.6941± 0.1125	0.88992± 0.2084
Z-score*	-	2.58	4.55	5.73	1.8828	0.2929
p-value	-	**0.00988**	**<0.00001**	**<0.00001**	0.0601	0.77182
Testosterone (ng/mL)						
Number	49	136	86	25	10	15
Mean±SD	0.262± 0.124	0.487± 0.25	0.523± 0.243	0.471± 0.238	0.559± 0.335	0.262± 0.098
Z-score*	-	6.56	7.19	6.86	-3.495	-0.444
p-value	-	**<0.00001**	**<0.00001**	**<0.00001**	**0.00046**	0.659
Free androgen index						
Number	47	111	73	18	7	13
Mean±SD	1.068± 0.74	4.84± 5.22	5.95± 5.77	3.09± 3.29	4.05± 4.34	1.47± 0.53
Z-score*	-	7.14	7.64	6.13	-2.95	-2.3
p-value	-	**<0.00001**	**<0.00001**	**<0.00001**	**0.00318**	0.02
Dehydroepiandrosterone sulphate (ng/mL)						
Number	49	133	86	24	10	13
Mean±SD	152.16± 78.45	216.27± 96.23	224.29± 98.43	213.40± 92.56	248.84± 92.16	143.39± 57.79
Z-score*	-	4.57	4.75	6.86	-3.323	0.103
p-value	-	**<0.00001**	**<0.00001**	**<0.00001**	**0.0009**	0.92

[Table/Fig-5]: Statistical comparisons of various androgens.
number varies between parameters due to failure of test/sample insufficiency
*Mann-Whitney U Test (2 tailed) Z-score (Analysis of 2-between-group data with a quantitative response variable)

Parameters	AUC	95% CI (lower bound)	95% CI (upper bound)
Dihydrotestosterone			
Total (101)	0.895	0.843	0.947
PhA (068)	0.945	0.908	0.982
PhB (016)	0.810	0.651	0.969
PhC (006)	0.803	0.610	0.996
PhD (011)	0.758	0.514	1.00
Androsterone			
Total (102)	0.630	0.538	0.722
PhA (070)	0.746	0.658	0.834
PhB (015)	0.359	0.194	0.524
PhC (007)	0.277	0.108	0.446
PhD (010)	0.469	0.278	0.661
Testosterone			
Total (136)	0.817	0.748	0.886
PhA (086)	0.873	0.811	0.936
PhB (025)	0.775	0.638	0.911
PhC (010)	0.854	0.722	0.986
PhD (015)	0.539	0.345	0.733
Free androgen index			
Total (112)	0.86	0.803	0.917
PhA (073)	0.915	0.861	0.969
PhB (018)	0.751	0.587	0.916
PhC (007)	0.85	0.688	1
PhD (013)	0.711	0.53	0.892
Dehydroepiandrosterone sulphate			
Total (134)	0.721	0.634	0.808
PhA (086)	0.747	0.657	0.837
PhB (024)	0.707	0.55	0.863
PhC (010)	0.837	0.721	0.953
PhD (013)	0.49	0.288	0.692

[Table/Fig-6]: Receiver Operating Characteristic (ROC) curve analysis of androgens.
AUC of ROC obtained using STATASE 12.1 (StataCorp, Texas, USA); AUC: (area under curve); Ph: (phenotype); CI (confidence interval); n=number (maximum; varies between parameters)
ROC value >0.9 is excellent, 0.8-0.9 is good, 0.7-0.8 is fair and 0.6-0.7 as poor

with BMI and no effect of BMI with DHT or FG score was observed. The Spearman's rank correlation coefficient of androgens with BMI, FGS and age detected negligible correlation coefficient with most but weak correlation coefficient with FGS (testosterone and DHEAS) and BMI (FAI) but not with DHT/androstenedione [Table/Fig-8].

BMI types (kg/m²)		BMI (kg/m²)	Age (years)	FG score (numeric)	DHT (pg/mL)
Normal BMI (18.5-24.9)	Mean	22.04	22.88	12.31	596.80
	SD	1.98	3.80	6.82	404.38
	Number	68	68	68	49
High BMI# ≥25	Mean	29.50	24.81	12.46	533.57
	SD	3.65	5.46	5.77	283.20
	Number	62	62	62	48
	*p-value	-	0.0100	0.8804	0.3246
Low BMI <18.5	Mean	16.77	22.57	10.14	479.92
	SD	1.01	3.20	3.33	196.64
	Number	6	7	7	5
	*p-value	-	0.8351	0.4096	0.5266

[Table/Fig-7]: Statistical comparisons of hyperandrogenism (DHT and FG Score) and age with Body Mass Index (BMI).
*Mann-Whitney U (2 tailed) Test (Social Science Statistics) comparisons with groups with normal BMI with high BMI and normal BMI with low BMI.
#High BMI (≥25) includes both overweight and obese categories

Androgens		BMI (kg/m²)	Age (years)	FGS (number)
Dihydrotestosterone (pg/mL)	Spearman's rho	0.1315	0.1099	-0.0534
	Prob > |t|	0.19	0.2739	0.5959
	Number of observations	101	101	101
	Interpretation/strength	negligible	negligible	negligible
Androsterone (ng/mL)	Spearman's rho	0.0228	-0.0511	-0.0607
	Prob > |t|	0.8199	0.6103	0.5448
	Number of observations	102	102	102
	Interpretation/strength	negligible	negligible	negligible
Testosterone (ng/mL)	Spearman's rho	0.1339	0.0789	0.2298
	Prob > |t|	0.1201	0.3613	0.0071
	Number of observations	136	136	136
	Interpretation/strength	negligible	negligible	weak
Free androgen index (ratio)	Spearman's rho	0.3389	0.1911	0.1458
	Prob > |t|	0.0003	0.0435	0.1252
	Number of observations	112	112	112
	Interpretation/strength	weak	negligible	negligible
Dehydroepiandrosterone sulphate (ng/mL)	Spearman's rho	-0.0266	0.0417	0.2206
	Prob > |t|	0.7618	0.6352	0.011
	Number of observations	132	132	132
	Interpretation/strength	negligible	negligible	weak

[Table/Fig-8]: Spearman's rank correlation coefficients of androgens with BMI, age and FGS.
Spearman's rank correlation coefficients using STATA SE 12.1 (StataCorp, Texas, USA) software; Prob > |t|: p-value for two tail test

DISCUSSION

The PCOS is a heterogeneous condition. It is characterised by clinical and/or biochemical hyperandrogenism (hirsutism and/or high testosterone), ovarian dysfunction (oligo/anovulation) and polycystic and/or enlarged ovary. Hyperandrogenism is regarded as key factor for the diagnosis of PCOS hence androgen excess should be evaluated in all women suspected of PCOS [15]. The serum testosterone is the most commonly used marker for hyperandrogenemia [7]. However, high testosterone is rarely observed in Indian women with PCOS despite clinical hyperandrogenism [6,16]. High level of testosterone (>0.51 ng/mL) was observed in only 35.3% total PCOS cases, mean testosterone was 0.487 ng/mL in study group whereas in control group 0.262 ng/ml and area under ROC curve for testosterone was 0.817 (total cases). Similarly, FAI is rarely used as diagnostic parameters of biochemical hyperandrogenemia in Indian women with PCOS due to complexity, cost and poor association [17]. Biochemical hyperandrogenism was conventionally measured as high testosterone (>0.6 ng/mL) [18,19] or high FAI (>4½) [19,20] whereas clinical hyperandrogenism measured as high FG score (hirsutism grade ≥9) [14] and/or other features of hyperandrogenism like acne, alopecia, acanthosis nigricans, hoarse voice, etc. As female reproductive hormone level, including androgen varies during menstrual cycle and at minimum in early follicular phase, the present study measured all androgens in early follicular phase (spontaneous or withdrawal cycle; within first five days of cycle).

During the study the authors observed mismatch between hirsutism/clinical hyperandrogenism (FG score ≥9 in 75.9% cases) and biochemical hyperandrogenism (testosterone >0.51 ng/mL in about 35.2% cases or FAI >2.55 in about 56.2% cases; present study cut-off values). This is also supported by weak correlation between FG score and testosterone and no correlation with FAI and FG score. The reasons for this in north Indian women with PCOS are not known but could be due to differences in the activity of the 5α reductase enzyme that converts testosterone to the more active metabolite DHT in skin [17]. Although testosterone (and/or FAI) is the central hallmark of hyperandrogenism, DHT measurement has been also advocated to enhance diagnostic performance in PCOS [21]. The FG score/hirsutism is directly related to androgen that mainly acts on skin, hair follicles etc. Among all androgens local DHT seems directly related to hirsutism [22,23]. As local skin DHT measurement is difficult hence authors tried to find out relationship with serum DHT and found no correlation with serum DHT and FG score. Although the study did not find any correlation between serum DHT and hirsutism but observed significantly higher value of serum DHT in PCOS women. Serum DHT probably reflects excess androgen synthesis in the ovary and/or adrenal (back door pathway) or peripheral tissues and justify to incorporate as biomarker for the androgen excess.

In this study, high FAI (>2.55) was observed overall in 56.25% total cases, mean FAI was 4.84 in study group whereas in control 1.068 and area under ROC curve for FAI was 0.86 (total cases). FAI at a cut-off value of 2.55 detects about 56% cases. However, much higher cut-off values (4.5 to 6.1) of FAI have been reported in literature [24-26]. Similarly, high DHT (>462 pg/mL) was observed in 61.38% total PCOS cases, mean DHT was 584.27 pg/mL in study group whereas in control 257.15 pg/mL and area under ROC curve for DHT was 0.895 {lower and upper bound 95% Confidence Interval (CI) 0.835-0.94}. ROC curve analysis suggests that DHT is superior to other androgens in the diagnosis of hyperandrogenemia in PCOS. The study observed area under ROC curve in PCOS cases for DHT as 0.895 with lower and upper bound 95% CI as 0.843, 0.947; p-value <0.0001 and for phenotype A as 0.945 with lower and upper bound 95% CI as 0.908, 0.982; p-value <0.0001. The best compromise was obtained with a cut-off value of 382 pg/mL for PCOS diagnosis with a sensitivity and specificity of 77% and 89% with total PCOS cases and 82% and 93% with phenotype A PCOS cases (cut-off value 413 pg/mL). ROC curve analysis revealed 279-413 pg/mL (depending upon phenotypes) as best cut-off value for DHT which is much lower than the value considered to define patients with high value (>462 pg/mL) in this study. This signifies that DHT is likely to be associated with PCOS in 77% total PCOS cases and 82% with phenotype A, considering 382 (total) and 413 (phenotype A) pg/mL cut-off value, instead 462 pg/mL cut-off value (mean+2SD). The DHT estimation seems comparatively simple (single test) and more sensitive. Androgen excess in many PCOS cases may have been missed if only T and/or FAI were determined, and hence the measurement of DHT is recommended for the detection of hyperandrogenemia in north Indian women with PCOS to improve diagnosis of hyperandrogenemia. This is also viewed by others [27,28]. Authors have also evaluated other potential androgens acting mainly on skin, hair follicles etc and implicated in PCOS like androstenedione but did not find any strong association. Similarly, comparisons of DHT, FG score and age with BMI did not find any statistical significance except high BMI with older age group.

The study has also analysed correlation of androgens with BMI, FG score and age and observed weak correlation between testosterone/DHEAS with FG score as well as FAI with BMI. The limitation of the use of FAI could be due to low serum SHBG with obesity which was evident in about 50% cases of PCOS. However, authors did not find any correlation with DHT or androstenedione and BMI or FG score. This indicates that serum DHT or androstenedione probably do not play a significant role in hirsutism or BMI change. The strength of the study was being prospective in nature and well characterised homogeneous group of PCOS cases. Another positive factor for the study was exclusion of cases with apparent secondary causes (CAH, gonadal tumour, premature ovarian failure) of PCOS.

Limitation(s)

Firstly, there were fewer number of controls and PCOS cases with Phenotype C and D besides younger age of study group. Secondly, the use of conventional chemiluminescent microparticle immunoassay to measure testosterone level in women against recommended Liquid Chromatography with tandem Mass Spectrometry (LC-MS). However, LC-MS is not easily available in India as well as expensive hence need a marker that can be tested easily and at the same time reasonably sensitive and specific.

CONCLUSION(S)

The present investigation establishes serum DHT immunoassay is a sensitive, specific and simple test of hyperandrogenemia in PCOS and may be implemented in routine hospital laboratories. Elevated serum levels of DHT (>462 pg/mL) can be introduced as hyperandrogenemia marker for PCOS in North Indian patients. However, more studies with larger cohort, including controls are needed for the validation of this result.

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