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Original Research Article

Establishment of age-specific reference intervals for AMH in Indian women and enhancing its use as a diagnostic marker in PCOS

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ABSTRACT

Background: Anti Mullerian hormone (AMH) level is a reliable marker of ovarian reserve. It is known to be influenced by factors like age, ethnicity, and ovarian pathology. Establishment of age-specific reference intervals for AMH, characteristic of different nationalities, is therefore of utmost importance. Serum AMH is known to be elevated in women with polycystic ovarian syndrome (PCOS). It is desirable to determine a population-specific cut-off of AMH, for it to be used as a diagnostic marker for PCOS.
Methods: Serum AMH, luteinizing hormone (LH), follicle-stimulating hormone (FSH), Estradiol, Progesterone and Testosterone assays were analyzed in 1978 Indian women, in the age range of 12–50 years. Age-specific reference intervals for AMH were derived for the study population. The cohort of study subjects were then divided into two groups, based on AMH values and clinical history: Control group, and patients with PCOS. The cut-off value of AMH in the study population, corresponding to the diagnosis of PCOS, was also established.
Results: Upper 95th percentile limits of reference intervals for the 18-25 26–30, 31–35, and 36–40, 41-45 and >45 age groups were 9.69, 7.60, 6.50, 6.1, 4.80 and 4.5 ng/ml respectively. In the PCOS group the 5th percentile value was 7.80 ng/ml and the upper 95th percentile was 21.81 ng/ml. The median percentile in PCOS group was 10.40 ng/ml. ROC analysis was done to obtain optimal cutoff values for each age group with better discriminative power than the reference limits. The best cut-off point of AMH value for PCOS in our study population was 7.51ng/ml. The sensitivity and specificity were 99.4% and 95.5%, respectively. The calculated area under the Receiver operating characteristic (ROC) curve was 0.988 (95% CI: 0.984-0.991, P <0.001). AMH, LH, and LH/FSH ratio was significantly higher in the PCOS group than in the control group ($p < 0.001$ for all comparisons). LH/FSH ratio was more than 2 in the PCOS group compared to controls. Serum Testosterone was significantly higher in PCOS.
Conclusions: The study aids to establish a biological reference interval for AMH, specific for different age groups in Indian women. 7.51ng/ml has been derived as a diagnostic cut-off of AMH for PCOS in our study population. The establishment of age-specific reference intervals, and syndrome-specific cut-offs in the Indian population will help overcome the influence of variables and broaden the use of AMH in women's health.

Keywords: Age-specific reference interval, Cut off value, Specificity and sensitivity, AMH, Diagnostic performance, PCOS

INTRODUCTION

The transforming growth factor beta family includes the anti-mullerian hormone (AMH), which gets its name from its function in male sex differentiation (by causing the mullerian ducts to regress). Granulosa cells of developing follicles from the primary stage up to the small antral stage of the ovary express AMH. AMH expression decreases

following FSH-dependent selection, with only a small amount of expression persisting in the cumulus cells of preovulatory follicles.

Since serum AMH levels represent the pool of developing follicles that may ovulate, they are used to evaluate the functional ovarian reserve. Since AMH does not exhibit cyclic changes across the menstrual cycle, it has gained favor as a serum marker for ovarian follicular reserve.[2] In adult women, serum AMH levels have been found to negatively correlate with age. Studies aimed at creating normative data for AMH have revealed that this correlation depended on the age group analyzed. Additionally, it appears that ethnicity is a significant contributor to the observed heterogeneity in AMH levels. Its usefulness in the general population has long been constrained by inter-assay variability, demographic features, and a lack of standardized population-specific AMH cutoff values.[3]

Serum AMH levels can be used as markers for ovarian pathology, such as polycystic ovary syndrome (PCOS). PCOS is the most common gynecological endocrine disorder affecting up to 10% of all women in their reproductive age.[4] Stein and Leventhal identified seven patients with amenorrhea, infertility, and hirsutism as having PCOS for the first time in 1935.[5] In young women, prolonged anovulation and hyperandrogenism are most frequently caused by PCOS.[6] According to three diagnostic characteristics from Rotterdam's criteria, the overall prevalence of PCOS in India is 11.34%.[7]

Since the pathophysiology of PCOS is not fully understood, making a diagnosis is not easy, leading to the creation of multiple sets of diagnostic standards. Clinical manifestations of hyperandrogenism include hirsutism, acne, or alopecia.[9] Radiological investigations like transabdominal and transvaginal ultrasonography have their own limitations, making the diagnosis of PCOS challenging. There is therefore a never-ending search for a superior or alternative diagnostic test or diagnostic criterion to facilitate the diagnosis of PCOS.[8]

Despite its high incidence, PCOS is underdiagnosed and frequently necessitates multiple visits or the involvement of multiple doctors, all of which typically take place over the course of several months to years.

Delay in diagnosis causes comorbidities, which makes it more difficult to implement lifestyle intervention, which is important for the improvement of features of PCOS and quality of life.[9] Serum AMH level is elevated in PCOS patients due to increased AMH production per follicle. Therefore, serum AMH has been proposed as a diagnostic marker of PCOS.[10]

To our knowledge, there is currently no data available on the biological reference interval of AMH in the Indian women population. Hence, data derived from the western population are still widely used as a reference for interpretation. Moreover, the cut-off of AMH which could be used as an adjunct to the existing Rotterdam criteria, for making an effective diagnosis of PCOS, is still elusive.

Objectives

The objectives of this study were to (a) establish age-specific reference intervals of serum AMH in apparently healthy women of Indian origin; (b) determine the sensitivity and specificity of AMH at 6.8 ng/ml cut off (derived from the assay literature) in the Indian women population; (c) establish the ROC based on our AMH data (best cut off for PCOS detection in our study population); and (d) compare the level of AMH and other hormones like FSH, LH, estradiol, progesterone and testosterone between women with, and without PCOS (controls).

METHODS

Subject characteristics

This was a prospective study performed at the National Reference Laboratory, Redcliffe Labs, India. Serum AMH, luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone, estradiol, and testosterone assays were analyzed in 1978 women, in the age group of 12-50 years.

The serum samples had been requested for hormonal assays on unspecified day of the menstrual cycle. For all patients having AMH values higher than the upper limit of the reference interval appropriate for age, a detailed clinical history was taken. The presence or absence of PCOS was confirmed by the referring gynecologists or patients themselves (also verified from their diagnostic reports shared with the laboratory).

The cohort of study subjects were then divided into two groups: (i) apparently healthy women without any diagnosed gynecologic diseases as control group (ii) patients with PCOS. Approval was taken from the Institutional Ethical Committee before commencing the study.

Serum sampling

Around 2.5 ml of blood was collected from each patient and transported to the laboratory under optimum conditions. The serum analysis was performed on arrival in the laboratory after ensuring the appropriateness of daily quality control runs.

Serum AMH was estimated on fully automated Cobas e 602 immunoassay analyzers using the Elecsys®AMH assay kit (Roche Diagnostics GmbH, Germany). The other hormones: LH, FSH, estradiol, progesterone and testosterone were also estimated using the respective Elecsys® assay kits on the same Cobas e 602

immunoassay analyzers. The methodology of estimation was Electrochemiluminescence (ECLIA).

Statistical analysis

Statistical Package for the Social Sciences (IBM SPSS) Statistics for Windows, Version 21.0 A was used for the statistical analysis. Descriptive statistics were used to express the data in terms of actual numbers, percentage, mean with standard deviation (SD) and median with percentile ranges at 95% confidence interval. Data was assessed for normality. Mann–Whitney U test was used to compare the median between the groups, as appropriate. The receiver operating curve (ROC) with area under the curve (AUC) and 95% confidence interval (CI) with lower and upper limits was determined. The ROC curve was used to calculate the cut off value of AMH, sensitivity, specificity for detection of PCOD. A p value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics

Among the 1978 subjects, 1817 (91.8%) were assigned to the healthy group, and 161 (8.13%) to the PCOS group.

Establishment of AMH reference intervals

The healthy population is defined as the reference group here. The reference intervals are set as nonparametric 5th to 95th percentiles.

The reference intervals along with the median and upper 95th percentile reference limits of AMH are presented for each age-specific in Table 1. The upper 95th percentile limits of reference intervals for the 18-25 26-30, 31-35, and 36-40, 41-45 and >45 age groups were 9.69, 7.60, and 6.50, 6.1, 4.80 and 4.5 ng/ml respectively (Table 1). In the PCOS group the 5th percentile value was 7.80 and the upper 95th percentile was 21.81.

The median percentile in PCOS group was 10.40 ng/ml. These age group values were employed in subsequent analyses for diagnostic performance (Figure 1 and 2).

AMH cut off value

In this study, the sensitivity, specificity, and diagnostic accuracy of AMH cut off value 6.8 ng/ml (as mentioned in the assay literature for AMH) for detection of PCOS were 100%, 93.23% and 93.78%.

Considering this cut off, 161 women with values above 6.8 were found to be diagnosed cases of PCOS, while 123 women with values above 6.8 were known not to be suffering from PCOS (Table 2).

ROC-AMH

We conducted ROC analysis to obtain optimal cutoff values for each age group with better discriminative power than the reference limits. The healthy groups were used as control groups individually and collectively.

The best cut-off point of AMH value for PCOS in our study population was 7.51 ng/ml. The sensitivity and specificity were 99.4% and 95.5%, respectively. The calculated area under the Receiver operating characteristic (ROC) curve was 0.988 (95% CI=0.984-0.991, p<0.001).

Comparison of AMH and hormonal levels in PCOS and non-PCOS

AMH, LH, and LH/FSH ratio were all significantly higher in the PCOS group than in the control group (p<0.001 for all comparisons) (Figure 4-6).

LH/FSH ratio was more than 2 in the PCOS group compared to the controls. FSH values were significantly lower in in the PCOS group than in the control group. The level of testosterone was significantly higher in the PCOS group, compared to controls. There was no statistically significant difference in the two groups with respect to estradiol and progesterone profile (Table 3).

Table 1: Normal Reference of AMH values as per the population data.

Age group (N=1817) (years)	Percentile in ng/ml (95 CI)						
	5th	10th	25th	Median	75th	90th	95th
18-25 (n=402)	0.60 (0.20-0.90)	1.10 (0.86 - 1.41)	2.20 (2.0-2.60)	3.80 (3.50-4.0)	5.90 (5.30-6.60)	8.60 (8.0-9.40)	9.69 (9.35-10.6)
26-30 (n=535)	0.30 (0.20-0.50)	0.70 (0.50-0.90)	1.70 (1.40-1.90)	2.80 (2.60-3.0)	4.70 (4.35-5.09)	6.10 (5.80-6.20)	7.60 (7.31-7.86)
31-35 (n=507)	0.10 (0.07-0.20)	0.35 (0.20-0.50)	1.00 (0.90-1.20)	1.98 (1.69-2.20)	3.60 (3.40-3.90)	5.02 (4.80-5.60)	6.50 (5.72-6.90)
36-40 (n=264)	0.03 (01-0.04)	0.04 (0.03-0.10)	0.20 (0.11-0.39)	1.00 (0.70-1.20)	2.00 (1.70-2.45)	3.60 (3.24-4.40)	6.1 (2.62-6.50)
41-45 (n=81)	0.1 (0.01-0.01)	0.02 (0.01-0.02)	0.07 (0.02-0.15)	0.30 (0.20-0.50)	1.20 (0.75-1.75)	2.86 (1.50-6.30)	4.80 (4.0-5.85

Continued.

Age group (N=1817) (years)	Percentile in ng/ml (95 CI)						
	5th	10th	25th	Median	75th	90th	95th
≥46 (n=28)	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.01 (0.01-0.02)	0.04 (0.01-0.10)	0.14 (0.05-0.50)	0.91 (0.17-5.90)	4.50 (0.44-5.90)
PCOS (n=161)	7.80 (7.50-8.10)	8.10 (7.80-8.30)	9.04 (8.50-9.40)	10.40 (10.0-10.80)	14.00 (13.15-15.12)	17.70 (15.80-19.47)	21.81 (18.56-23.10)

Table 2: Diagnostic utility of AMH cut off value 6.8 ng/ml for detection of PCOS.

Diagnostic utility of AMH cut off 6.8 ng/ml		PCOS		Total
		Present	Absent	
AMH value	6.8 and above	161	123	284
	Below 6.8	0	1694	1694
Total		161	1817	1978

Table 3: Comparison of AMH and hormonal levels in PCOS and non-PCOS.

Parameters		N	Mean	SD	Median	P value
AMH	Control	1817	2.84	2.37	2.30 (1.0-4.2)	<0.001
	PCOS	161	11.90	4.02	10.4 (9.04-14)	
LH	Control	1817	8.09	7.13	7.13 (4.8-8.9)	<0.001
	PCOS	161	12.77	12.11	9.40 (7.02-14.2)	
FSH	Control	1817	7.84	7.31	7.15 (5.3-8.7)	<0.001
	PCOS	161	5.53	3.54	4.50 (3.2-9.4)	
LH/FSH ratio	Control	1817	1.31	1.51	0.99 (0.67-1.43)	<0.001
	PCOS	161	2.65	4.11	2.18 (1.9-2.6)	
TESTO	Control	1817	21.12	10.91	17.2 (14.8-25.3)	0.046
	PCOS	161	25.12	21.11	19.0 (14.0-26.65)	
E2	Control	1817	89.55	189.51	49.6 (39.2- 77.35)	0.325
	PCOS	161	91.10	237.94	46.80 (38.50-71.25)	
PROG	Control	1817	2.27	8.96	0.18 (0.13-0.36)	0.199
	PCOS	161	1.75	5.53	0.17 (0.12-0.36)	

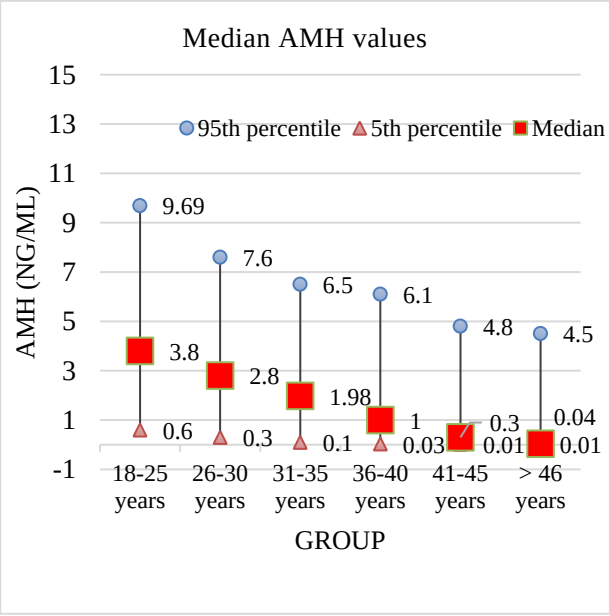

Figure 1: Normal Reference of AMH values as per the population data.

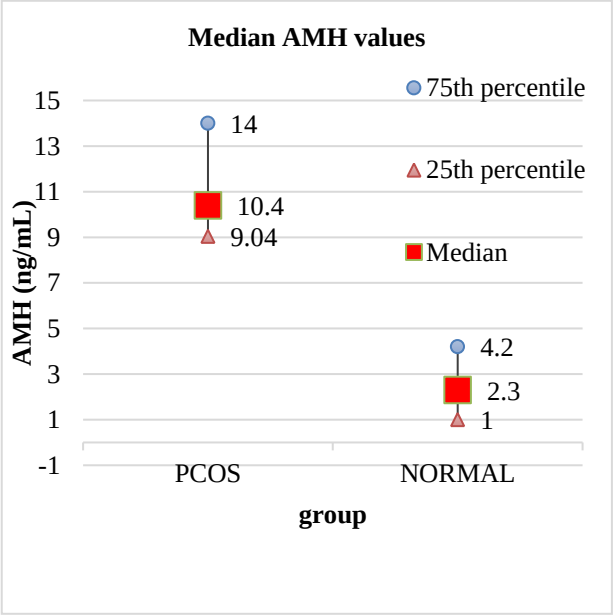

Figure 2: Median AMH levels: in PCOS & controls.

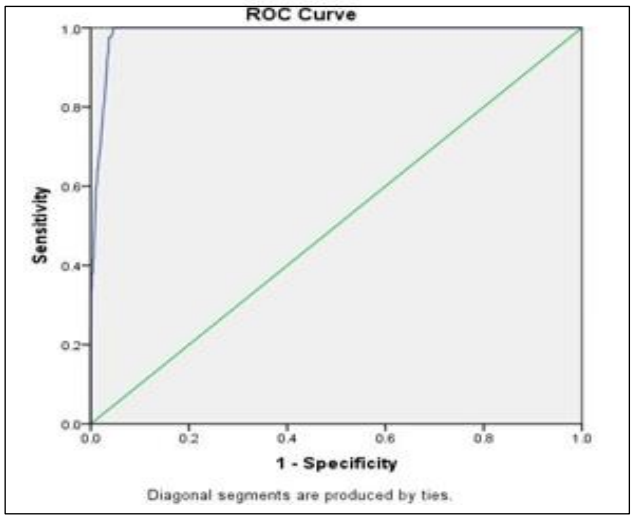

Figure 3: Receiver operating characteristic curve for cut off of 7.51 ng/ml.

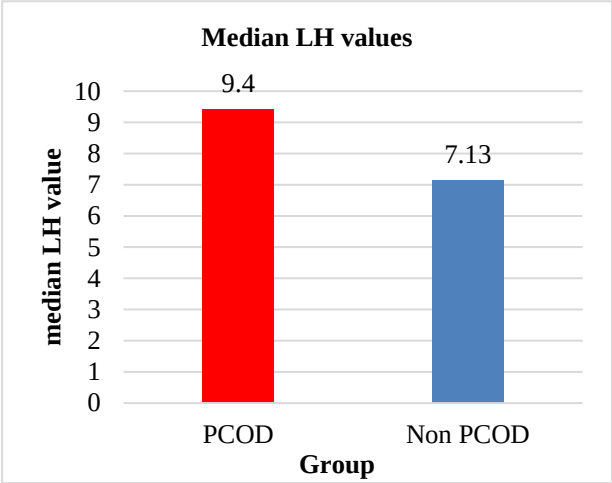

Figure 4: Mean LH levels in PCOS versus non-PCOS.

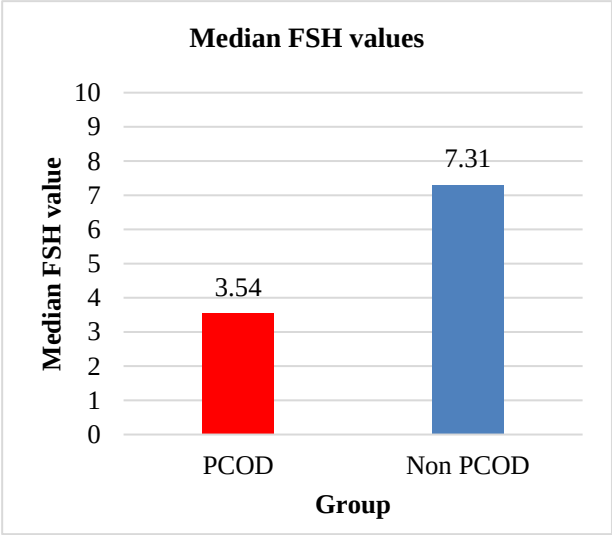

Figure 5: Median FSH levels in PCOS versus non-PCOS.

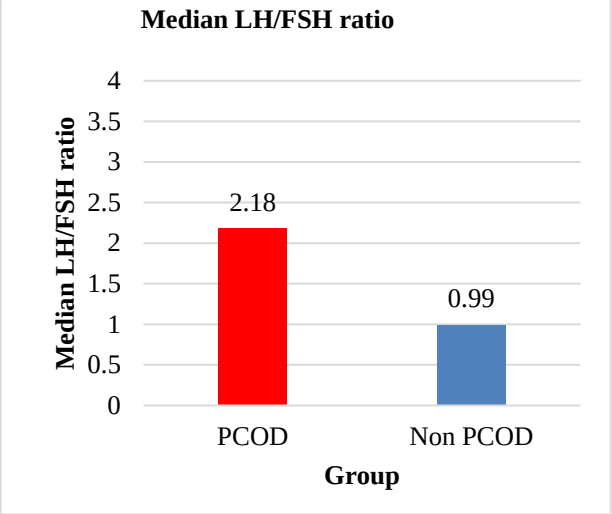

Figure 6: Median LH/FSH ratio in PCOS versus non-PCOS.

DISCUSSION

In this study, 1978 women, who got themselves tested for serum AMH (through self-checks, clinician's referrals etc.) were considered for deriving its age-specific reference interval in the Indian population. Since samples are received at the National Reference Laboratory of Redcliffe Labs from different parts of the country, this sample group may be representative of a varied section of the Indian women population.

The upper 95th percentile limits of reference intervals for the age group of 18-25 26-30, 31-35, 36-40,41-45 and >45 years were 9.69, 7.60, 6.50, 6.1, 4.80 and 4.5 ng/ml respectively. There is a linear decrease in AMH with age, indicating that AMH exactly mirrors the decrease in the follicular pool with time, and may be used as a reliable ovarian marker. The data is in accordance with references where AMH levels show a steady decrease with age.[11]

Studies have shown that AMH levels vary across races and geographical areas. AMH levels in African and Hispanic women are lower when compared to Caucasian women of the same age. Chinese women aged 25 years showed substantially higher AMH levels than Caucasian women.[12,13] In subpopulations within ethnicities, the study found that Maya women had lower AMH levels than other Hispanic women. Although peak AMH levels at age 25 years were higher in Chinese women compared with Europeans, the age-related decline in Chinese women was greater, leading to 28% and 80% lower AMH levels at age 30 and 45 years, respectively.[14]

In addition, African American women appeared to have lower serum AMH levels compared with white women but with a slower age-dependent decline.[15]

Furthermore, Gromski et al reported that fertile and infertile women from one Indian town had significantly

lower AMH levels than selected European women, and the rate of decline in AMH levels was faster in the former.[16]

There are numerous reasons why this disparity could exist. First, racial/ethnic variations in the research populations could have an impact on AMH levels. Genetic and environmental variables may also be a contributory factor. There are contradictory views regarding intra and inter-cycle variability of AMH levels.[17] Some studies show these to be limited and represent fluctuations by chance, possibly related to gradual changes in the number of antral follicles present in both ovaries.[18] Other studies have however, demonstrated substantial fluctuations in the menstrual cycle which would favor measuring AMH levels in the early follicular phase.[19] Clinical conditions under which the samples were drawn, like use of GnRH antagonists or oral contraceptives also variably influence the level of AMH.[20]

Other factors which have been recently described to influence absolute AMH concentrations include weight, Vitamin D status, smoking, polymorphisms of AMH and its receptor, and genetic variations.[21-24] The limited use of standardized automated methods for the analytical measurement of AMH across the diagnostic spectra has also resulted in the variability of test results. Concerns about the stability of the AMH assay, specifications regarding optimal storage and handling, assay variability, unavailability of an international reference material, and assay standardization, have all contributed to its restricted utilization.[25-27]

To our knowledge, there is no existing study in India, which has established age-specific reference intervals for AMH in the Indian women population. Most of the available studies are limited in their scope, because their sample source is restricted to specific geographical areas, women seeking fertility consultation, or data from assisted reproductive institutes. The AMH cut-offs representative of the Caucasian population are still majorly used as a reference in Indians. Hence this study was conducted to address the need for deriving an age-specific reference interval for AMH, which would specifically be reflective of Indian women.

Table 4: Current diagnostic criteria of PCOS according to different societies.

Critreia	Hyperand-rogenism	Oligom-enorrhoea	PCOM
NIH	Both criteria required		
Rotterdam	Two of three required		
AE-PCOS society	Required	One of either of them required	

Note: Exclusion of other etiologies of hyperandrogenism oligomenorrhoea required.

Table 5: Threshold serum AMH levels for detection of PCOS in various studies.

Authors	Type of study	Number of participants	Threshold AMH ng/ml	AUC	Sensitivity (%)	Specificity (%)
Ahmed et al	Case-control	Case-control	3.19	0.938	81	100
Saxena et al	Prospective case-control	Cases 45 Controls 45	3.44		77.78	68.89
Saxena	Cross-sectional		3.44	NA	86.66	100
Sahmay et al	Cross-sectional	PCOS 419 Controls 151	3.94	NA	89.8	80
Sharma et al	Cross-sectional	Cases 45 Controls 45	3.98	NA	82.2	93.3
Wiweko et al	Case-control	Cases 71 Controls 71	4.45	NA	76.1	74.6
Dewailly et al	Prospective case-control	Non PCOS 105 PCOS with HA/OA 73	5	NA	92	97
Mahajan et al	Case-control	-	5.03	0.826	70.68	79.91
Chao-Yan Yue et al	Case-control	-	8.16	0.846	78.4	80.9
Homburg et al	Prospective case-control	PCOS 90	6.72		60	98.2
Woo et al	Prospective cross section	-	7.82		75.9	8.62
Our study	Prospective case-control	PCOS 161 Control 1817	7.51	0.988	99.4	95.5

It is now well recognized that serum AMH is elevated in women with PCOS. Polycystic ovary syndrome is a complex, heterogeneous, endocrine disorder that affects women from adolescence to menopause. Many groups have attempted to define the diagnostic criteria for PCOS. However, the widely-used criteria is the Rotterdam criteria. The current diagnostic criteria of PCOS according to different societies are as shown in Table 4.

Due to the simultaneous activation of several antral follicles and higher AMH production per follicle, serum AMH levels are said to be high in PCOS women. However, the unavailability of a uniform cut-off for AMH in PCOS has limited its use as a diagnostic tool. In this study, clinical history was taken for all patients having AMH levels above the upper limit of the reference interval appropriate for age. On application of the assay cut-off of 6.8 ng/ml for detection of PCOS, the sensitivity, specificity, and diagnostic accuracy of AMH cut off was 100%, 93.23% and 93.78% respectively. 161 women with values above 6.8 were found to be diagnosed cases of PCOS, while 123 women with values above 6.8 were known not to be suffering from PCOS. A need to establish a cut-off for PCOS derived from women of Indian origin, was therefore felt.

We conducted ROC analysis to obtain optimal cutoff values for each age group with better discriminative power than the reference limits. The healthy groups were used as control groups individually and collectively. The best cut-off point of AMH value for PCOS was 7.51 ng/ml in which sensitivity and specificity were 99.4% and 95.5%, respectively. The calculated area under the Receiver operating characteristic **(**ROC) curve was 0.988 (95% CI= 0.984-0.991, p<0.001). According to some studies, an AMH level greater than 3.8-5 ng/ml can be utilized as a diagnostic indicator for PCOS.[11] Simultaneous use of the Rotterdam criteria and AMH levels have been suggested by some studies for a prompt and accurate diagnosis. In our study, the 5th percentile value was 7.80 while the upper 95th percentile limit was 21.81 ng/ml in the PCOS group. The median percentile in the PCOS group is 10.40 ng/ml.

These figures are similar to certain studies where in, the median AMH level was significantly higher in the PCOS group 7.59±4.61 ng/ml compared to controls. Another study by Liu et al showed that PCOS women with PCOM had significantly greater serum AMH levels compared with those without PCOM (7.60 ng/ml).[16] We compared the cut off values with certain other studies which showed similar results. However as observed from the table the sensitivity and specificity of other studies were lower than our study. Multiple authors have reported moderate to good sensitivity and specificity in predicting PCOS at levels ranging from 3.44 to 8.16 ng/ml (Table 5).[28-37] However, a consensus on a single cut-off value has remained elusive for long.

In our study, LH levels were significantly higher in the PCOS group than in the healthy group (p<0.001 for all comparisons). LH/FSH ratio was more than 2 in the PCOS group versus controls. Serum testosterone was significantly higher in the PCOS group as compared to the controls.

There was no statistically significant difference in the two groups with respect to estradiol and progesterone profile in this study population.

The LH/FSH ratio was almost double in the PCOS group compared to controls. Consistently rapid GnRH pulsatility, which promotes pituitary LH synthesis over FSH, and leads to elevated LH concentrations and consequently changed LH: FSH ratios, is a neuroendocrine feature typical of PCOS. Follicular development is hampered by low FSH levels, whereas ovarian androgen production is enhanced by high LH levels.[38,39]

Malini and George et al stated that the most prevalent clinical symptom in women with PCOS was a greater LH/FSH ratio and a difference in the range of LH and FSH production.[40] 70.58% of women with PCOS had an increased LH/FSH ratio, according to Nath et al.[41] As a result, many studies propose increased LH/FSH ratio is an as an important indicator of PCOS. Hyperandrogenism is a key feature in the diagnosis of PCOS. The increased concentration of total or free testosterone levels is an important diagnostic characteristic of biochemical hyperandrogenism. Our study also shows a significant rise in Testosterone levels in PCOS, as compared to the control group.[42]

The establishment of cut-offs specific to a particular population will help overcome variables related to geography and ethnicity and help AMH emerge as a reliable marker in the diagnosis of PCOS.

Limitations

The limitations of this study include recording of clinical history related to PCOS only in women above the recommended reference interval of AMH for age. A few cases could have been missed in the process. The reference interval of AMH and cut-off for PCOS established by our study is representative of our patient population, and should be further expanded to include more factors like BMI, individual ethnicity, specific geographical locations, and different methodologies, to help enhance the diagnostic value of AMH.

CONCLUSION

Our study helped establish biological reference intervals for AMH, specific for different age groups in Indian women. It is unique in terms of its representation of a large section of the Indian women population, and the large sample size on which the study was based; thereby taking into consideration the influence of several variables, which have an impact on the value of AMH. Our research also confirmed the diagnostic value of AMH in PCOS. With a

cutoff value of 7.51 ng/ml in our study population, AMH had a sensitivity of 99.4% and specificity of 95.5% for diagnosis of PCOS. The specificity and sensitivity were both high, and the study concludes its use as a reliable indicator in the diagnosis of PCOS.

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