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ORIGINAL RESEARCH

Anti-Mullerian Hormone Levels in Regularly Menstruating Nigerian Women

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Abstract

Background: Studies have shown that serum levels of Anti-Mullerian Hormones (AMH) decrease with age as it is also an early and sensitive marker of ovarian reserve in women in the North American, European and Asian regions. Various research works have also generated data about AMH in the Caucasian, Americans and Asians There was a need to compare these known data with African data.
Objectives: To assess the serum levels of AMH in healthy women of reproductive age and determine the relationship between AMH, age, Body Mass Index, parity and menstrual cycle in healthy regularly menstruating women.
Methods: A cross-sectional study of 200 apparently healthy women aged 21-45 years was carried out between January and May 2014. Serum AMH and FSH levels were measured in the participants using Enzyme-Linked Immunosorbent Assay.
Results: The median AMH value was 4.07ng/mL, while the median FSH value was 9.65mIU/mL. The reference 90% CI of AMH was 0.60 -9.71 ng/ml. There was a significant negative correlation between serum level of AMH and age ($r = -0.718$, $p<0.001$).
Conclusion: The serum AMH levels gradually declined throughout the reproductive lifespan of a woman.

Keywords: Anti-Mullerian Hormones, Fertility, Follicle Stimulating Hormone, Women, Menstruation, Menopause.

Introduction

The Anti-Mullerian Hormone (AMH) is a peptide growth factor and a member of the large family of TGF-beta growth and differentiation factors. Anti-Mullerian Hormone was originally known for its unique effect in males with the regression of the Mullerian ducts during embryonal

development leading to the initiation of further development towards the male phenotype.[1]

AMH is first expressed in the human female foetus after 36 weeks of gestation *in-utero* and it is produced in ovarian granulosa cells, by the primary follicles, secondary follicles and small antral follicles. Serum AMH concentrations are almost undetectable at birth, reach the highest values during late puberty and then show a progressive decline during reproductive life as the follicular reserve decreases, becoming undetectable after menopause.[2]

AMH is expressed in the growing follicles in the ovary until they have reached the size and differentiation stage at which they are to be selected for dominance by the action of pituitary Follicle Stimulating Hormone (FSH). [3] AMH has an inhibitory role in primordial follicle recruitment [4] and a regulatory role in the FSH sensitivity of large antral follicles reaching the stages of sensitivity to pituitary gonadotropins. [5] Serum AMH concentrations do not change significantly throughout the menstrual cycle when compared to sex steroids, gonadotrophins and peptides such as inhibin-B. [6] AMH acts as a paracrine factor and is not involved in the feedback mechanisms of the hypothalamo-pituitary-gonadal axis, thus it is reasonably stable and convenient to measure. [7]

Serum AMH level on day 3 of the menstrual cycle, decreases progressively with age suggesting that peripheral AMH levels are a valuable parameter to monitor the relative follicular exhaustion due to ovarian ageing. [8] Serum AMH declines to undetectable levels (<1ng/mL), about five years before the final menstrual period, thereby serving as a predictor of the onset of menopause. [8] There has been conflicting data regarding Body Mass Index and AMH levels in healthy reproductive women but several studies have shown that healthy, obese late reproductive-age women (35-49 years) have lower serum AMH levels when compared with their non-obese counterparts. [9, 10]

AMH has been recognized as a diagnostic means in reproductive medicine due to its capability to measure ovarian reserve and response to controlled ovarian stimulation before assisted reproduction treatments. It is also useful in monitoring therapeutic interventions in ovarian cancer (chemotherapy or radiotherapy) as well as diagnosing disorders of the menstrual cycle. Therefore, it is necessary to establish its values in the healthy female population in Nigeria where there is a paucity of published data on the level of circulating AMH in healthy menstruating women and determine the relationship between AMH concentrations, anthropometric and reproductive characteristics among Nigerian women. This study assessed the serum level of AMH and FSH at the same time to determine the correlation between these two biochemical markers in regularly menstruating women. It also looked at the correlation of these two biomarkers with age, Body Mass Index (BMI), parity, menarche and menstrual cycle.

Methods

Study design and participants

This was a cross-sectional study carried out among 200 apparently healthy, regularly menstruating women who were staff of the University College Hospital, Ibadan, Oyo state between January and May 2014.

Sampling method

The multi-stage sampling method was used as follows:

Stage One: A sampling frame of all the departments in the University College Hospital was drawn and ten departments were selected. These departments included the following: Internal Medicine, Physiotherapy, General

Outpatient, Staff Medical Service, Family Medicine, Medical Microbiology and Parasitology, Chemical Pathology, Periodontology and Community Dentistry, Clinical Nursing and Medical/Health Records.

Stage Two: A sampling frame of each selected department was drawn and female health workers from the chosen departments were selected using a simple random sampling technique. Healthy regularly menstruating women with normal menstrual cycle aged 21 -45 years with no history of hysterectomy, myomectomy, oophorectomy or any other surgery on their ovaries, chronic, systemic, endocrine, metabolic disease, no signs of hyperandrogenism nor galactorrhoea, current hormone therapy or drug that will interfere with menstrual cycle was recruited on their first visit after granting written informed consent.

Questionnaires were administered in an interview-based manner to each volunteer before blood sample collection was done. Five millilitres of blood sample were collected from each healthy participant on the third day of their menstrual cycle and the blood sample analysed in the Chemical Pathology Department of the University College Hospital.

Ethical considerations

Ethical approval was granted by the University of Ibadan/ University College Hospital (UI/UCH) Health Research Ethics Committee with number: UI/ EC/13/0133. The purpose of the study, the risks and the benefits involved were explained to each of the participants and informed consent was obtained.

Data collection

Baseline demographic data and fertility characteristics of all the subjects were obtained using a semi-structured questionnaire.

Anthropometry

Weight and height were measured in all the subjects. The weight was measured in kilograms to the nearest 0.05kg using an RGZ 160 Med –Lab Scientific Company England Qty weighing scale using standard procedures. The height was measured with an RGZ 160 Med –Lab Scientific Company England Qty stadiometer (measuring range 20 cm to 190 cm) according to standard procedure. The Body Mass Index (BMI) was derived using the formula: Weight/Height^2 (kg/m^2).

Laboratory measurements.

Specimen collection

Five millilitres of blood was drawn aseptically from the antecubital fossa vein with minimal stasis using pyrogen-free disposable needles and syringes into a labelled serum separator gel bottle and was taken to the laboratory.

Specimen Separation and Storage

The blood was allowed to clot and retract after 30 minutes of collection. Sera were separated by centrifugation at room temperature at 2000 (g) for ten minutes and dispensed into two different cryovials for AMH and FSH testing. The cryovials were labelled with study numbers for identification and were analysed in three batches. The samples were stored at -20°C which was monitored using a minimum and maximum freezer thermometer checked twice daily for four months.

Specimen Analysis

Serum AMH Immunoassay

Serum AMH levels were assayed using the Ultrasensitive AMH /MIS ELISA kit (AL -105), manufactured by ANSH LAB, 445 Medical Center Blvd. Webster, TX 77598-4217, USA. The kit's detection limit was 0.023ng/ml. The absorbance of the solution to each well was read off using a Stat Fax 4200 Awareness Technology Microplate reader set to a wavelength of 450nm after following the manufacturer's protocol. All standards, controls and samples were assayed in duplicate.

FSH Enzyme Immunoassay

Serum FSH levels were assayed using a solid phase enzyme-linked immunosorbent assay (ELISA) kit (Cat # 230CH) from International Immuno Diagnostics, 1155 Chess Drive #121 Foster City, CA 99404. The kit had a minimum detectable concentration of 2.5miu/ml. The assay results were read off using a Stat Fax 4200 Awareness Technology at a wavelength of 450nm after following the manufacturer's protocol. All standards, controls and samples were assayed in duplicate. The mean absorbance for each point of the standard curve and each sample were calculated and the mean values of absorbance of the standards were plotted against concentration. The concentration of the unknown samples was extrapolated from the standard curve.

Data management and analysis

The data were subjected to descriptive and correlation statistical analysis using SPSS version 15 software (SPSS Inc., Illinois, USA). The mean, median, standard deviation was derived for continuous variables (age, menstrual cycle, menarche, AMH and FSH) while categorical variables (tribe, religion, occupation, level of education) were summarized as proportions.
AMH and FSH values were presented as means and 95% CI of the mean. Comparisons between age groups were done using one-way analysis of variance. AMH and FSH values for the total population did not have a normal distribution so the values were log-transformed for them to be normally distributed before any correlation testing was done as required by linear regression. Pearson's correlation tests were performed between log AMH, log FSH and the independent variables (age, body mass index, parity, menarche and menstrual cycle.). A p-value <0.05 was set as statistically significant.

Results

The median age of the two hundred subjects was 33 years. The median age at menarche was 13 years while the median age at first pregnancy was 27 years. The median duration of menstrual flow was 4 days and the median menstrual cycle was 28 days. The median AMH concentration was 4.07ng/mL while the median FSH concentration was 9.35mIU/mL. The demographic and reproductive characteristics of the subjects are shown in Table I.

Serum AMH

The 2.5th to 97.5th percentiles for serum AMH at 90% CI for the whole population studied were 0.6 -9.71ng/mL while the lower reference and upper reference limit were 0.6 (0.31-0.79) ng/mL and 9.71 (8.86-10.5) ng/mL. Serum AMH showed a significant negative correlation with age ($r = -0.718$, $p<0.001$) and serum FSH showed a positive correlation with age ($r = 0.265$, $p <0.001$) suggesting that AMH progressively decreased with age while FSH increased with age. Linear regression between log AMH and age performed to determine the age-related changes in AMH concentration were best fitted by the equation: $y = 1.9153 + - 0.04253 x$ as shown in Figure 1 where the coefficient of determination was $r^2 = 0.5158$.

The study population was subdivided as follows: 21-25 years, 26-30 years, 31-35 years, 36-40 years and 41-45 years in order to determine age-specific AMH values as shown in Table II. The mean values of AMH for each age group showed a significant negative decrease with age ($p = 0.001$).

Serum FSH

The mean serum FSH levels of each age group increased with increasing age. The corresponding lower and upper limits for serum FSH values according to the age groups are shown in Table III. The mean values for serum

FSH for the age groups were 8.64±2.13 mIU/mL, 8.62±2.7 mIU/mL, 9.31± 2.96 mIU/mL, 10.84±3.88 mIU/mL and 11.77±3.89 mIU/mL respectively suggesting significant increase with age (p= 0.047).

Table I: Demographic and fertility characteristics of the subjects

Variable	*Frequency*	*Percentage*
Age group (Years)		
21-25	24	12.0
26-30	59	29.5
31-35	49	24.5
36-40	45	22.5
41-45	23	11.5
Body Mass Index (Kg/m²)		
18.5-24.5	93	46.5
25.0-29.5	70	35.0
≥30.0	37	18.5
Parity		
Nulliparous	86	43.0
Multiparous	103	51.5
Grandmultiparous	11	5.5
Age at Menarche (Year)		
10	6	3.0
11	15	7.5
12	32	16.0
13	61	30.5
14	42	21.0
15	25	12.5
16	8	4.0
17	5	2.5
18	6	3.0

Serum FSH

The mean serum FSH levels of each age group increased with increasing age. The corresponding lower and upper limits for serum FSH values according to the age groups are shown in Table III. The mean values for serum FSH for the age groups were 8.64±2.13 mIU/mL, 8.62±2.7 mIU/mL, 9.31± 2.96 mIU/mL, 10.84±3.88 mIU/mL and 11.77±3.89 mIU/mL respectively suggesting significant increase with age (p= 0.047).

Table II: Mean AMH values of 200 healthy regularly menstruating women by age group.

Age groups (Years)	*Frequency*	*Mean Serum AMH (ng/mL)*	*95%CI*
21-25	24	6.42 ± 2.02	5.58-7.28
26-30	59	5.82±2.30	5.25-6.40
31-35	49	4.28±1.90	3.78-4.83
36-40	45	2.55 ± 1.80	1.99-3.11
41-45	23	1.35±1.48	0.7-1.99

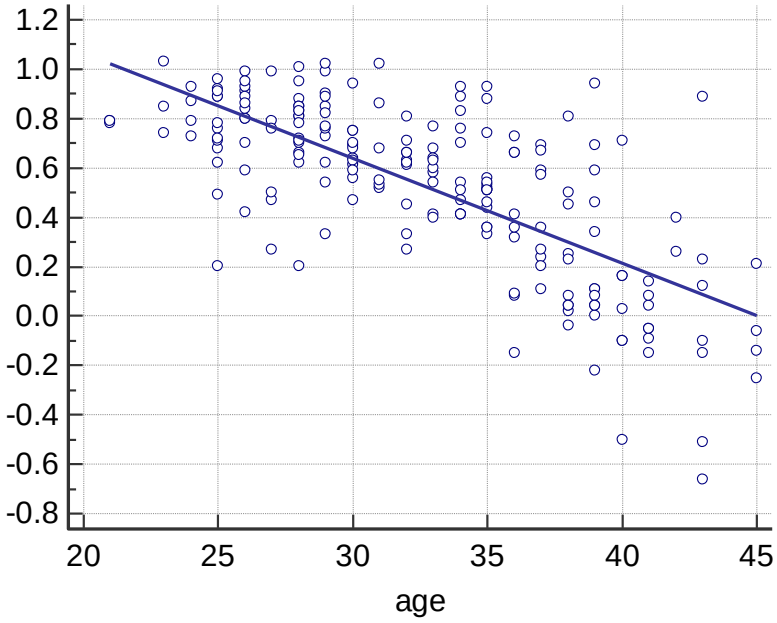

Figure 1: Linear regression of log AMH values and the age of subjects

Where r^2 = Coefficient of determination and age in years.
r^2 = 0.5158.

Table III: Mean FSH values among the subjects distributed by age group

Age groups (years)	*Frequency*	*Mean FSH (mIU/mL)*	*95% CI*
21-25	24	8.64 ±2.13	7.86-9.60
26-30	59	8.62±1.22	7.92-9.33
31-35	49	9.31±2.97	9.31-12.28
36-40	45	10.84±3.88	9.68-12.42
41-45	23	11.77±3.89	10.01-13.46

AMH and Body Mass Index (BMI)

There was a significant but weak correlation between serum AMH and BMI (r = - 0.203, p = 0.04).

AMH and reproductive characteristics (parity, menarche and menstrual cycle)

Table IV shows that there was no correlation between serum AMH and age at menarche (r = - 0.104, p = 0.14) and menstrual cycle (r = 0.111, p = 0.12). However, a weak correlation was found between serum AMH and parity (r = - 0.485, p = 0.00).

Table IV: Pearson Correlation of log AMH and other parameters.

AMH and parameters	*Pearson's Correlation Coefficient*	*p-value*
Log AMH		
Age	-0.718	0.00
BMI	-0.203	0.04
Menarche	-0.104	0.14
Menstrual Cycle	0.111	0.12
Parity	-0.485	0.00

Discussion

In this study, the 2.5th and 97.5th percentiles for AMH at 90% Confidence Interval for the whole population studied were 0.6ng/mL and 9.71ng/mL respectively as generated in accordance with the IFCC recommendation for the determination of range. [11] The mean serum AMH values decreased significantly as age increased and are similar to the serum AMH values generated in a study conducted in Seoul, South Korea among a similar group of 1,298 women. [12] The Korean study sub-divided the study population into six groups within the age range of 20 - 43 years and showed a gradual decrease in median serum AMH values from 4.2ng/mL to 0.60ng/mL whereas the present study showed a gradual decrease in the mean serum AMH values from 6.42±2.02 ng/mL to 1.35±1.48ng/mL. The serum AMH levels obtained in the present study are also very similar to those obtained by Eddie Racoubian, *et al*. In a similarly designed study of Lebanese women.[13] The similar serum AMH values trend generated in these three studies suggest that race may not affect the age-related decline in AMH over time. These values, however, are different from what was observed in a study done in New York where variations in serum AMH between white, black, and Hispanic women were described. It was discovered in that study, that there is an independent effect of race and ethnicity on the age-related decline in serum AMH over time. [14]

Lower serum AMH values compared to values observed in the present study were obtained from a study done among 442 women who were attending an In-Vitro Fertilization (IVF) centre in New York for reasons which included diminished ovarian reserve and/or premature ovarian ageing (54.7%), tubal infertility (19.6%), male factors (17.9%) and others (7.8%).[15] The diminished ovarian reserve could be responsible for the lower mean serum AMH values generated in their study compared to the serum AMH values obtained among 277 healthy women with regular menses in Modena, Italy.[16]

The variation in the reference or mean serum AMH values noticed in these studies is likely due to the selection of different population used in determining these values and the non-standardization of the analytic methodologies used in the different studies. In determining reference values, the reference population, as well as the criteria used to determine health in individuals selected from this population, must be defined. The population to be used should be healthy reference individuals appropriately chosen (which was what was done in this study). The pre-analytical and analytical procedures should be standardized consistent with the methods used for the assay according to IFCC recommendation on reference intervals determination.[11]

It has been shown that there is a strong association between age and serum AMH levels and a better correlation between age and serum AMH than other known markers of ovarian reserve. [8, 17] A group of 81 women were studied over four years in Rotterdam, Netherlands [9] in whom it was discovered that serum AMH had a better correlation with age than serum FSH until age 40 years. Serum FSH values were not correlated with age in the present study, confirming the finding of an earlier study reporting the lack of correlation of FSH and inhibin B with age in women aged 20 to35 years.[17] At 40 years and even more so at 45 years of age, serum FSH started to increase substantially, and the highest levels were recorded among women with irregular menstrual cycles. These endocrine changes seem only to occur when the quantities of follicles are strongly reduced [8] just before or during the menopausal transition. In the present study, serum AMH showed a significant negative correlation with age which supports the findings of the study that determined the age-specific serum AMH levels in 1,298 Korean women with regular menstruation. [12] An ideal marker reflecting the decline of reproductive function should be clearly associated with age and should demonstrate a change over time preferably from approximately 30 years until 50 years of age and not only during or just before the episode of the menopausal transition. From the information demonstrated from all the studies mentioned above, serum AMH shows a progressive decline in follicular reserve during reproductive life as women approach menopause.[8]

Serum AMH reflects better the continuous decline of the oocyte/follicle pool with age than serum FSH because its serum level decline in all age classes and appears to be the better marker of the gradual dwindling of follicle numbers. More importantly, as the changes over time in individuals show consistency concerning the mean decline, serum AMH gives a reliable reflection of individual reproductive ageing and is expected to give better predictions concerning the extent of the decline in the future.[8] Subjects with serum AMH concentrations in the lowest quartile <0.6ng/ml are considered to have evidence of diminished ovarian reserve and to be at risk for diminished ovarian function. Similarly, subjects with serum AMH in the highest quartiles >9.71ng/ml are at risk for unusually high oocyte yields and therefore possibly suffering from polycystic ovarian syndrome and potentially at risk of developing ovarian hyperstimulation syndrome. The lower limit (2.5 percentile) in the study conducted in 277 women with regular menses [16] was 0.52ng/mL and it was suggested under this value reduced ovarian reserve should be suspected. Diminished ovarian reserve is a relevant condition in infertility clinics and now generally accepted that poor ovarian reserve is the main reason explaining the occurrence of poor response to ovarian stimulation in IVF settings. A strong relationship has been shown to exist between reduced ovarian reserve, reduced ovarian response to FSH and the risk of early menopause, [16] and studies done on serum AMH's role in predicting poor response in IVF put the range of 0.3ng/mL to 0.75ng/mL as clinically useful in predicting poor responders.[2, 18] The present study's lower limit at 90% CI ranged from 0.3-0.71ng/mL in predicting poor responders.

Studies have shown that women with Polycystic Ovarian Syndrome (PCOS) have increased serum AMH concentrations when compared with controls. [19-21] The mean serum AMH reported in the literature for PCOS patients ranged between 5.3 and 8.1ng/mL. [19] These high values are considered to be normal in this study and were determined in women in the younger age group (21-25 years). These can be attributed to the presence of a larger number of small antral follicles which are the main sources of AMH.
Moreover, only healthy regularly menstruating women were included in the present study and

the possibility that a woman with a normal menstrual cycle and without hirsutism has PCOS is very remote. [16]

The relationship between high serum AMH concentrations and the risk of hyper-response and Ovarian Hyperstimulation Syndrome (OHSS) in IVF is an additional remarkable clinical utility of serum AMH determination. A high number of antral follicles, high serum AMH concentrations and PCOS are risk factors for both hyper response and OHSS. [22] It was shown in the study done in 277 healthy women with regular menses that all the cases with ovarian hyper response to ovarian stimulation were in the highest serum AMH quartile. The reported serum AMH value in that study was 7ng/mL which is close to what was obtained in the present study (6.08ng/mL). However, the occurrence of OHSS drops with increasing age because the proportion of women with serum AMH concentrations higher than the 75th percentile for young women constantly diminishes.

Serum AMH is an excellent marker for the extremes of the broad spectrum of the ovarian reserve and the adoption of serum AMH measurement to make different ovarian stimulation strategies is appropriate.
The associations between serum AMH, BMI and the reproductive characteristics of the healthy women were also assessed in the present study. There was no significant correlation between serum AMH levels and BMI in the healthy women who participated in the present study. This is similar to the findings obtained in a large population-based study conducted among 2,320 premenopausal women in the Netherland [23] as well as in the study that examined the effect of obesity on parameters of ovarian reserve in premenopausal women.[24] Another study which was conducted among late reproductive-age women and young women on oral contraceptive pills showed a negative correlation between BMI and serum AMH levels.[9, 25]

The association between serum AMH levels and reproductive characteristics examined in the present study showed that there were no correlations between serum AMH levels, menarche and menstrual cycle. This finding was also noted in the large population-based study conducted among 2,320 premenopausal women in Netherland. [23] There was a weak correlation between parity and serum AMH levels in the group studied. This was also in keeping with the finding obtained from the study conducted among young Filipino women in whom it was discovered that women with higher parity had lower serum AMH than nulliparous women. [26] This was also noticed in another study conducted on 277 healthy women with regular menstruation. [16] However, the multiparous women were significantly older than nulliparous ones further emphasizing the inverse relationship between age and serum AMH levels.

Conclusion

Serum AMH progressively decreased with age in this study and, therefore, would be a sensitive marker of ovarian reserve. Serum AMH also had a weak correlation with BMI and parity and had no association with menarche and menstrual cycle. The determination of serum AMH values will produce clinically useful information on ovarian reserve and responsiveness. Larger population studies in the Nigerian populations to establish Reference Intervals will increase the clinical relevance of serum AMH as a biomarker for the diagnosis and treatment of fertility disorders.

Authors' Contributions: OOO, AFM, DVO conceived and designed the study and participated in data acquisition and analyses. DVO, EOO participated in

the literature review. All the authors participated in the drafting of the manuscript. All the authors approved the final version of the manuscript.

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