International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

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Serum Vitamin D and anti-mullerian hormone levels in infertile women in Calabar, Cross River State, Nigeria

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International Journal of Science and Research Archive, 2024, 13(02), 3124-3132

Publication history: Received on 29 October 2024; revised on 18 December 2024; accepted on 20 December 2024

Article DOI: https://doi.org/10.30574/ijsra.2024.13.2.2418

Abstract

Aim: To determine anti-mullerian hormone (AMH) and vitamin D (25(OH)D) levels in infertile women in Calabar.

Study design: A comparative cross sectional study was used.

Methodology: A total of ninety (90) participants which comprises of thirty (30) menorrheic infertile women, thirty (30) amenorrheic infertile women and thirty (30) controls were enrolled into the study. The infertile women were further grouped into twenty three (23) primary and thirty seven (37) secondary infertile women. Blood samples were collected from menorrheic infertile women and control on third day of the menstrual cycle. AMH was analyzed using Enzyme Link Immunosorbent Assay Technique and 25(OH)D was analyzed using High Performance Liquid Chromatography on day three serum samples. Random blood samples were collected from amenorrrheic infertile women and were assayed for AMH and 25(OH)D. Anthropometric indices were obtained using standard methods. Data were analyzed using student t-test, one-way analysis of variance (ANOVA), and Pearson's correlation. Significant difference was considered at $p < 0.05$.

Results: The mean levels of AMH and vitamin D were significantly lower ($p=0.001$) in infertile women when compared to control. The waist hip ratio (WHR) was significantly higher ($p=0.037$) in the secondary infertile women compared to the primary infertile women. There was a significant negative correlation between serum AHM & age ($r = -0.861$, $p= 0.001$) in infertile women.

Conclusion: In this study, the serum vitamin D and AMH levels were significantly lower in the infertile women than in the fertile women. A large number of studies are still required to assess the relationship between vitamin D and AMH and other factors that contribute to infertility.

Keywords: Vitamin D; Anti-mullerian hormone; Vitamin D receptor; Infertility

1. Introduction

Vitamin D is a group of fat-soluble secosteroids responsible for increasing intestinal absorption of calcium, magnesium, and phosphate, and for many other biological effects (19). In humans, the most important compounds in this group are vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) (3). The major natural source of vitamin D is synthesis of cholecalciferol in the lower layers of the epidermis of the skin, through a photochemical reaction with Ultraviolet B (UV-B) radiation from sun exposure or UV-B lamps. Cholecalciferol and ergocalciferol can be ingested from the diet and supplements (19).

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Vitamin D from the diet, or from skin synthesis, is biologically inactive. It is activated by two protein enzyme hydroxylation steps, the first in the liver and the second in the kidneys (3). Because vitamin D can be synthesized in adequate amounts by most mammals if they get enough sunlight, it is not essential and therefore is technically not a vitamin. Instead it can be considered a hormone, with activation of the vitamin D pro-hormone resulting in the active form, calcitriol, which then produces effects via a nuclear receptor in multiple locations (18).Cholecalciferol is converted in the liver to calcifediol (25-hydroxycholecalciferol); ergocalciferol is converted to 25-hydroxyergocalciferol. These two vitamin D metabolites (called 25-hydroxyvitamin D or 25(OH)D) are measured in serum to determine a person's vitamin D status (24). Calcifediol is further hydroxylated by the kidneys and some of the immune system cells to form calcitriol (1,25-dihydroxycholecalciferol), the biologically active form of vitamin D. Calcitriol circulates as a hormone in the blood, having a major role regulating the concentration of calcium and phosphate, and promoting the healthy growth and remodeling of bone (17). Calcitriol also has other effects, including some on cell growth, neuromuscular and immune functions, and reduction of inflammation (19).

Vitamin D is required not only for calcium and phosphorus homeostasis regulation, but also for a variety of other functions, including female reproduction. Vitamin D receptor (VDR) and 1-hydroxylase are found in reproductive tissues such as the ovary, uterus, placenta, testis, and hypophysis (6). As a result, a link between vitamin D and reproductive health appears to be almost inevitable. The importance of 25 hydroxyvitamin D (25(OH) vitamin D) in female reproduction was initially demonstrated in mice lacking in either 25(OH) vitamin D or VDR, which developed uterine hypoplasia and ovulatory dysfunction, resulting in infertility (8).

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting hormone (MIH), is a glycoprotein hormone structurally related to inhibin and activin from the transforming growth factor beta superfamily, whose key roles are in growth differentiation and folliculogenesis (21d). In humans, it is encoded by the AMH gene, on chromosome 19p13.3 (3) while its receptor is encoded by the AMHR2 gene on chromosome 12 (11).

AMH is also a product of granulosa cells of the preantral and small antral follicles in women. As such, AMH is only present in the ovary until menopause (20). So AMH makes it possible to predict the age at which menopause will occur. Production of AMH regulates folliculogenesis by inhibiting recruitment of follicles from the resting pool in order to select for the dominant follicle, after which the production of AMH diminishes (15). As a product of the granulosa cells, which envelop each egg and provide them energy, AMH can also serve as a molecular biomarker for relative size of the ovarian reserve (25). AMH can also be used as a marker for ovarian dysfunction, such as in women with polycystic ovary syndrome (PCOS).

Therefore, the aim of this study is to compare ovulatory fertile females to infertile females in terms of serum AMH and vitamin D status, as well as to determine vitamin D levels in infertile females

2. Material and methods

2.1. Study Area

The subjects of the study were recruited from the Gyneacology Clinics of the University of Calabar Teaching Hospital, Bakor Medical Centre and Arubar Specialist Hospital. Calabar is the capital city of Cross River State, Nigeria. It was originally named Akwa Akpa, in the Efik language. The city is adjacent to the Calabar and Great Kwa rivers and creeks of the Cross River (from its inland delta).

Calabar is often described as the tourism capital of Nigeria (Achum, 2017). Administratively, the city is divided into Calabar Municipal and Calabar South Local Government Areas. It has an area of 406 square kilometres (157 sq mi) and a population of 371,022 as at 2006 census. (22)

2.2. Study Design

The study design was a comparative cross sectional study.

2.3. Study Population

A total of ninety (90) participants were enrolled for the study using simple random sampling technique to give each participant equal chance of being selected. Thirty (30) were menorrheic infertile women, thirty (30) were amenorrheic infertile women attending Gyneacology Clinics of the University of Calabar Teaching Hospital, Bakor Medical Centre and Arubar Specialist Hospital and thirty (30) were enrolled as control (i.e. women who have given birth in the last two years). The infertile women were further grouped into twenty three (23) primary and thirty seven (37) secondary

infertile women. The diagnosis and selection was done with the help of a Gynaecologist in the Obstetrics and Gynaecology Department of the Hospitals.

2.4. Inclusion and Exclusion Criteria

2.4.1. Inclusion Criteria

- Infertile women between the age of 18 to 45 years were recruited as test subjects
- Apparently healthy fertile women (i.e. women who have given birth in the last two years) between the age of 18 to 45 years were recruited as controls
- Women who gave informed consen

2.4.2. Exclusion Criteria

- Women who were on contraceptive drugs were not included in the study.
- Women who were breast feeding were excluded from the study.

2.5. Data Collection

Detailed information about the study was explained to the participants and consent was obtained before patients sample and data were collected.

2.6. Sample Size Determination

The sample size was calculated from the formula (4).

$$N = Z^2pq/d^2$$

Where

N = minimum sample size ghg
Z = area under normal curve corresponding to 95% confidence interval
p = 6% (Okonufua & Odunsi, 2003).

$$q = 1- p = 0.87$$

d = the level of statistical significance = 0.05

$$N = 80$$

An attrition of 10% was added, a total of 90 was used

2.7. Sample Collection, Processing and Storage

A standard venepuncture method was used to obtained threemillilitres (3 ml) of whole blood each from menorrheic infertile women and control on day three (3) of the menstrual cycle and three millilitres (3ml) of random blood samples from amenorrheic infertile women into plain tubes. They were labelled appropriately and allowed to clot at room temperature. The sera were separated from the red cell by spinning at 3,000 rpm for 5 minutes. The supernatant obtained was stored frozen at -20 ℃ until the day of analysis. AMHand vitamin D were analysed on day three serum samples and on random serum samples.

2.8. Anthropometric Parameters

The Height (Metres) and weight (Kg) of participants were measured using a stadiometer and digital weighing scale. Body mass index (BMI) was calculated using the formula: weight/height^2 in the morning on same day of biochemical sample collection. The waist circumference in centimetre was measured in the distance around the smallest part of the waist, just above the belly button. The hip circumference in centimetre was determined by measuring the distance around largest part of the hips. Waist-to-Hip (W/H) ratio was obtained by dividing the waist circumference by the hip circumference (26).

2.9. Estimation of Anti-mullerian hormone

Serum AMH c oncentration was determined using the enzyme linked immunosorbent assay (ELISA) reagents obtained from CUSABIO: 7505 Fannin St., Ste 610, Room 322 (CUBIO Innovation Center), Houston, TX 77054, USA.

2.10. Principle

This assay employs the quantitative sandwich enzyme immunoassay technique.

2.11. Assay procedure

The wells for standard, blank and sample were determined. 100µL of standard and sample was added per well. They were covered with adhesive strip and incubated at 37 ℃ for 2 hours. The liquid of each well was removed and was not washed. 100µL of Biotin antibody was added to each well. A new adhesive strip was used to cover the well and was incubated at 37 ℃ for 1 hour. Biotin-antibody may appear cloudy. They were warm up to room temperature and mixed gently until solution appears uniform. Each well was aspirated and washed, and the process was repeated two times for a total of three washes. They were washed by filling each well with wash buffer (200µL) using a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer, and allowed to stand for 2 minutes. After the last washed, any remaining wash buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels. 100µL of HRP-avidin was added to each well. The micro titre plate was covered with a new adhesive strip and incubated at 37 ℃ for 1 hour. The aspiration and wash process was repeated for five times as before. 90µL of TMB substrate was added to each well and incubated at 37 ℃ for 15-30 minutes. 50 µL of stop solution was added to each well, and the plate was gently tapped to ensure thorough mixing. The optical density of each well was determined within 5 minutes, using a micro plate reader set to 450nm.

2.12. Normal range

1.5-3.5ng/ml

2.13. Analysis of Vitamin D (25- hydroxycholecalciferol) using high performance liquid chromatography (HPLC) (Waters 616/626)

The HPLC system consists of water 616/626 LC-10 AD pump with reference RF- 5300 fluorescence detector and waters CTO-6A column oven. The analytical column is a reverse phase hydrosphere C 18 column with (internal diameter 4.6 x 50mm, 5μm).

2.14. Sample extraction

0.15ml of samples were pipetted or weighed into a set of extraction tubes. A solution of trifluroacetic acid is mixed with sodium hydroxide and ultra-pure water and prepared in the ratio of 0.5:40ml (10ml). Then 10ml of the extraction solution was dispensed into each of the extraction cups containing the samples. The mixture was shaken for 20min with a shaker. Later centrifuged for 10min at 3500rpm. The supernatant was transfered to a set of HPLC autoanalyser cups for deterioration of the analytes (25(OH)D) at the wavelength of 345nm.

2.15. Preparation of standard

The working standard ranges of 0.0, 2.0, 4.0, 6.0, 8.0$\mu g/ml$ was prepared from the stock standard, using ultra pure water, methanol and acetonitrile.The working standards were arranged in tubes in the HPLC autoanalyser tray driven by the proportioning pump.

The analysis took place at 25 ℃ under isoelectric condition.

The column temperature was maintained at 40 ℃. The mobile phase consist of a mixture of acetone nitrile and methanol/ ultra pure water in the ratio of (35:10:20 v/v) ml. Sample flow rate was 1.5 ml/ minute. The sample injector volume was 50 microL. The respective samples and standard were read at 345nm. The spreadsheet software of the instrument was prepared with the following ways: samples serial number, samples date of analysis, the sample weight, and the samples dilution factor. The working standards were 0.0$\mu g/ml$, 2.0$\mu g/ml$, 6.0$\mu g/ml$, 8.0$\mu g/ml$.

Different tubing were connected to the various prepared reagent solutions for the delivering of the solutions to the instrument such as the mobile phase solution bottle, buffer solution and ultra-pure water as well as acetonitrile solution. The working standards were arranged on the autoanalysis cups and mounted on the sampler that is connected to the pump. With the mechanism of the pump the prob gets to the sample cups, pick the sample solution into the instrument

where the samples were drawn to mobile phase in the column. Considering the affinity and other chemical relationship factors of the analyte with the stationary phase, the analyte with smaller molecular weight elutes first followed by the large mass (weight) ones.

At the end of the runs (analysis), the solution automatically calculate the standard curve from the relationship between outcomes of the concentration versus the intensity of the respective working standard graph or equation and use it to automatically calculate and display on the read out the concentration of the samples of the unknown molecular concentrations. Final results are displayed on the read out of the interphased computer or system for reporting.

2.16. Data Analysis

Data were analysed using Statistical Packages for Social Sciences (SPSS) version 23.0 software, Chicago, IL, USA. Difference between means of the groups was analysed using Analyses of variance (ANOVA), and Student t-test. was performed to study the relation between parameters. Significant difference was considered at P <0.05

3. Results

Table 1 Comparison of mean age, BMI, WHR, AMH and Vitamin D levels in infertile women and control

Parameter	Infertile Women n = 60	Control n = 30	t-value	P – value
AGE (years)	32.13±4.97	30.57±4.46	1.458	0.148
BMI (kg/m2)	26.56±6.13	26.27±6.17	0.212	0.833
WHR	0.79±0.07	0.77±0.06	1.189	0.237
AMH(ng/mL)	2.12±0.50	2.73±0.43	-5.681	0.001*
Vitamin D (ng/ml)	29.00±8.24	33.43±3.88	-3.469	0.001*

Values are expressed as mean ± SD. Where BMI = Body mass index, WHR = Waist hip-ratio, AMH =Anti-mullerian hormone * significant at P < 0.05

Table 1 shows the comparison of the mean values of age, body mass index, waist-hip ratio, anti-mullerian hormone (AMH), and vitamin D between the infertile women and control. The mean levels of AMH, and vitamin D were significantly lower (P = 0.001) in infertile women when compared to control. However, the mean age, BMI, and WHR in infertile women were not significantly different (P>0.05) from the control subjects.

Table 2 Comparison of mean age, BMI, WHR, AMH and Vitamin D levels in menorrheicinfertile women, amenorrheicinfectile women and control

Parameter	Menorrheic Infertile women n = 30	Amenorrhei infertile women n = 30	Control n = 30	F-ratio	P- –value
AGE (years)	32.0±5.21	32.13±4.97	30.57±4.46	1.074	0.346
BMI (kg/m^2)	26.36±6.04	26.75±6.32	26.27±6.17	0.052	0.949
WHR	0.79±0.05	0.79±0.09	0.77±0.06	0.748	0.476
AMH(ng/mL)	2.09±0.51	2.16±0.49	2.73±0.43	16.194	0.001*
Vitamin D (ng/ml)	28.07±9.11	29.93±7.30	33.43±3.88	4.416	0.015*

Values are expressed as mean ± SD. Where BMI = Body mass index, WHR = Waist hip-ratio, AMH = Anti-mullerian hormone * significant at P < 0.05

Table 2 shows the comparison of the mean values of age, body mass index, waist-hip ratio, anti-mullerian hormone (AMH), and vitamin D among menorrheic infertile women, amenorrheic infertile women and control. Significant variations (P < 0.05) were observed among the groups in AMH, and vitamin D. No significant variations were found in age, body mass index, and waist-hip ratio.

Table 3 Comparison of mean age, BMI, WHR, AMH and Vitamin D levels in infertile women based on the type of infertility

Parameter	Primary 23	Secondary 37	t-value	P – Value
AGE (years)	32.00±5.17	32.22±4.91	0.163	0.871
BMI (kg/m^2)	25.91±6.62	26.96±5.86	0.647	0.520
WHR	0.77±0.06	0.81±0.07	2.137	0.037*
AMH(ng/mL)	2.14±0.49	2.10±0.49	0.231	0.819
Vitamin D (ng/ml)	29.43±7.62	28.73±8.69	0.320	0.750

Values are expressed as mean ± SD. Where BMI = Body mass index, WHR = Waist hip-ratio, AMH = Anti-mullerian hormone * significant at $p < 0.05$

Table 3 shows the comparison of the mean values of age, body mass index, waist-hip ratio (WHR), anti-mullerian hormone (AMH), and vitamin D in infertile women based on the type of infertility. The WHR was significantly higher ($p = 0.037$) in the secondary infertile women compared to the primary infertile women. There were no significant differences ($p > 0.05$) in other parameters.

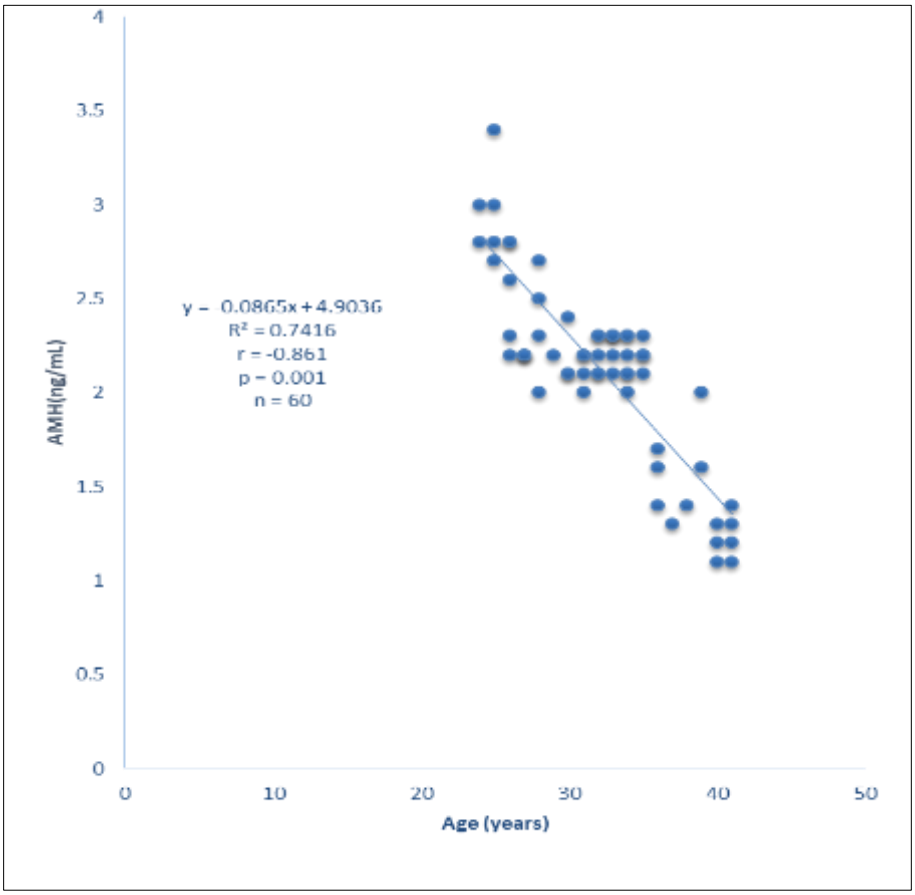

Figure 1 Correlation plot of Anti-mullerian hormone against age in infertile women

Figure 1 shows the correlation plot of anti-mullerian hormone against age in infertile women. There was a significant negative correlation ($r = -0.861$, $p = 0.001$) between anti-mullerian hormone and age in infertile women.

4. Discussion

In this study, it was found that vitamin D level was lower in infertile women compared to control, and was statistically significant. Vitamin D is also known as "anti-ricketic factor or sunshine vitamin." Dietary intakes generally have only a minor influence on serum levels outside of the consumption of vitamin D supplements (5). Even in tropical countries,

despite of ample sunlight (required for the synthesis of vitamin D endogenously), Vitamin D deficiency is prevalent in range of 50 to 90% among all the age groups (16). Nigeria is a tropical country. Though there is no scarcity of sunlight, Vitamin D deficiency is prevalent in women as mostly they stay indoors (9).This result agrees with the findings of (1) who reported a higher percentage of low vitamin D among subfertile Parkistani women in the reproductive age group. This was associated to lack of sun exposure, especially for women, due to religious oblications, poor dietary intake, and environmental factors , especially pollution (1). Another similar findings was reported in Iraq by (10), which showed a significantly lower serum vitamin D level among infertile and fertile women. There is some evidence that vitamin D deficiency and its effects on fertility may be indirect. Without vitamin D, the body absorbs up to 30% less calcium and 20% less phosphorus. In experimental conditions, when the hypocalcaemia and hypophosphatemia were corrected in the female their fertility returned (14). Its possible the primary cause of infertility may be hypocalcaemia and/ or hypophosphatemia. On the contrary, it was found that vitamin D level was lower in fertile females than infertile females, which was significant (12). Other findings also showed that vitamin D level in the case and control groups was not significantly different ([20], [23]). Even though the case group levels were lower, implying that vitamin D may play a function in fertility (23).

This study showed that the mean value of AMH was significantly lower in infertile women when compared with the control. The difference in the mean value of AMH could be due to early reduction in the number of primordial follicles left in the ovaries, thus, leading to reduction in ovarian reserve in the infertile women. This was similar to the findings of (12) where AMH levels were lower in cases than control groups, which might be a cause of infertility in infertile females. AMH is a predictor of ovarian reserve and ovarian responsiveness that directly affect the fertility of a woman, excluding the other causes of infertility (12). The result of serum AMH in this study was also in line with other studies ([7], [10], [23]). In contrast, the result disagrees with the findings of (13) who found no significant difference in serum AMH among infertile women and control.

Although women with primary infertility had slightly higher mean levels of AMH and vitamin D than women with secondary infertility, the differences observed were not statistically significant. This study is in consistent with the works of (7) who had elevated serum AMH level in women with primary infertility when compared with secondary infertility, but was not statistically significant. It also disagrees with the works of (27), who found slightly lower vitamin D level in women with primary infertility when compared with secondary infertility but was not statistically significant.

AMH negatively correlated with age among the infertile women. This was comparable to the correlation documented by (7). This indicates that there was a decline in serum AMH as the age of study participants increases. This further buttress the fact that there was a linear relationship between age and AMH than other known markers of ovarian reserve.

5. Conclusion

This study has shown that infertile women have significantly lower levels of vitamin D and anti-mullerian hormone.

Recommendations

A large number of prospective studies and clinical trials are still needed to assess the causal relationship between adequate vitamin D, AMH and other factors that contribute to infertility.

Compliance with ethical standards

Acknowledgments

The authors acknowledge all the women who gave consent to participate in the study.

Disclosure of conflict of interest

The authors have declared that no conflicts of interests exist.

Statement of ethical approval

Ethical approval for the study was obtained from Cross River State Health Research Ethics Committee, Ministry of Health.

Statement of informed consent

Informed consent was obtained from all participants before they were enrolled for the study.

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