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Original Article

Correlation of serum vitamin D levels with ovarian reserve markers in patients with primary ovarian insufficiency

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Abstract: The cause of primary ovarian insufficiency (POI) is unknown for many women. Previous studies have suggested that vitamin D plays an important role in reproduction, but there have been few studies to investigate possible correlation between serum vitamin D levels and ovarian reserve markers in patients with POI. In this study, 33 women with POI and 72 women with normal menstrual cycles were recruited. Serum levels of follicle stimulating hormone (FSH), anti-Müllerian hormone (AMH), 25-hydroxyvitamin D (25(OH)D and total cholesterol (TC) were measured. Correlations of 25(OH)D with AMH and FSH levels were assessed using regression models. There was a statistically significant difference in 25(OH)D levels between the POI group and the control group. The 25(OH)D levels were inversely correlated with log-transformed FSH and positively correlated with log-transformed AMH. However, no statistical significance was found. The results show low levels of vitamin D may contribute to decreased ovarian reserve. Additional nationwide studies should be conducted to clarify whether appropriate vitamin D levels reduce the risk of developing POI.

Keywords: 25(OH)D, FSH, AMH, primary ovarian insufficiency

Introduction

Primary ovarian insufficiency (POI) is a clinical syndrome defined by loss of ovarian activities before the age of 40. POI is one of the major causes of female infertility, and the prevalence is approximately 1%. POI is characterized by menstrual disturbance (amenorrhea or oligomenorrhea) with raised gonadotrophins and low oestradiol [1]. Anti-Müllerian hormone (AMH) is a protein hormone and a well-recognized biomarker of ovarian reserve. AMH is mainly expressed in granulosa cells of growing antral and pre-antral follicles [2]. AMH regulates follicle growth by inhibiting their sensitivity to follicle-stimulating hormone (FSH) [3]. Thus, reduced AMH serum levels in patients is an important marker of POI [2]. Several causes of POI have been identified and include genetic, autoimmune, metabolic, toxic, infectious and iatrogenic factors [1, 4]. However, the precise cause of most POI cases has not been elucidated [4].

Vitamin D is a fat-soluble steroid hormone precursor that is mainly synthesized in the skin upon ultraviolet light exposure. In addition to its skeletal effects, vitamin D plays a role in several cellular functions, including cell differentiation, apoptosis, decreased proliferation, immune-suppression, and reduced inflammation [5]. The biological actions of vitamin D are mediated through the vitamin D receptor (VDR), which is expressed in many reproductive organs such as ovary, placenta and uterus [6]. Previous studies have suggested that vitamin D plays an important role in reproduction. Expression of VDR in the ovaries suggests there is a potential

role of vitamin D in the ovarian reserve [7-9]. A prior study confirmed that vitamin D stimulated the production of progesterone, oestrone and oestradiol in cultured human ovarian cells [10]. Serum 25(OH)D is the major circulating form of vitamin D and is used by clinicians to determine vitamin D status [11]. A previous study found that low levels of 25(OH)D were associated with POI patients [12]. However, another study showed no difference in 25(OH)D levels between control and POI patients [13].

This study sought to investigate the correlation between serum levels of vitamin D and ovarian reserve markers (such as AHM and FSH) in patients with POI because the relationship between vitamin D and POI is unclear. This case-control study recruited 33 women with POI and 72 healthy women as controls. The serum levels of FSH, AMH, 25(OH)D and total cholesterol (TC) were measured. The correlations of 25(OH)D with AMH and FSH levels in women were assessed using binary logistic regression models.

Materials and methods

Subjects

The initial sample consisted of 110 women with POI and 179 healthy women of whom 174 were excluded (Figure S1). This case-control study recruited 33 women with POI from the Gynaecology Endocrinology Outpatient Clinic at Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, China from September 2015 to April 2016. There were 72 healthy women recruited as the control group during the same study period. This study was approved by the Ethics Committee of Women's Hospital of Zhejiang University. All patients included in the study provided informed consent before participating in the study. The following inclusion criteria were used: 1) diagnosis of primary ovarian insufficiency: (i) oligo/amenorrhea for at least 4 months and (ii) an elevated FSH level > 25 IU/l on two occasions > 4 weeks apart [1]; 2) no iatrogenic cause or known chromosomal abnormality; 3) age between 18 and 40; 4) no hormone therapy for at least 6 months; and 5) the control group consisted of individuals with regular menstruation cycles and without history of infertility. The study exclusion criteria included the following: 1) taking vitamin D supplements or using medication likely to affect the levels of vitamin D and ovarian reserve determinants; 2) history of hysterectomy, oophorectomy, ovarian surgery, chemotherapy and/or radiotherapy; 3) cigarette smoking and 4) women with autoimmune diseases (e.g., autoimmune thyroid disease, autoimmune liver diseases, systemic lupus erythaematosus).

Blood collection

Each patient in the POI group provided a blood sample on the day of their first consultation. The control group blood samples were collected from the first day to the fifth day of spontaneous bleeding episodes during the menstrual cycle. The participants completed questionnaires addressing socio-demographic characteristics, gynecological, and medical histories, and lifestyle factors.

Hormones and 25(OH)D measurement

Serum samples were separated by centrifugation at 4,000 rpm for 10 minutes within 30 minutes of blood sampling. The samples were then stored in polypropylene tubes at -80°C until the time of analysis. The serum levels of FSH and AMH were determined using an electro-chemiluminescence immunoassay (cobas e602, Roche). Serum 25(OH)D levels were measured with mass spectrometry (API 3200, SCIEX). The serum total cholesterol (TC) levels were measured with enzymatic colorimetric methods with commercial kits (cobas c701, Roche). Pro-vitamin D3 is a precursor in the cholesterol biosynthetic pathway, and there is a significant association between serum 25(OH)D and serum lipids [14]. Therefore, the plasma levels of 25(OH)D were adjusted by TC. The intra- and inter-assay coefficients of variation (CVs) were both < 10% based on laboratory controls.

Statistical analysis

The data were analysed using SPSS statistical software for windows version 17.0. The descriptive statistics and continuous data are presented as the mean ± SD. The categorical data are described by number and percentage of cases [15, 16]. The continuous variables were analysed using the independent *t*-test or Mann-Whitney *U* test depending on the normality of the variables. The normality of the variables was tested using the Kolmogorov-Smirnov test.

Table 1. Demographic characteristics of the POI group and the control group

Variable	POI (n = 33)	Control (n = 72)
Age, y		
< 20	1 (3%)	0
20-30	7 (21.2%)	27 (37.5%)
31-40	25 (75.8%)	45 (62.5%)
BMI, kg/m^2		
< 18.5	4 (12.1%)	13 (18.1%)
18.5-24	23 (69.7%)	49 (68.1%)
≥ 24	6 (18.2%)	10 (13.8%)
Gravidity		
0	9 (27.3%)	17 (23.6%)
≥ 1	24 (72.7%)	55 (76.4%)
Infertility	3 (9%)	2 (2.8%)
Education		
≤ Primary school	3 (9.1%)	4 (5.6%)
High school	8 (24.2%)	6 (8.3%)
≥ College	22 (66.7%)	62 (86.1%)
Annual household income (CNY ¥)		
5,000-30,000	1 (3%)	1 (1.4%)
30.000-100,000	16 (48.5%)	10 (13.9%)
> 100,000	16 (48.5%)	61 (84.7%)

Table 2. Biochemical parameters of the POI group and the control group

Variable	POI (n = 33)	Control (n = 72)	P
FSH (IU/L)	59.56 ± 32.67	7.05 ± 2.90	< 0.001
AMH (ng/ml)	0.02 ± 0.004	3.27 ± 2.33	< 0.001
25(OH)D (nmol/L)	92.38 ± 31.07	96.76 ±33.12	0.523
TC (mmol/L)	5.04 ± 0.838	4.30 ± 0.732	< 0.00
25(OH)D/TC (nmol/mmol)	18.47 ± 6.03	22.79 ± 7.90	0.006

Values are expressed as mean ± SD. Testing for differences used student's *t* test.

The odds ratios (OR) for POI associated with 25(OH)D were examined by binary logistic regression analysis before and after adjusting for potential confounders. The log transformation of AMH and FSH was performed to assess the plausibility of linear regression. Correlations for 25(OH)D, log-transformed FSH, and log-transformed AMH levels were examined by multivariate linear regression before and after adjusting for potential confounders. All covariates (age, BMI, education, household income) were simultaneously entered into the multivariate linear regression model to identify regression coefficients for factors related to the log-transformed AMH and log-transformed FSH levels. All *P*-values < 0.05 were considered statistically significant.

Results

Demographic characteristics

The demographic characteristics of the women included in this study are presented in **Table 1**. The majority of women were older than 30 years of age (POI 75.8%, control 62.5%). Additionally, more than half (POI 69.7%, control 68.1%) of the women had BMI values ≥ 18.5 and < 24 kg/m^2. The majority of women in this study had been pregnant once (POI 72.7%, control 76.4%). There were 3 infertile women in the POI group but only 2 in the control group. The participants were generally highly educated, and 66.7% of cases in POI group and 86.1% of subjects in the control group had at least a college education. Participants in the control group (84.7%) had higher annual household income than in the POI group (48.5%).

Biochemical parameters

The FSH levels in the POI group (59.56 ± 32.67 IU/L) were significantly higher than those in the control group (7.05 ± 2.90 IU/L) (P < 0.001). The AMH levels in the POI group (0.02 ± 0.004 ng/ml) were significantly lower than those in the control group (3.27 ± 2.33 ng/ml) (P < 0.001). The 25(OH)D levels did not differ between the POI (92.38 ± 31.07 nmol/L) and the control groups (96.76 ± 33.12 nmol/L) (P = 0.523). However, the adjusted 25(OH)D levels were significantly lower in POI (18.47 ± 6.03 nmol/mmol) group compared to controls (22.79 ± 7.90 nmol/mmol) (P = 0.006) (**Table 2**).

Odds ratio (OR) for POI associated with 25(OH)D

The logistic regression models were used to assess the OR and the corresponding 95% CI for the relationship between the adjusted 25(OH)D levels and POI risk. There was a signifi-

Table 3. Odds Ratio (OR) (95% CI) for POI Associated with 25(OH)D

Variable	Unadjusted Model		Adjusted Model[a]	
	95% CL (0.857, 0.978)		95% CL (0.861, 0.991)	
25(OH)D[b]	*OR* = 0.915	*P* = 0.009	*OR* = 0.924	*P* = 0.026

[a]The adjusted model included age, BMI, annual household income and education.
[b]Serum 25(OH)D levels were adjusted for TC (nmol/mmol).

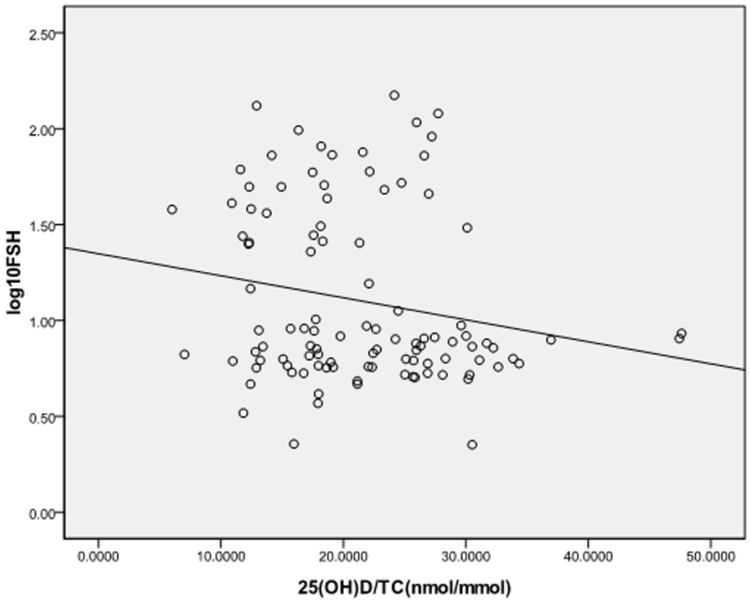

Figure 1. Correlation between 25(OH)D/TC ratio and log-transformed FSH in all participants.

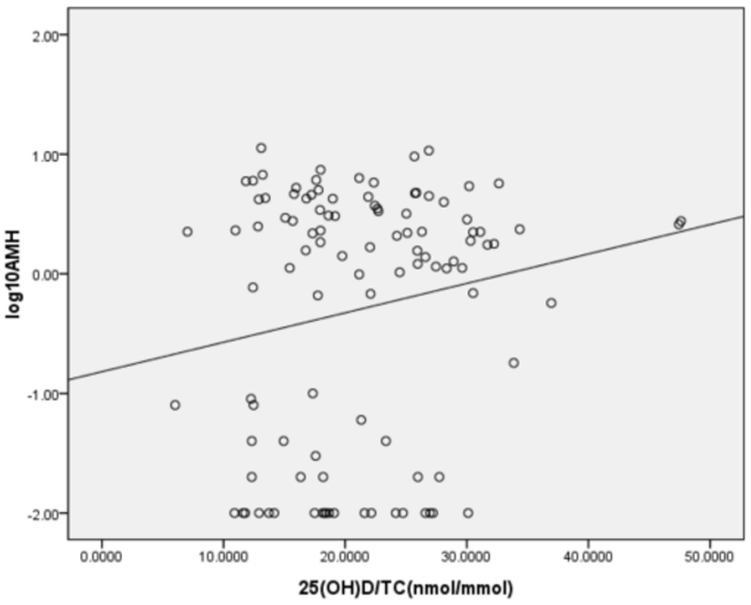

Figure 2. Correlation between serum 25(OH)D/TC levels and log-transformed AMH in all participants.

cant negative association observed between POI risk and 25(OH)D levels in the unadjusted analysis (OR = 0.915, CI: 0.857-0.978, P = 0.009). After adjusting for potential confounders (age, BMI, education, household income) the negative correlation between POI risk and 25(OH)D levels was similar (OR = 0.924, CI: 0.861-0.991, P = 0.026) (**Table 3**).

The relationship between 25(OH)D and ovarian reserve markers

A multiple linear regression analysis was conducted to evaluate the relationships between the 25(OH)D levels and the log-transformed FSH or log-transformed AMH. The 25(OH)D level was inversely correlated with log-transformed FSH (r = -0.192, P = 0.05) (**Figure 1**) and positively correlated with log-transformed AMH (r = 0.175, P = 0.075) (**Figure 2**). However, there was no statistically significant difference. Additional adjustments for age, BMI, education, and household income did not yield a significantly statistical difference between 25(OH)D and log-transformed FSH (r = -0.175, P = 0.071) or log-transformed AMH (r = 0.153, P = 0.120) (**Table 4**).

Discussion

The cause of POI is unknown for many women, and these cases are described as having unexplained or idiopathic POI [4]. Chromosome abnormalities were discovered in several POI patients. Previous studies also reported that autoimmunity, metabolism, infection, smoking, and iatrogenic causes may contribute to the development of POI [17].

Table 4. Relationship between 25(OH)D and log-transformed FSH or log-transformed AMH after adjusting for potential confounders (age, BMI, education and annual household income) in all participants

Variable	25(OH)D[a]	
	r	p
Log-transformed FSH	-0.175	0.071
Log-transformed AMH	0.153	0.120

[a]Serum 25(OH)D levels were adjusted for TC (nmol/mmol).

Serum 25(OH)D is the major circulating form of vitamin D. According to the US Preventive Services Task Force guidelines [18] and the Endocrine Society guidelines [19] vitamin D deficiency is generally recognized as a 25(OH)D level below 20 ng/mL and vitamin D insufficiency has been defined as a serum 25(OH)D level of 21-29 ng/mL. In our study, the mean 25(OH)D levels was (92.38 ± 31.07 nmol/L, 26.40 ± 8.88 ng/mL) in POI cases and (96.76 ± 33.12 nmol/L, 27.65 ± 9.46 ng/ml) in the control groups. The results indicate that most of the women in the study had inadequate 25(OH)D levels. Our study also found the infertility rate in the POI group was very high (9%) and the adjusted 25(OH)D levels were significantly lower in POI. All these findings improved the power of the study to detect an association between low vitamin D and ovarian reserve markers.

The biological actions of vitamin D are mediated through VDR, which is expressed in many reproductive organs such as ovary, placenta and the uterus [6]. Previous studies found the promoter region of the AMH gene contained a vitamin D response element [20]. This discovery prompted researchers to focus on the relationship between vitamin D and ovarian reserve markers. Recent evidence from animal and human studies suggests vitamin D is involved in many functions of the reproductive system. Vitamin D deficiency reduced overall fertility by 75% in female rats when diet interventions reduced levels [21]. Furthermore, VDR null mutant mice showed gonadal insufficiencies [22]. Uterine hypoplasia and impaired folliculogenesis were observed in female VDR-defective mice [22]. In a previous human study involving 35 women with POI and 28 control women, there were significantly lower levels of serum vitamin D found in the POI group compared with the healthy control group with normal menstrual cycles [12]. This study found an association of lower 25(OH)D levels with increased risk of POI in women, which is consistent with prior observations.

The occurrence of POI is accompanied by high levels of FSH, and the FSH elevation is usually used as a diagnostic basis for POI. Several studies showed a link between vitamin D levels and FSH. Vitamin D levels were found to be inversely correlated with FSH levels in POI women [12]. Another study demonstrated that vitamin D was inversely related to urinary levels of FSH in older premenopausal women [23]. In this study, 25(OH)D was negatively correlated with log-transformed FSH which confirmed that low levels of vitamin D play a role in the aetiology of POI.

AMH is an important marker of ovarian reserve, and multiple studies have reported a relationship between vitamin D and AMH in women. In a study of 33 premenopausal women, the change in AMH level was correlated with the magnitude of change in vitamin D levels [24]. In this study, a similar trend for the positive correlation of 25(OH)D and AMH levels. However, there was no significant statistical difference.

Although these previous studies and our observations suggest that low vitamin D levels might have an adverse effect on ovarian reserve, several previous studies failed to identify the association between vitamin D and ovarian reserve markers. For example, a recent study showed that 25(OH)D levels in follicular fluid were negatively correlated with AMH mRNA levels in human granulosa cells of small follicles [25]. A prospective cross-sectional study showed that vitamin D was not associated with the ovarian reserve markers such as AMH and antral follicle count in infertile women [26]. Another study of 130 women reported that 25(OH)D3 levels did not differ between the premature ovarian failure (POF) group and the control group [13].

There are several limitations of our study. First, the women we recruited were mainly residing in Zhejiang province, whose sun-light exposure was similar. Another limitation is the small study sample. Future studies should be randomized studies with larger samples and have patients with various concentrations of serum vitamin D.

In conclusion, this study reveals that low levels of vitamin D may contribute to decreased ovarian reserve and induce POI. Further investigations in a larger population are needed to corroborate these observations and determine the mechanism(s) by which vitamin D interacts with POI.

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Disclosure of conflict of interest

None.

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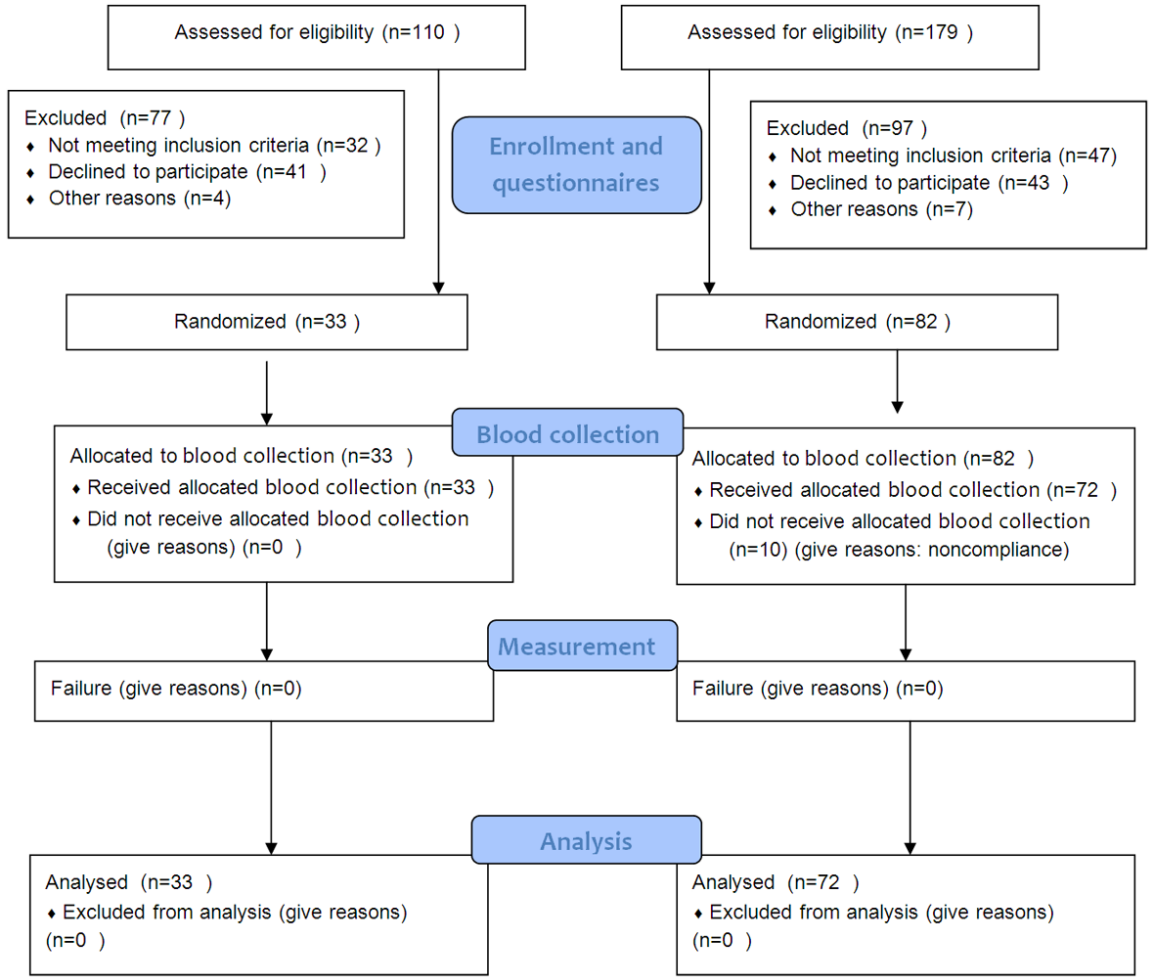

Figure S1. Flowchart of our study strategy.