

ORIGINAL

Serum estradiol level during withdrawal bleeding as a predictive factor for intermittent ovarian function in women with primary ovarian insufficiency

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Abstract. The objective of this study was to assess the potential predictive factors for follicle growth, ovulation, and pregnancy rate in patients with primary ovarian insufficiency/premature ovarian failure (POI/POF). We enrolled 25 POI patients with desired fertility who were treated and monitored for a minimum of 7 months between the years of 2000-2009 into this retrospective study. The clinical, endocrinologic, chromosomal, and autoimmune parameters of these patients were collected. Furthermore, hormonal backgrounds on each of 620 treatment cycles were investigated. The main outcome measures were follicle growth, ovulation, and pregnancy rate. Four of 25 patients (16%) conceived while being monitored and undergoing treatment. Follicle growth, ovulation, and pregnancy rate were not significantly different as a function of parity, iatrogenic history (*e.g.*, chemotherapy), age of disease onset, serum estradiol (E_2)/follicle stimulating hormone (FSH) level at the time of diagnosis, chromosomal abnormality, and positive autoantibody titer. The serum E_2 levels on days 1-5 of withdrawal bleeding (Day 1-5 E_2) were significantly higher in the cycles with successful follicle growth and ovulation than unsuccessful cycles ($P < 0.05$). Receiver-operator characteristic curve analysis revealed the cut-off value of the Day 1-5 E_2 to be 15.5 pg/mL, and an area under the curve (AUC) value of 0.674 for follicle growth and 0.752 for ovulation. The results suggest that cycles with a Day 1-5 $E_2 \geq 15.5$ pg/mL have a higher rate of follicle growth and ovulation in patients with POI.

Key words: Primary ovarian insufficiency, Premature ovarian failure, Follicle growth, Ovulation, Pregnancy

OVARIAN function is established in adolescence, but declines with age due to bi-exponential consumption of primordial follicles [1]. Women undergo menopause when the primordial follicles are completely depleted, and the median age at menopause for Japanese woman is 50 years of age [2]. Some women undergo premature dysfunction and depletion of follicles, and complain of menopausal symptoms with hypergonadotropic hypogonadism before 40 years of age. Such patients, however, should not be diagnosed with early menopause because ovarian insufficiency in this syndrome is not always permanent. Indeed, some women with premature ovarian insufficiency are known to experience intermittent and unpredictable ovarian function [3-7],

with ovulation in 11%-46% of primary ovarian insufficiency (POI) patients undergoing weekly measurement of estradiol and progesterone, as well as an ultrasound examination during the 2-6-month follow-up period [4, 6, 8].

POI affects approximately 1% of women < 40 years of age, and 0.1% of women < 30 years of age [9, 10]. Although autoimmunity, monosomy X, and environmental factors are considered factors that can cause POI, the etiology in most cases remains unknown [11, 12]. POI patients need hormone replacement therapy (HRT), which consists of combination estrogen-progesterone to minimize bone loss and decrease the risk of cardiovascular events [13, 14] whether or not they desire fertility. Women with POI rarely ovulate and achieve pregnancy spontaneously while on HRT; however, it is believed that 5%-10% of such women will conceive and deliver a child after the diagnosis is established [13, 14]. Indeed, there are several case reports in the literature involving POI patients who

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spontaneously and unexpectedly conceived on HRT [6, 8, 15-23]. For couples affected by POI, the only practical alternatives for having a child are adoption or oocyte or embryo donation [13]. Both of these options, however, are extremely limited in Japan due to ethical problems [24]. We previously reported 4 women with POI who conceived by timed intercourse (TI) or intra-uterine insemination (IUI) in combination with close monitoring of follicle growth [25]. All the patients gave birth to healthy babies with an uncomplicated perinatal course. Our previous report clearly demonstrated that POI patients experience intermittent ovarian activity. Prediction of such phenomena can enable more efficient management of POI patients.

In this retrospective study we investigated the relationship between clinical, endocrinologic, chromosomal, and autoimmunologic parameters and intermittent ovarian activity, including follicle growth, ovulation, and pregnancy rate, of 25 POI patients with desired fertility; a total of 620 treatment cycles were evaluated.

Subjects and Methods

Diagnosis and management of patients

Twenty-five patients with POI were referred to our center between 2000 and 2009, and studied retrospectively. POI was defined as at least 3 months of amenorrhea, 2 serum FSH readings > 40 mIU/mL, and 2 serum estradiol (E_2) readings < 10 pg/mL. Patients with Turner's syndrome were excluded from this study. None of the patients had male factor infertility or a history of pelvic radiotherapy. Four of the 25 patients who gave birth to healthy babies were described previously [25]. Clinical, ultrasound, and endocrine evaluations, and karyotype determinations were performed. Briefly, clinical screening included: presumed age at the onset of POI; age at menarche; age at the initial visit; personal history of autoimmunity; history of pregnancy and/or delivery; iatrogenic history, including chemotherapy or surgery on the ovary; hormonal evaluation, including determination of E_2 and FSH; and systematic screening for thyroid autoimmunity. The onset of POI was presumed based on irregular menstruation or amenorrhea. Pelvic ultrasound screening included the presence or absence of follicles. Follicle growth was defined as the presence of follicle(s) of any size in the ovary with a serum $E_2 > 25$ pg/mL, or the presence of a follicle(s) in which the mean diameter was > 14 mm with or without an E_2 measurement. Ovulation was

defined as the disappearance of the follicle(s) and/or formation of a corpus luteum after confirmation of follicle growth with or without administration of human chorionic gonadotropin.

As previously described [25], patients with POI desiring pregnancy with their own oocytes provided a semen specimen from their partner for analysis and underwent hysterosalpingography to rule out other causes of infertility. They were then most often treated with cyclic hormone therapy (cyclic EPT) using estrogen (conjugated equine estrogen [CEE], 1.25-2.5 mg [Premarin®, 2-4 tablets]/day for 7-28 days, representing an absolute time period) followed by estrogen in combination with progestin (0.5 mg norgestrel and 0.05 mg ethinylestradiol [Planovar®, 1 tablet] or 2.00 mg chlormadinone acetate and 0.05 mg mestranol [Lutedion®, 1 tablet]/day for 10-12 days, representing an absolute time period), or in some occasions, human menopausal gonadotropins with or without estrogen or gonadotropin-releasing hormone agonist. Basal serum levels of FSH and E_2 were measured on cycle days 1-5 after withdrawal bleeding. While receiving cyclic EPT, follicle size and number, and ovulation were closely monitored biweekly or twice a month. The Keio University School of Medicine Institutional Review Board approved this retrospective study.

Hormone measurements

Blood was collected, and serum was immediately separated by centrifugation for 6 min at room temperature. Serum E_2 and FSH were measured by electrochemiluminescence immunoassay (Roche Diagnostics, Basel, Switzerland). The intra- and inter-assay coefficients of variation were 1.07-3.5% and 2.03-2.55% for E_2 and 0.73-1.24% and 2.10-2.40% for FSH at all ranges, respectively. The detection limits for E_2 and FSH were 5 pg/mL and 0.1 mIU/mL, respectively.

Statistical analysis

Statistical analysis was carried out using IBM-compatible SPSS for Windows (version 21.0.0; SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean $\pm$ SD. The distribution of each sample was assessed with the Shapiro-Wilk test. When the distributions of both samples were normal, the dispersions were assessed with the Levene test. We compared homoscedastic samples using a two-sample *t*-test or the Welch test for heteroscedastic samples. When at least one of the samples was not normally-distributed, they

were compared using the Mann-Whitney test. The frequencies of categorical variables were compared using the chi-square test. When the cells with an expected frequency < 5 comprised > 20% of the total number of cells in the contingency table, we used the Fisher exact test. A receiver-operator characteristic (ROC) curve was plotted to determine the appropriate cut-off value with maximum sensitivity and specificity for the endpoint. The correlations between the incidence of follicle growth, ovulation, and pregnancy were calculated using Spearman's rank correlation coefficient. *P* values < 0.05 were considered statistically significant.

Results

Patient characteristics

At baseline, the mean presumed age of onset of POI for these 25 patients was 28.6 ± 7.6 years. All of our patients presented with secondary amenorrhea; the mean age of menarche was 13.1 ± 1.5 years. The mean age at the initial visit was 33.2 ± 5.6 years. The duration of ovarian dysfunction (DOD), defined as the period from the onset of POI to the initial visit, was an average of 4.7 ± 4.6 years. Four patients had a history of pregnancy. Five patients had an iatrogenic history, two patients had histories of chemotherapy for Hodgkin's disease, and three patients had histories of surgery on the ovary. The clinical and hormonal backgrounds of the POI patients are listed in Table 1.

Chromosomal examinations were performed in 15 of 25 patients, 3 of whom had karyotypic abnormalities {46,XX,inv(9)(p11q13),46XX,inv(9)(p12q13)(29)/45X,inv(9)(p12q13)(1), and 46XX(28)/45X(1)/47XXX(1),ishX(DXZ1x1)[2]/X(DXZ1x3)[1]/X(DXZ1x2)[97]}. Elevated serum autoantibodies were detected in 8 patients, 2 of whom were positive for thyroglobulin antibodies with elevated thyroid stimulating hormone and diagnosed with Hashimoto's thyroiditis, and 6 of whom only had elevated antinuclear, anticardiolipin, or anti-Sjogren's syndrome A/B antibody titers without diagnosis of specific autoimmune diseases. As anticipated, the mean FSH level at the time of initial diagnosis was high (72.6 ± 44.0 mIU/mL).

Outcomes of POI patients for intermittent ovarian activity

During the follow-up, some patients exhibited intermittent ovarian activity; 17 POI patients (68%) had follicle growth on at least one occasion. Ovulation was

Table 1 Clinical and hormonal backgrounds of POI patients (n = 25)

Presumed age of POI onset (years)	28.6 ± 7.6
Age of menarche (years)	13.1 ± 1.5
Age at the initial visit (years)	33.2 ± 5.6
Duration of ovarian dysfunction (years)	4.7 ± 4.6
Serum E ₂ at the initial diagnosis (pg/mL)	29.6 ± 28.0
Serum FSH at the initial diagnosis (mIU/mL)	72.6 ± 44.0
Pregnancy history	4
Delivery history	3
Iatrogenic history	5
Chromosomal abnormality	3
Elevated serum autoantibody	8

observed in 15 patients (60%). Four (16%) patients conceived and all gave birth to healthy babies. The relationship between each parameter and intermittent ovarian activities is demonstrated in Table 2.

The mean presumed age of onset of POI in patients with follicle growth was comparatively higher than in patients without follicle development, although the trend was not significant (30.3 ± 6.3 years vs. 24.9 ± 9.3 years, *P* = 0.10). The median DOD in ovulatory patients was significantly shorter than that in anovulatory patients (*P* = 0.01; Table 2). The DOD in patients with follicle growth and pregnancy was comparatively shorter than that in patients without follicle growth and pregnancy, although the trend was not significant (*P* = 0.07 and 0.10, respectively; Table 2). None of the other clinical parameters were significantly different between patients with and without intermittent ovarian activity, as well as laboratory factors, including serum E₂ and FSH levels at the time of diagnosis.

The mean incidence of follicle growth, ovulation, and pregnancy in POI patients included in the current study was 1.7 ± 1.8 /person/year, 1.3 ± 1.6 /person/year, and 0.1 ± 0.1 /person/year, respectively. The incidence of follicle growth was significantly correlated with ovulation (*R* = 0.94, *P* < 0.001), but not pregnancy (*R* = 0.31, *P* = 0.14). There was a slight correlation between the incidence of ovulation and pregnancy, although the trend was not significant (*R* = 0.35, *P* = 0.09).

Cycle-based analysis for prediction of intermittent ovarian activity

For further assessment, additional analysis on each of 620 treatment cycles was performed because the hormonal background varied according to cycle, even within the same patient.

Table 2 Relationship between clinical and hormonal parameters and resumption of ovarian function in POI patients

	Follicle growth		<i>P</i> -value	Ovulation		<i>P</i> -value	Pregnancy		<i>P</i> -value
	Yes (n = 17)	No (n = 8)		Yes (n = 15)	No (n = 10)		Yes (n = 4)	No (n = 21)	
Presumed age of POI onset (years)	30.3 ± 6.3	24.9 ± 9.3	0.10*	30.3 ± 6.5	25.9 ± 8.8	0.16*	27.5 ± 8.2	28.8 ± 7.7	0.77*
Age of menarche (years)	13.2 ± 1.6	12.9 ± 1.4	0.72†	13.2 ± 1.7	12.9 ± 1.2	0.82†	13.2 ± 1.6	12.9 ± 1.4	0.20†
Age at the initial visit (years)	33.4 ± 5.8	33.0 ± 5.6	0.89*	33.2 ± 6.1	33.3 ± 5.2	0.97*	29.5 ± 7.0	34.0 ± 5.2	0.15*
Duration of ovarian dysfunction (years)	3.1 ± 1.9	8.1 ± 6.7	0.07**	2.9 ± 1.9	7.4 ± 6.1	0.01†	2.0 ± 1.6	5.2 ± 4.8	0.10†
Serum E ₂ at the initial diagnosis (pg/mL)	26.9 ± 24.4	35.3 ± 35.7	0.86†	27.9 ± 25.9	32.2 ± 32.1	0.86†	14.5 ± 5.1	32.5 ± 29.7	0.22†
Serum FSH at the initial diagnosis (mIU/mL)	77.6 ± 44.5	62.0 ± 43.8	0.45†	78.2 ± 47.3	64.3 ± 39.4	0.45*	107.9 ± 70.1	65.9 ± 35.9	0.08*
Pregnancy history	3/17	1/8	0.62‡	3/15	1/10	0.47‡	1/4	3/21	0.53‡
Delivery history	2/17	1/8	0.70‡	2/15	1/10	0.65‡	1/4	2/21	0.42‡
Iatrogenic history	4/17	1/8	0.48‡	4/15	1/10	0.31‡	0/4	5/21	0.38‡
Chromosomal abnormality	3/9	0/6	0.19‡	3/8	0/7	0.12‡	0/4	3/11	0.36‡
Elevated serum autoantibody	6/17	2/8	0.49‡	5/15	3/10	0.61‡	3/4	5/21	0.08‡

Statistical procedures: * *t*-test, ** Welch test, † Mann-Whitney test, ‡ Fisher exact test.

Table 3 Cycle-based analysis for prediction of intermittent ovarian activation

	Follicle growth		<i>P</i> -value	Ovulation		<i>P</i> -value	Pregnancy		<i>P</i> -value
	Yes	No		Yes	No		Yes	No	
Age during the cycle (years)	32.3 ± 4.0 (n = 142)	32.5 ± 4.2 (n = 478)	0.51*	30.9 ± 4.1 (n = 118)	32.7 ± 4.2 (n = 502)	0.27*	32.0 ± 4.2 (n = 4)	32.5 ± 4.2 (n = 616)	0.89*
Day1-5 E ₂ (pg/mL)	135.5 ± 205.8 (n = 29)	14.6 ± 19.4 (n = 144)	0.002*	193.0 ± 229.4 (n = 20)	14.6 ± 18.9 (n = 153)	< 0.001*	17.0 ± 9.9 (n = 2)	34.6 ± 95.7 (n = 171)	0.65*
Day1-5 FSH (mIU/mL)	22.8 ± 15.2 (n = 47)	23.3 ± 14.3 (n = 195)	0.65*	18.8 ± 12.3 (n = 36)	23.8 ± 14.6 (n = 206)	0.37*	31.6 ± 20.2 (n = 2)	23.1 ± 14.4 (n = 240)	0.34*

Statistical procedure: *Mann-Whitney test.

For each cycle, Day 1-5 E₂ and Day 1-5 FSH averaged 34.1 ± 94.6 pg/mL and 22.6 ± 14.0 mIU/mL, respectively. The average age at the time the treatment cycle was 35 ± 5.3 years. Comparison of the mean value of each parameter between cycles with and without intermittent ovarian activity is shown in Table 3. Evaluating intermittent ovarian activity from cycle-based perspectives, follicle development was observed in 142 cycles (22.9%). Ovulation was confirmed in 118 cycles (19.0%). Pregnancy occurred in 4 cycles (0.6%). Day 1-5 E₂ were significantly higher in the cycles with successful follicle growth and ovulation than cycles without ovarian activity (135.5 ± 205.8 pg/mL vs. 14.6 ± 19.4 pg/mL, *P* < 0.01 and 193.0 ± 229.4 pg/mL vs. 14.6 ± 18.9 pg/mL, respectively; *P* < 0.01), although a difference in Day 1-5 E₂ was not observed

between cycles with and without pregnancy (17.0 ± 9.9 pg/mL vs. 34.6 ± 95.7 pg/mL; *P* = 0.65).

ROC curve analysis on prediction of follicle growth and ovulation revealed that an optimal cut-off value of 15.5 pg/mL for Day 1-5 E₂ had sensitivities of 55.2% and 70.0%, and specificities of 83.3% and 83.0%, respectively (Fig. 1). The AUC was 0.674 for follicle growth and 0.752 for ovulation.

To address the relationship between Day 1-5 E₂ and intermittent ovarian activity, the patients were divided into two groups based on the Day 1-5 E₂ (< 15.5 pg/mL and ≥ 15.5 pg/mL). Patients with Day 1-5 E₂ ≥ 15.5 pg/mL were more likely to have follicle growth and ovulation than patients with Day 1-5 E₂ < 15.5 pg/mL (*P* < 0.001; Table 4).

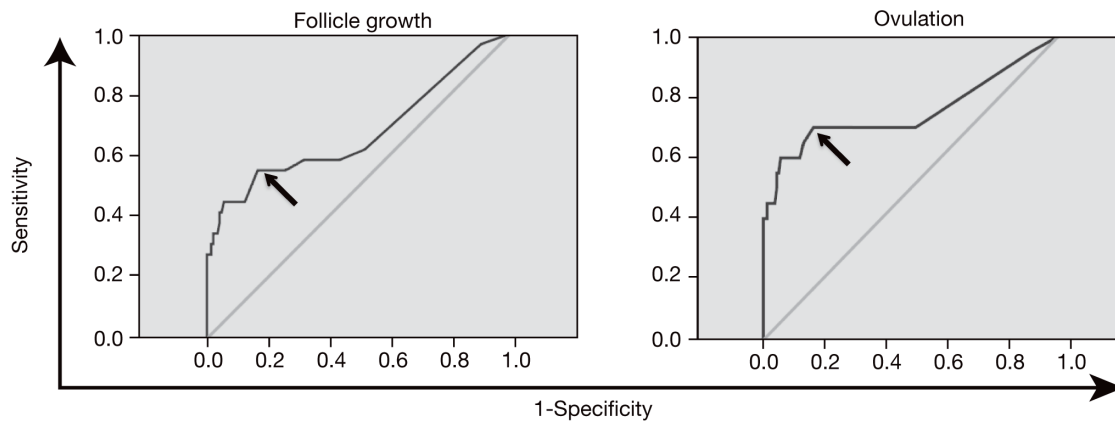


Fig. 1 ROC analysis of Day 1-5 E_2 for prediction of the cycle with intermittent ovarian function. $N = 173$; AUC for follicle growth = 0.674, AUC for ovulation = 0.752. Arrows show the cut-off points of 15.5 pg/mL for Day 1-5 E_2 .

Table 4 Relationship between Day 1-5 E_2 and intermittent ovarian activity

Cycles with	Follicle growth		<i>P</i> -value	Ovulation		<i>P</i> -value
	Yes (n = 29)	No (n = 144)		Yes (n = 20)	No (n = 153)	
Day 1-5 $E_2 < 15.5$ pg/mL	13/29	120/144	< 0.001*	6/20	127/153	< 0.001**
Day 1-5 $E_2 \geq 15.5$ pg/mL	16/29	24/144		14/20	26/153	

Statistical procedure: *chi-square test, **Fisher exact test.

Discussion

In this study we searched for a potential factor(s) that could predict intermittent ovarian function in POI patients. The results suggested that the cycle in which Day 1-5 E_2 was ≥ 15.5 pg/mL had a higher rate of follicle growth and ovulation in patients with POI. The precise mechanism(s) underlying the association between recovery of ovarian function and high E_2 levels in cycles with follicle growth or ovulation on cycle days 1-5 remains to be elucidated; however, one possible mechanism is as follows: HRT containing estrogen and progesterone down-regulates FSH production through a negative feedback loop; subsequent cessation of hormonal supplementation results in the release of the negative feedback loop, and thereby increases FSH production, which in turn may stimulate the growth of a follicle and its E_2 production when a FSH-responsive competent follicle is present; a high level of basal FSH may strongly and immediately stimulate E_2 production from the competent follicle; and serum E_2 could reach a high level on cycle days 1-5, which is an indicator of a competent follicle and the subsequent recovery of intermittent ovarian activity.

A shorter DOD can also be considered a favorable

factor for ovulation (Table 2). The DOD also has a negative relationship with follicle growth and pregnancy, although a significant difference was not proved, presumably due to the small sample size. There are several case reports in the literature involving POI patients who spontaneously and unexpectedly conceived [6, 8, 15-23], but very few had reported statistical analysis of this population due to the rarity of POI.

Bidet *et al.* [26] reported predictive factors for spontaneous resumption of ovarian function in POI patients. A retrospective and prospective study was performed, and included 358 consecutive POI patients in their center. Multivariate analysis showed that a family history of POI, secondary amenorrhea, the presence of follicles on ultrasound, and inhibin B and E_2 levels were significantly predictive of resumption of ovarian function [26]. In particular, it is interesting that serum E_2 , but not serum FSH, is important as a predictive factor for follicular development, as indicated in the multivariate analysis [26] and our current study. In contrast, it has been reported that POI patients with a FSH level < 15 mIU/mL before treatment can ovulate in response to exogenous gonadotropins [27]. These results collectively suggest that each patient may have an optimal level of serum FSH for follicle growth in each cycle.

Anti-müllerian hormone (AMH) is a widely accepted marker for ovarian reserve [28-30], but is not reliable for predicting follicular development in POI patients [31-33] because most POI patients have undetectable AMH levels.

Bidet *et al.* [26] also reported that during the 1-423 months of follow-up (58.8 ± 57 months [mean $\pm$ SD]) of 358 patients with idiopathic POI, 86 patients (24%) had features indicating resumption of ovarian function, and 21 spontaneous pregnancies (16 births and 5 miscarriages) occurred in 15 patients (4.4%). We showed that during the 7-97 months of follow-up (45.5 ± 25.7 months [mean $\pm$ SD]) of 25 patients with idiopathic POI, 17 patients (68%) had features indicating resumption of ovarian function, and spontaneous pregnancies (4 births and no miscarriages) occurred in 4 patients (16%). Although these two studies cannot be directly compared, it is possible that the high rates of spontaneous resumption of ovarian function and conception obtained in our study may be, at least in part, due to our close and continuous monitoring of follicle growth to identify very rare ovulatory events.

The fundamental dogma for the mammalian ovary is now changing due to the emerging novel concept of oogonial stem cells (OSCs); OSCs have been recently reported in several studies [34-36]. Isolation and expansion of OSCs from POI patients might be the key to a new strategy for fertility treatment. Furthermore, Kawamura *et al.* [37] established the method of *in vitro* activation (IVA) of primordial follicles in mouse ovaries; 13 patients with POI were treated using this technique, and 1 patient gave birth to a healthy baby. These unique techniques may be able to offer a new fertility

treatment for patients with POI in the future.

Currently, however, POI patients have limited options for conceiving. For POI patients with desired fertility, clinicians should suggest options for treatment, including oocyte donation, which is not permitted in Japan, and close monitoring of the follicle, because there are very few who will achieve pregnancy with her own oocytes. In our current study, the importance of Day 1-5 E₂ for predicting intermittent ovarian function was suggested. To shorten the DOD of potential patients with POI, it is important to widely educate women to seek evaluation when they have irregular menstruation or amenorrhea. Though the Day 1-5 E₂ had less statistical power due to the small sample size in POI subjects and further study in a large scale study will be required, the results are helpful for managing POI patients who have a strong desire to conceive.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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