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Clinical Applications of Serum Placental Protein 14 (PP14) Measurement in the IVF-ET Cycle

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Abstract

Objective: Placental protein 14 (PP14) is known to be one of the endometrial proteins that reflect endometrial functioning throughout the menstrual cycle. In this study, we examined PP14 as a marker for human endometrial receptivity in order to predict the outcome of *in vitro* fertilization and the embryo-transfer (IVF-ET) cycle.

Patients and Methods: The subjects were 72 women who had 96 IVF-ET cycles and who were examined at Tokyo Medical University Hospital during the period of January 1998 to June 1998 because of mechanical or unexplained infertility for a duration of at least 2 years. Serum samples were collected from all patients during treatment cycles, and serum PPI4 concentrations were measured by a newly established enzyme-linked immunosorbent assay (ELISA).

Results: In the pregnant group, serum PP14 concentrations were markedly increased after ET, and a significant difference between the pregnant group and the nonpregnant group was observed 8 days following ET ($p < 0.01$). PP14 concentrations were higher in patients with endometria that exhibited homogenous patterns and that were more than 7 mm thicker than in other patients, as determined by ultrasound on the day of oocyte collection ($p < 0.005$). The pregnancy rates of patients with homogeneous patterns were lower than those of patients showing a trilaminar pattern. No pregnancies were observed when serum PP14 concentrations were greater than 6.85 U/*l* on the day of oocyte collection.

Conclusion: PP14 might be a useful marker for human endometrial receptivity to predict the outcome of IVF-ET cycles.

Key words: placental protein 14, IVF-ET, endometrium, receptivity, pregnancy outcome

Introduction

Recently treatments for infertile couples have advanced rapidly. Assisted reproductive technology (ART), such as *in vitro* fertilization and embryo transfer (IVF-ET), gamete intrafallopian transfer (GIFT), and intracytoplasmic sperm injection (ICSI), and so on has become more commonly used worldwide. But in spite of the popularity of ART, pregnancy rates are still low, and

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the optimal conditions for successful implantation and inducing pregnancy by assisted reproduction in humans are still poorly understood. Decreased implantation rates of human embryos are probably the result of genetically or morphologically abnormal embryos and/or nonreceptive endometria.[1-3] We believe that establishing the receptivity of the endometrium is one of the most important factors when considering implantation. Although there have been numerous reports concerning human endometrial receptivity,[4-7] no beneficial clinical markers for receptivity have yet been found. In one receptivity study, ultrasonographic examination of the thickness and echogenic determination of the endometrium were performed.[8-11] However, the prognostic value of measurements of endometrial thickness and/or the appearance of the endometrium in conception and non-conception IVF-ET cycles remains controversial. Nonetheless biochemical studies concerning the many proteins produced in the human endometrium indicate that placental protein 14 (PP14) might serve as the most promising indicator of endometrial functioning.[12-16] PP14 was originally isolated from human term placenta by Bohn *et al.*[17] in 1982, but in 1988 was shown by Julknen *et al.* to derive predominantly from the endometrium and decidua.[18] It is a glycoprotein, having a molecular weight of 28–30 kDa, and containing 17.5% carbohydrate. The isoelectric point of PP14 is 4.6, and its electrophoretic mobility is intermediate between $\alpha 1$ and $\alpha 2$ globulin. The precise biological role of PP14 remains unknown. However, PP14 is considered to be one of the most important endometrial proteins and to have important immunosuppressive activity during human reproductive life.[14,19-20] In this study, we established a newly devised enzyme-linked immunosorbent assay (ELISA) for PP14, and in this report we discuss the usefulness of PP14 measurement as a marker for receptivity in the human endometrium.

Materials and Methods

1. *Patients, Treatment for IVF-ET and Serum Samples*

The subjects were 72 women (31 ± 5 years old) who had 96 IVF-ET cycles and who were examined at Tokyo Medical University Hospital during the period of January 1998 to June 1998. All of the subjects had a history of normal ovulation and underwent IVF-ET because of mechanical or unexplained infertility for a duration of at least 2 years.

All patients underwent our standard "long" protocol of pretreatment with a GnRH analogue (Suprecure, Hoechst Japan Co., Japan) followed by ovarian stimulation with pure-FSH (Fertinorm P, Serono Japan Co., Japan). Briefly, GnRH-a, 0.5 mg/day, was administered from Day 21 of the previous cycle until administration of human chorionic gonadotropin (hCG). On Day 3 of the treatment cycle, transvaginal ultrasonography was performed, and the serum estradiol and progesterone levels were assessed. When the serum estradiol levels were below 10 pg/m*l* and the serum progesterone levels were below 2.0 ng/m*l*, concomitant pure-FSH was given, 4 ampules per day, starting on Cycle Day 3 with the dosage being increased as indicated by a patient's response. Human chorionic gonadotropin (HCG Mochida, Mochida Pharmaceutical Co., Japan) 10,000 IU was administered when at least 2 follicles were more than 18 mm. From 32 hours to 36 hours after hCG administration, oocytes were harvested by sonographically guided transvaginal follicular aspiration. Embryo transfer was performed 2 days after oocyte aspiration, and a maximum of 3 embryos were transferred. All of the patients included in this series received progesterone support for 2 weeks, starting on the day prior to embryo transfer.

Serum samples were collected from all patients at the following times: 1) start of pure-FSH administration, 2) hCG administration, 3) oocyte collection, 4) embryo transfer (ET), 5) 4th day after ET, 6) 8th day after ET, 7) 12th day after ET, and 8) 16th day after ET. Approval for use of the sera in this study was granted by the Ethics Committee of Tokyo Medical University.

2. *Enzyme-Linked Immunosorbent Assay (ELISA) for PP14*

The IgG fraction of the anti-PP14 antibody and the biotinylated affinity-purified anti-PP14 antibody were purified as described previously.[21-22]

ELISA was performed using Maxisorp flat-bottom microtitre plates (Nunc, Roskilde, Denmark). Coating (100 μl/well) was performed with the IgG fraction of anti-PP14 diluted to a concentration of 40 μg/m*l* in a carbonate buffer (15 mM Na_2CO_3, 34.9 mM $NaHCO_3$), pH 9.6, overnight at 4°C. All washing procedures were performed in PBS + 0.37 M NaCl, 0.05% Tween 20, pH 7.3. The standards, controls, and samples were diluted in PBS containing 1% normal rabbit serum and 0.05% Tween 20 (dilution buffer), and were incubated (100 μl/well, in duplicates) overnight at 4°C. The standards were calibrated using

a pool of second-trimester amniotic fluid, which was assigned an arbitrary value of 10,000 U/*l*. The biotinylated, affinity-purified anti-PP14 antibody (diluted to 0.3 μg/m*l* in dilution buffer) was added (100 μ*l*/well) and incubated at room temperature for 1 hour. Streptavidin-labelled horseradish peroxidase (Zymed Laboratories Inc., San Francisco, USA; 100 μ*l*/well, diluted 1:1000) was added. After incubation (25 minutes at room temperature) and washing, the H_2O_2-orthophenylene-diamine (OPD) substrate indicator solution (0.4 μ*l* H_2O_2 and 0.4 mg OPD/m*l* 0.1 M citrate buffer, pH 5.0) was added (100 μ*l*/well) followed by incubation (in the dark at room temperature) for 15 minutes. The color reaction was stopped by the addition of 150 μ*l* of 1 M H_2SO_4 to each well. All chemicals, unless otherwise stated, were purchased from Sigma, St Louis, MO, USA. ELISA plates were read by an ELISA reader at 492 nm.

3. Assays for Estradiol and Progesterone

Estradiol concentrations were measured by a commercially available EIA kit (AIA-PACK E_2, TOSOH Co. Japan), in accordance with the manufacturer's recommendations. The sensitivity of the assay was 10 pg/m*l* (intra-assay coefficient of variation = 9.1%, inter-assay coefficient of variation = 10.3%).

Progesterone concentrations also were measured by a commercially available EIA kit (AIA-PACK P, TOSOH Co. Japan). The sensitivity of the assay was 0.1 ng/m*l* (intra-assay coefficient of variation = 8.7%, inter-assay coefficient of variation = 10.5%).

4. Vaginal Ultrasound Scanning

Sonographic assessment was performed for all patients transvaginally, using a Sonovista-MSC (Mochida Pharmaceutical Co., Japan) 5-MHz endovaginal probe on the day of oocyte collection. The endometrium was imaged in a longitudinal section and its thickness was measured at the greatest anteroposterior dimension of the endometrium. The endometrial echogenicity pattern was then determined. The echogenicity of the endometrium was compared with that of the surrounding myometrium and 2 patterns were noted. A homogeneous pattern was defined as a single hyperechoic layer. A trilaminar pattern was identified as a hypoechoic layer with a central hyperechoic line or an isoechoic layer with a central echogenic line. Therefore, the endometrial images observed were classified by ultrasound into 4 groups (Group A, Group B, Group C, and Group D). In short, the endometrial shape was classified into 2 groups: trilaminar pattern and homogenous pattern, and its thickness was classified into 2 groups: less than 7 mm and greater than 7 mm. The basis on which the endometrial thickness was classified by use of 7 mm as the dividing point was determined by our clinical experience concerning the relationship between the endometrial thickness and the IVF-ET outcome. All follicles had to appear consistent with the preovulatory status.

5. Statistical Analysis

Data for the concentrations of serum PP14, estradiol, and progesterone are presented as means ± SD.

An analysis of variance comparing the pregnant and nonpregnant groups was performed by repeated measurements of the fluctuations in serum PP14 concentrations during the IVF-ET treatment cycles. A grouped *t*-test was used to compare the pregnant and nonpregnant groups, and p values < 0.01 were considered statistically significant. Also, a Scheffe-type multiple comparison test was performed on the concentrations of serum PP14, estradiol, and progesterone in Groups A, B, C, and D at the time of oocyte collection, and p values < 0.005 were considered statistically significant.

Results

The characteristics of the 72 women included in this study are shown in Table 1. Pregnancies occurred in 33 of the 96 (34%) IVF-ET cycles.

1. Validation of PP14 ELISA

The PP14 assay was designed using a pool (*n* =

Table 1. Characteristics of 72 women monitored for serum PP14 and hormone analysis and examined by ultrasound scanning throughout IVF-ET cycles

No. of women	72
Median (range) age (years)	31 (23–39)
Median (range) duration of infertility (years)	4 (2–8)
Primary infertility rate (%)	73
Median (range) menstrual cycle interval (days)	29 (25–33)
Cause of infertility (%)	
Tubal	67
Unexplained	33
Treatment cycles	96
Pregnancy cycles (rate)	33 (34%)

16) of second-trimester amniotic fluid for calibration. Amniotic fluid was arbitrarily assigned to a concentration of 10,000 U/*l* of PP14. The calibration curve was a dilution series of amniotic fluid (6 steps; in duplicate) ranging from 24.8 to 0.6 U/*l*. This calibration curve was almost linear from 0 to 25 U/*l*, and the detection threshold was 0.1 U/*l*. Sera ($n = 3$, Day 16 after ET), amniotic fluid, and PP14 purified from endometrial extract revealed parallel titration curves by ELISA (Fig. 1). Cross-reactions between the anti-PP14 antibody and major pregnancy-associated proteins [human chorionic gonadtropin (HCG), schwangerschafts protein 1 (SP1), pregnancy associated plasma protein A (PAPP-A), placental protein 12 (PP12), placental protein 13 (PP13), placental protein 19 (PP19), fetal antigen 1 (FA1), and fetal antigen 2 (FA2)] were insignificant. Moreover, normal male serum was undetected, when applied undiluted to the assay ($n = 5$), indicating that the assay was not influenced by "normal" circulating antigens. The coefficients of variation for inter-assays were 10.2% at 26.3 U/*l*, 9.7% at 8.7 U/*l*, and 9.3% at 1.2 U/*l*, and for intra-assays they were 9.8% at 25.4 U/*l*, 9.1% at 9.2 U/*l*, and 8.4% at 1.1 U/*l*.

2. *Serum PP14 Concentrations in 96 IVF-ET Cycles*

The variations in serum PP14 concentrations (mean ± SD) throughout the 96 IVF-ET cycles are shown in Fig. 2. The serum PP14 concentrations at the time of pure-FSH administration were 1.2 ± 0.8 U/*l* in the pregnant group and 1.1 ± 0.7 U/*l* in the nonpregnant group. In both the pregnant and nonpregnant groups the PP14 concentrations gradually increased from the time of pure-FSH administration to the time of embryo transfer (ET), and no significant differences between the groups were detected before ET. However, in the pregnant group the PP14 concentrations steeply increased after ET, and a significant difference between the 2 groups was observed after the 8th day following ET ($p < 0.01$).

3. *Sonographic Imaging and Serum PP14, Estradiol and Progesterone Concentrations*

The concentrations of serum PP14, estradiol, and progesterone (mean ± SD) in the 4 groups, A, B, C, and D, which were classified by sonographic endometrial images at the time of oocyte collection, are shown in Fig. 3.

The PP14 concentrations in the trilaminar groups (Groups A and B) were lower than in the homogenous groups (Groups C and D), and the trilaminar group showed a higher pregnancy rate. Within the trilaminar group, no significant difference in PP14 concentrations between Groups A and B were observed. However, within the homogeneous group, the serum PP14 concentrations in Group D, which had endometrial thicknesses greater than 7 mm, were much higher than those of Group C, which had endometrial thicknesses of less than 7 mm ($p < 0.005$). The highest pregnancy rate was observed in

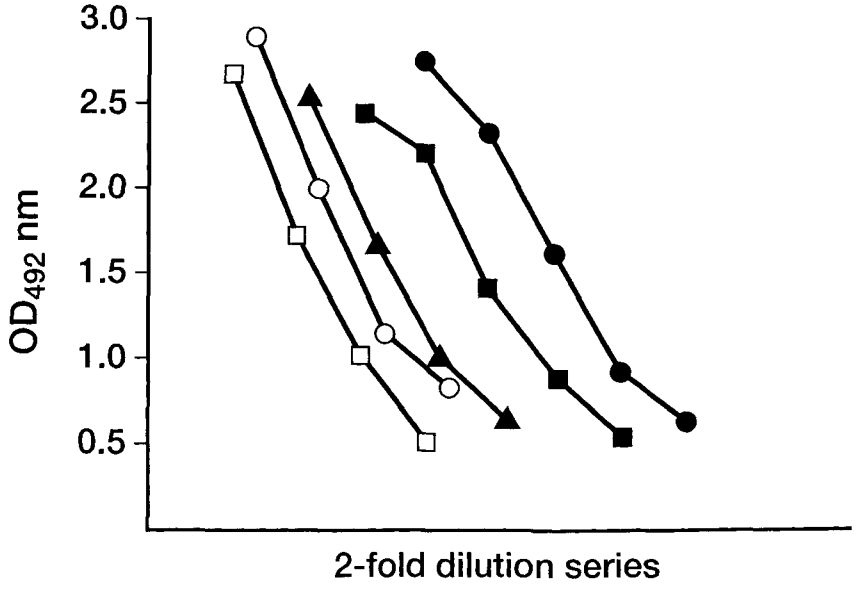

Fig. 1. Comparison of the optical density (OD) 492 nm signal of 2-fold dilution series of amniotic fluid (□), PP14 purified from endometrial extracts (○), and sera from 3 patients on the 16th day after ET (▲, ■, ●) respectively.

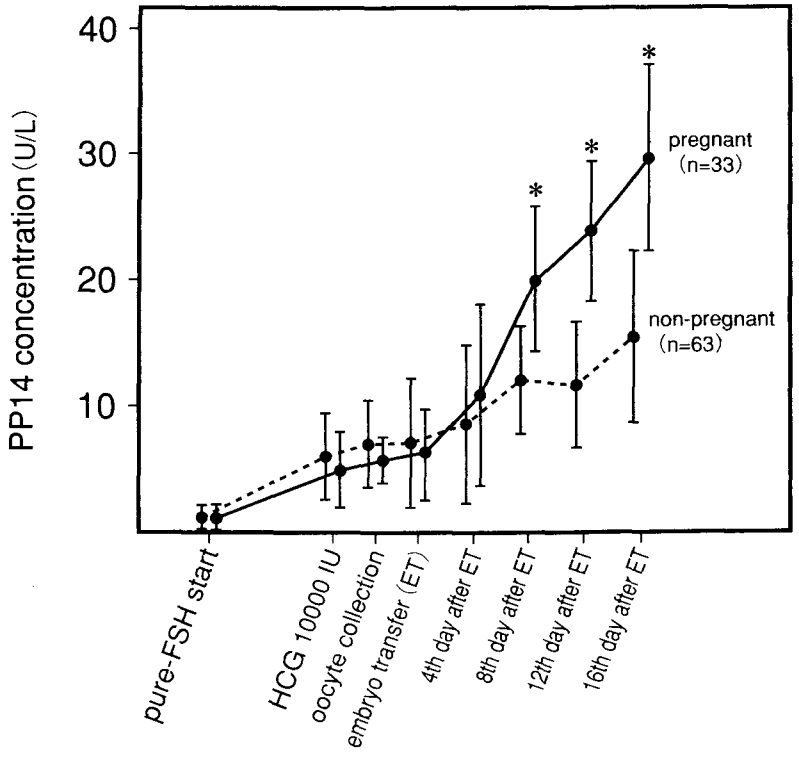

Fig. 2. The dynamics of serum PP14 concentrations in 72 patients with 96 IVF-ET cycles. Significant differences were observed between the pregnant group and non-pregnant group after the 8th day following ET (*p < 0.01).

Group	Sonographic image	Endometrial shape	Endometrial thickness	PP14 (U/L)	Estradiol (pg/ml)	Progesterone (ng/ml)	Pregnancy rate (%)
A (n=19)		 Trilaminar	< 7mm	3.68±1.35	564±246	2.1±1.7	26.3
B (n=39)			≧ 7mm	4.52±1.48	872±394	2.4±1.8	46.2
C (n=10)		 Homogeneous	< 7mm	4.98±1.68	627±303	3.1±2.1	10.0
D (n=28)			≧ 7mm	*7.13±2.65	764±411	*4.8±2.7	32.1

Fig. 3. The relationship between sonographic endometrial images and serum PP14, estradiol and progesterone concentrations at the time of oocyte collection during IVF-ET cycles. PP14 and progesterone concentrations in group D were statistically higher than in the other groups (*p < 0.005). No significant differences in the serum estradiol concentrations were observed between the 4 groups. Group B showed the highest rate of pregnancy, and group C showed the lowest rate.

Group B (46.2%), and Group C showed the lowest pregnancy rate (10%). A comparison of serum PP14 concentrations in Groups A and C, which had endometrial thicknesses less than 7 mm, revealed that Group A whose pregnancy rate was higher than that of Group C, showed lower PP14 concentrations, though significant differences were not observed. In addition, a comparison of serum PP14 concentrations in Groups B and D, whose endometrial thicknesses were greater than 7 mm, revealed that group B, whose pregnancy rate was higher than that of Group D, showed significantly lower PP14 concentrations ($p < 0.005$).

The serum progesterone concentrations in Group D were significantly higher than those in the other groups ($p < 0.005$). No significant differences in the serum estradiol concentrations were observed between the 4 groups.

Discussion

One of the current goals of clinicians working in the field of reproductive medicine is to determine the parameters of endometrial receptivity with prognostic value, so as to better predict the outcome of IVF-ET. For this reason, in the past decade many different parameters have been investigated, and in particular hormonal determinations and ultrasound examinations have been proposed as beneficial parameters for evaluating endometrial receptivity. Nonetheless, endometrial receptivity is a poorly understood factor that influences the likelihood of conception, and truly beneficial parameters for clinical application have not yet been established.

Numerous papers have reported on the relationshoip between ultrasonic findings and endometrial receptivity, as we have described in the introduction of this report. Abdalla *et al.*[23] reported only 2 pregnancies in 15 patients who had an endometrial thickness of less than 7.5 mm, and no pregnancies when the endometrial thickness was less than 5 mm. They therefore proposed that endometrial thickness is related to the functional receptivity of the endometrium. Indeed, a poor prognosis can be easily assumed when the endometrium is thin, although we often treat patients who have never succeeded in achieving pregnancy in spite of having a sufficiently thick endometrium. In this study, we classified endometrial images into 4 groups according to their shape and thickness. As one result, we showed that the pregnancy rate was higher when the endometrial shape displayed a trilaminar pattern and the endometrial thickness was greater than 7 mm. However, it seems difficult to assess endometrial receptivity only by ultrasound examination.

In order to assess endometrial receptivity, biochemical markers currently being investigated by many clinicians might be utilized in the near future. For example, special proteins that are produced and secreted from the human endometrium are regarded as potentially effective markers for predicting IVF-ET outcome.

It has recently been suggested that CA-125 measurement in a patient's serum might be efficiently employed as a preretrieval pregnancy predictor in human IVF-ET. In fact, it has been reported that higher serum CA-125 concentrations on the day of hCG administration[24] or on the day of oocyte collection[25] are associated with a higher pregnancy rate. However, Noci *et al.*[26] suggest that CA-125 levels on the day of oocyte collection are lower in the conceptional cycles than in nonconceptional cycles, and that CA-125 concentrations on the day of oocyte collection are not predictive of subsequent pregnancy. For this reason, CA-125 determinations before embryo transfer in IVF patients do not seem to be useful for predicting the outcome of IVF-ET.

PP14 represents the most abundant protein product of the human endometrium, and PP14 has been proposed as the most reliable biochemical marker of endometrial functioning in women.[12] PP14 is a glycoprotein, being secreted by proliferative and secretory endometria and decidua in women. PP14 has been implicated in the process of endometrial preparation for blastocyst implantation under estrogen and progesterone dominance in women.[14,27,28]

In this study, serum PP14 concentrations in the trilaminar group at the time of oocyte collection, which showed higher pregnancy rates, were statistically lower than in the homogenous group. We propose that the high PP14 concentrations in group D, which demonstrated homogeneous endometrial images and thicknesses greater than 7 mm, were associated with increased serum progesterone concentrations, because it is known that PP14 production is related to progesterone *in vitro* and *in vivo*.[29–31] We suggest that increased serum PP14 concentrations with increased progesterone before oocyte collection might affect the endometrial function and might close an implantation window in the early luteal phase after oocyte collection. Moreover we observed no pregnancies when the serum PP14 concentration was greater than 6.85 U/*l* on the day of oocyte collection. Westergaard *et al.*[22] also reported that serum PP14 concentrations in patients who underwent IVF-ET treatment were lower in the pregnant subjects than in the nonpregnant group. Furthermore, Yding *et al.*[32] reported that serum PP14 concentrations in IVF-ET cycles were significantly lower during the follicular phase in conceptional cycles than in nonconceptional cycles. Thus, it seems that serum PP14 concentrations at preovulatory phases might be a better indicator of optimal endometrial functioning and the probability of implantation. It might also be worthy pointing out that high PP14 concentrations before oocyte collection can indicate a poor

prognosis for pregnancy. Therefore, for patients undergoing IVF-ET who display high serum PP14 concentrations on the day of oocyte collection, it might be practical to cryopreserve the embryos and to perform ET in a future cycle when the endometrium displays a more favorable pattern.

The data in this study showing lower PP14 concentrations in conceptional compared to nonconceptional cycles are in agreement with the data reported on PP14 fluctuation throughout the menstrual cycle. Among the investigators who reported on this subject in the literature the only point of agreement is that PP14 concentrations rise during the menstrual period as a consequence of endometrial disruption.[33,35] These data suggest that endometrial shading is directly responsible for the rise in serum concentrations of PP14. Because it is reported here that pregnancy is associated with a lower concentration of PP14 than is the case in the nonpregnant state, it is possible that the measurement of PP14 on the day of oocyte collection reflects the actual state of the endometrium and its shading tendency. So it seems likely that the endometrium is more compact, without any shading trend, and somehow is more receptive to embryo implantation when PP14 concentrations are low.

Although the height and pattern of the estradiol response during hMG stimulation during IVF have been found to be associated with higher pregnancy rates,[36] in this study we did not find them to be useful indicators of clinical pregnacy, but the concentration of PP14 seems to be a more sensitive indicator of pregnancy before oocyte collection.

In conclusion, we have succeeded in establishing a highly sensitive, specific, and reproducible ELISA technique for PP14. Second, in the IVF-ET cycle, significant differences in serum PP14 concentrations were observed between the pregnant and nonpregnant groups from the 8th day after ET. Third, because serum PP14 concentrations were lower in the pregnant group than in the nonpregnant group on the day of oocyte collection, we believe that PP14 might be a useful marker reflecting receptivity of the human endometrium. In addition, prospective studies, including a much larger number of patients than in the present study, and different stimulation regimens, are required in order to precisely define the threshold concentrations of serum PP14 with a sufficiently high degree of predictability. Also a combination of biochemical markers like PP14 and ultrasound examination might be more effective for evaluating endometrial receptivity. Such studies are currently in progress in our department.

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