Original Article

# Does Follicle-Stimulating Hormone Receptor Polymorphism Status Affect *In vitro* Fertilization-Intracytoplasmic Sperm Injection Results and Live Birth Rate? A Retrospective Study

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**Abstract**

**Background:** Follicle-stimulating hormone (FSH) plays a key role in fertility and shows its effect through the FSH receptor (FSHR), which is localized in cells. **Aims:** The aim of this study was to examine pregnancy outcomes and responses to controlled ovarian stimulation according to FSHR polymorphism types. **Study Setting and Design:** The study was retrospective, and included patients who applied to the University of Health Sciences Tepecik Training and Research Hospital in vitro fertilization (IVF) Unit during 2018 and 2019. **Materials and Methods:** Patients who underwent IVF-intracytoplasmic sperm injection and at the same time studied FSHR gene polymorphism in the genetic unit of our hospital were included in the study. **Statistical Analysis:** The Kruskal–Wallis test was used for multiple comparisons of continuous variables. The Chi-square test was used for categorical variables between groups. **Results:** A total of 143 patients who met our criteria were included in the study. 14% ($n$ = 20) of the patients are also homozygous natural (Asn/Asn) type; 44.7% ($n$ = 64) of the heterozygous mutant (Asn/Ser) type; 41.3% ($n$ = 59) of them were homozygous mutant (Ser/Ser) type. There was no statistically significant difference between the groups in terms of pregnancy rate per started cycle, ongoing pregnancy per started cycle, ongoing pregnancy per embryo transfer and live birth per embryo transfer. A significant difference was observed between peak E2 and peak progesterone levels between Asn/Ser and Ser/Ser groups, and the levels of these hormones were lower in the Ser/Ser group ($P$ = 0.018 and $P$ = 0.016, respectively). Ovarian responses were classified as poor (≤3 oocytes), normal (4-20 oocytes) and hyperresponse (≥20 oocytes) according to the oocyte count. Accordingly, the number of patients with poor response was higher in the Ser/Ser group ($P$ = 0.011). **Conclusions:** Ser/Ser polymorphism is characterised by a poor ovarian response. Despite this, polymorphisms in the FSHR gene do not seem to affect the results of pregnancy per started cycle, ongoing pregnancy per started cycle, ongoing pregnancy per embryo transfer and live birth per embryo transfer.

**Keywords:** *Assisted reproductive techniques, follicle-stimulating hormone receptor, in vitro fertilization, infertility, intracytoplasmic sperm injection, polymorphism*

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## Introduction

Follicle-stimulating hormone (FSH) is produced by the anterior pituitary gland in response to gonadotropin-releasing hormone (GnRH) released from the hypothalamus. FSH is essential for gonadal development, access to sexual maturity and oocyte development and maturation during fertility. FSH plays a key role in fertility and shows its effect through the FSH receptor (FSHR), which is localized in cells.[1,2] In the ovaries, the FSHR is expressed on granulosa cells.

Controlled ovarian hyperstimulation (COH) protocols applied during *in vitro* fertilization (IVF) are based on the use of gonadotropins. By using GnRH agonists and antagonists, premature ovulation and luteinization are prevented ahead of time and better control of the cycle is aimed. After COH, the goal is to get mature quality and enough follicles. In patients with similar characteristics, the unpredictability of different responses to COH has triggered pharmacogenetic research. Predicting the response of ovaries to COH is of great importance for the success of IVF. With pharmacogenetic studies, more than 1300 single-nucleotide polymorphisms (SNPs) have been detected on the FSHR gene in various ethnic groups, in encoded and non-coded regions.[3] Compared with Caucasians and Mediterraneans, Asians have a significantly lower variant of Ser680Ser polymorphism.[4] In recent years, the focus has been on Thr307Ala (rs6165) and Asn680Ser (rs6166), two polymorphisms thought to be important, and are the most studied polymorphisms.[2,3]

Polymorphisms in the FSHR gene have been shown to affect the results of COH.[5-9] On a molecular basis, perhaps the rates of conception,[10-12] ongoing pregnancy and live birth may also vary according to the types of polymorphism.[13-15] This study aims at examining cycle results and live birth rates according to FSHR polymorphism types in women who underwent IVF by the intracytoplasmic sperm injection (ICSI) method. In addition, basal hormone levels according to polymorphism types and ovarian responses to COH were also investigated in our study.

## Materials and Methods

### Study design

The study was retrospective, and the included patients who applied to the University of Health Sciences Tepecik Training and Research Hospital IVF Unit during 2018 and 2019, and underwent IVF-ICSI and at the same time studied FSHR gene polymorphism in the genetic unit of our hospital. Information and data were obtained from the hospital information management system and the medical records of the patients. The research was conducted in accordance with the 1964 Helsinki Declaration and its later amendments. Ethics committee approval for the study was obtained from the Ethics Committee of the University of Health Sciences Tepecik Training and Research Hospital (approval number: 2018/16-8). Written informed consent for future data use was routinely obtained from all participants at admission.

### Study participants

According to the inclusion criteria; the first IVF transfer was included, IVF application with ICSI technique, age between 18 and 40 years, body mass index (BMI) <30 kg/m$^2$, presence of both ovaries, normal karyotype of women and men were included in the study.

The exclusion criteria included; women who conceived with non-IVF-assisted reproductive techniques, women who had previous IVF transfer (without the first transfer), women who underwent conventional IVF technique (without ICSI method), diminished ovarian reserve and male infertility cases, patients with uterine anomaly (women with abnormal hysterosalpingography), and patients whose information could not be obtained and/or whose information was incomplete in the records.

On the twelfth day of transfer, β-hCG ≤5 mIU/ml was defined as negative pregnancy if β-hCG between 5 and 30 mIU/ml was defined as chemical pregnancy (net result: negative pregnancy) and β-hCG ≥30 mIU/ml was defined as positive pregnancy. Pregnancy losses before 12 weeks were defined as miscarriage, while pregnancies reaching 12 weeks and later as ongoing pregnancy. Newborns with a positive heart rate and a weight of ≥500 g were defined as live births.

### Controlled ovarian hyperstimulation and *in vitro* fertilization-ET protocols

During COH, patients who used GnRH antagonist or long GnRH agonist protocols were evaluated. Our IVF unit mainly uses antagonist protocol.

Following the GnRH antagonist protocol in our clinic; on the 2$^{nd}$ or 3$^{rd}$ day of the menstrual cycle, the number of antral follicles is evaluated by transvaginal ultrasound (TVUS). Blood samples are taken for basal FSH and E2. Considering the patient's age, BMI and the number of antral follicles (AF), 150–300 IU recombinant (GONAL-f ®; Merc-Serono, Darmstadt, Germany) or (Puregon ®; NV Organon, Oss, The Netherlands) or urinary (Fostimon ®; IBSA Institut Biochimique SA, Lugano, Switzerland) FSH is started. On the 6$^{th}$ day of the cycle, 0.25 mg cetrorelix (Cetrotide ®; Merc-Serono, Idron, France) is started. FSH and GnRH antagonists are continued until the day of the human chorionic gonadotrophin (hCG) administration.

Following the long GnRH agonist protocol in our clinic; the number of AF is evaluated by TVUS on the 2nd or 3rd days of the cycle before treatment. Blood samples are taken for basal FSH and E2. The patient is administered oral contraceptive tablet and 1 mg leuprolide acetate (Lucrin ®; AbbVie, Saint-Remy-sur-Avre, France) is given daily for downregulation from the 21st day of the same cycle. With menstruation, the leuprolide acetate dose is reduced by 50% on the 2nd day of the cycle, 225–300 IU recombinant (GONAL-f ®; Merc-Serono, Darmstadt, Germany) or (Puregon ®; NV Organon, Oss, The Netherlands) or urinary (Fostimon ®; IBSA Institut Biochimique SA, Lugano, Switzerland) FSH is started. FSH and leuprolide acetate are continued until the day of hCG administration.

When three or more follicles are ≥18 mm, ovulation is triggered by 10.000 IU hCG (Ovitrelle ®; Merc-Serono, Modugno (Bari), Italy). Oocyte pick up (OPU) is performed 36 h after hCG injection since OPU, the luteal phase is supplemented intravaginally with daily progesterone (Crinone 8% ®, Merc-Serono, Watford (Hertfordshire), United Kingdom). Embryo transfer is performed on the third or fifth day after OPU (one embryo for patients <35, two embryos for patients ≥35 years old). On the twelfth day of transfer, β-hCG ≤5 mIU/ml was defined as negative pregnancy if β-hCG between 5 and 30 mIU/ml was defined as chemical pregnancy (net result: negative pregnancy) and β-hCG ≥30 mIU/ml was defined as positive pregnancy. Daily progesterone is continued until the 11th week of pregnancy.

### Analysis of follicle-stimulating hormone receptor gene polymorphism

For DNA isolation, approximately 350 µl of peripheral blood is taken into sterile tubes containing K3EDTA and isolated with MagPurix Blood DNA Extraction Kit by MagPurix 12 Series (Zinexts Life Science Corp., Taiwan). Polymerase chain reaction (PCR) amplification was performed in a volume of 25 µl containing 100 ng of sample DNA. HelixAmp™ Ready-2x-MultiPlex v2.0 (Nanohelix Corp., South Korea) master mix was used: 25 nM of each primer and 1 U of Taq polymerase were added to the master mix. The amplification protocol conditions selected were as follows: Initial denaturation at 95ºC for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 45 s and a final extension at 72°C for 7 min. PCR products were purified as follows: 5 µl PCR products were treated with 2 µl of ExoSAP-IT enzyme (USB Affymetrix, USA) at 37 ºC for 30 min and at 80 ºC for 15 min. Sequence PCR (cycle sequencing) was done by reverse PCR primer (5 pmol) and BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA). The PCR conditions were as follows: At 96°C for 1 min, at 96°C 10 s, at 50°C for 5 s, at 60°C for 4 min the cycle was repeated 25 times. Products of sequence PCR were purified (second purification) by spin colon (ZR DNA Sequencing Clean-up Kit™, Zymo Research, USA). Sanger sequencing was performed by capillary electrophoresis after 5 min denaturation (3500 Genetic Analyzer, Life Technologies, USA). The resulting sequences were analysed using SeqScape® Software v3.0 (Applied Biosystems, USA).

### Statistical analysis

The Statistical Package for the Social Sciences 22.0 version (IBM Corporation, Armonk, New York, US) package programme was used in the analysis of the data. Numerical data are presented as mean ± (Standard deviation) or *n*,%. Qualitative data were calculated as a percentage. The Chi-square test was used for categorical variables between groups. The Kruskal–Wallis test was used for multiple comparisons of continuous variables. If a significant difference was found as a result of the analysis, the homogeneity of the variances was checked to determine between which groups the difference was. If the variances were homogeneous, the Scheffe test was used. In cases where the Scheffe test did not determine between which groups the difference was, the Bonferroni test was used. Tamhane T2 test was used if the variances were heterogeneous. A 95% significance level (or $\alpha = 0.05$ margin of error) was used to determine differences in the analyzes. The number of people required for this was made by power analysis with G-Power, which required a minimum of 19 people in each group.

## Results

A total of 143 patients who met our criteria were included in our study. Of these number, 14% ($n = 20$) of the patients were homozygous natural (Asn/Asn) type; 44.7% ($n = 64$) were heterozygous mutant (Asn/Ser) type; 41.3% ($n = 59$) of them were homozygous mutant (Ser/Ser) type.

The demographic and medical characteristics of the patients are presented in [Table 1]. In our study, no significant difference was observed between the groups in terms of female age, advanced female age (≥35 years), male partner age and BMI. The basal FSH, E2 and AF values (measured on the 2nd or 3rd day of the menstrual cycle) were statistically similar between the groups. Similarly, AF values were statistically similar between the groups.

The treatment characteristics of the patients are given in [Table 2]. Accordingly, a significant difference was observed between peak E2 (estradiol levels on

**Table 1: Demographic and medical characteristics of women involved in the study**

| | Asn/Asn (*n*=20; 14%) | Asn/Ser (*n*=64; 44.7%) | Ser/Ser (*n*=59; 41.3%) | *P* |
|---|---|---|---|---|
| Age (years), mean±SD | 30.7±5.1 | 33±4 | 31.6±4.6 | 0.071 |
| Advanced age ≥35, *n* (%) | 4 (20) | 25 (39) | 19 (32.2) | 0.277 |
| Male age (years), mean±SD | 35.3±5.6 | 35.5±5.3 | 35.7±4.4 | 0.928 |
| BMI (kg/m$^2$) | 23.3 | 23.8 | 22.8 | 0.987 |
| Infertility factor, *n* (%) | | | | 0.947 |
| Primary | 8 (40) | 28 (43.7) | 26 (44) | |
| Secondary | 12 (60) | 36 (56.3) | 33 (56) | |
| Antral follicle count, mean±SD | 10.3±2.61 | 9.9±2.41 | 9±2.30 | 0.117 |
| Basal FSH (IU/l), mean±SD | 9.69±5.26 | 8.37±3.40 | 9.91±4.18 | 0.128 |
| Basal E2 (pg/ml), mean±SD | 42.55±22.17 | 49.85±35.59 | 51.44±53.42 | 0.720 |

SD=Standard deviation, BMI=Body mass index, FSH=Follicle-stimulating hormone

**Table 2: Treatment characteristics of women participating in the study**

| | Asn/Asn (*n*=20; 14%) | Asn/Ser (*n*=64; 44.7%) | Ser/Ser (*n*=59; 41.3%) | *P* |
|---|---|---|---|---|
| Ovarian stimulation protocol, *n* (%) | | | | |
| Long GnRH agonist | 1 (5) | 3 (4.7) | 3 (5.1) | 0.994 |
| GnRH antagonist | 19 (95) | 61 (95.3) | 56 (94.9) | |
| Days of FSH stimulation (day), mean±SD | 8±0.8 | 8.2±1.8 | 8.5±1.4 | 0.362 |
| Total dose of FSH (IU), mean±SD | 1875±345 | 2100±515 | 2150±550 | 0.121 |
| Peak E2=hCG injection day estradiol (pg/ml), mean±SD | 1633±1150 | 2177±1379[b] | 1561±1111[b] | 0.018 |
| Peak P=hCG injection day progesterone (ng/ml), mean±SD | 0.93±0.52 | 1.33±0.92[b] | 1.01±0.40[b] | 0.016 |
| E2 level on OPU day (pg/ml), mean±SD | 1025±700 | 1412±1112[b] | 939±712[b] | 0.014 |
| Endometrium thickness on transfer day (mm) | 10.2±1.9 | 9±1.3 | 10.1±1.1 | 0.856 |
| Oocyte count retrieved in the OPU, mean±SD | 9.4±7.9 | 10.8±6.3[b] | 7.4±5.1[b] | 0.009 |
| Ovarian response, *n* (%) | | | | |
| Poor response (≤3 retrieved oocytes) | 3 (15) | 3 (4.7)[b] | 13 (22)[b] | 0.011 |
| Normal response (4-20 retrieved oocytes) | 15 (75) | 55 (85.9) | 45 (76.3) | 0.323 |
| Hyperresponse (≥20 retrieved oocytes) | 2 (10) | 6 (9.4) | 1 (1.7) | 0.164 |

[b]The difference between Asn/Ser group and Ser/Ser group is significant. SD=Standard deviation, FSH=Follicle-stimulating hormone, OPU=Oocyte pick up, hCG=Human chorionic gonadotrophin, GnRH=Gonadotropin-releasing hormone

the day of hCG injection), and peak progesterone (progesterone levels on the day of hCG injection) levels between Asn/Ser and Ser/Ser groups, and the levels of these hormones were lower in the Ser/Ser group ($P$ = 0.018 and $P$ = 0.016, respectively). The E2 level measured on the day of OPU was significantly lower in the Ser/Ser group compared to the Asn/Ser group ($P$ = 0.014). The number of oocytes collected on the day of OPU was the lowest in the Ser/Ser group, and the results were statistically significant ($P$ = 0.009). Ovarian responses were classified as poor (≤3 oocytes), normal (4–20 oocytes) and hyperresponse (≥20 oocytes) according to the oocyte count. Accordingly, the number of patients with poor response was higher in the Ser/Ser group ($P$ = 0.011).

The treatment results of the patients are examined in [Table 3]. Accordingly, there was no statistically significant difference between the groups in terms of pregnancy rate per started cycle, ongoing pregnancy per started cycle, ongoing pregnancy per embryo transfer and live birth per embryo transfer.

## Discussion

In this study, clinical parameters, responses to COH and pregnancy outcomes of 143 patients who underwent IVF-ICSI were compared according to Asn680Ser polymorphism types. There was no statistically significant difference between the groups in terms of pregnancy rate per started cycle, ongoing pregnancy per started cycle, ongoing pregnancy per embryo transfer and live birth per embryo transfer.

There are 3.2 billion base pairs in the human genome, and the structure of the genome shows a 99.9% similarity between individuals.[16] SNPs are largely responsible for the 0.1% difference.[16] SNPs provide different results in the same environment. The population frequency of a particular polymorphism may vary by gender, race/ethnicity and geographic location. The FSHR gene is a 54 kb-sized single-copy gene. It is localized on chromosome 2p21-16.[1,2] Many SNPs have been detected on the FSHR gene in encoded and non-encoded regions.[3] In recent years, the focus has been on two polymorphisms that are thought to be important: Thr307Ala (rs6165)

**Table 3: Treatment outcomes of women participating in the study**

| | Asn/Asn (*n*=20; 14%), *n* (%) | Asn/Ser (*n*=64; 44.7%), *n* (%) | Ser/Ser (*n*=59; 41.3%), *n* (%) | *P* |
|---|---|---|---|---|
| Pregnancy per started cycle | 25 (5/20) | 34.3 (22/64) | 22 (13/59) | 0.297 |
| Ongoing pregnancy per started cycle | 10 (2/20) | 20.3 (13/64) | 16.9 (10/59) | 0.564 |
| Ongoing pregnancy per embryo transfer | 8 (2/25) | 16.25 (13/80) | 14.7 (10/68) | 0.590 |
| Live birth per embriyo transfer | 8 (2/25) | 15 (12/80) | 14.7 (9/68) | 0.667 |

and Asn680Ser (rs6166), and these polymorphisms are the most studied types.[2,3] The presence of both polymorphisms on exon 10 causes a strong linkage disequilibrium (non-random combination of alleles) between them.[7] The fact that both polymorphisms are located in the same exonic region, combinations of p. Thr307-p. Asn680 and p. Ala307-p. Ser680 are inherited together, they bind to each other during recombination and do not show random distribution.[7] For this reason, we have focussed on only Asn680Ser polymorphism in this study.

Behre *et al*. prospectively evaluated the patient group who underwent conventional IVF and IVF-ICSI in their study in 2005. Accordingly, in terms of clinical pregnancy rate, all three groups were statistically similar, similar to our study.[10] Loutradis *et al*. in their prospective study in 2006, performed conventional IVF and IVF-ICSI on their patients, found no statistically significant difference between the groups in terms of clinical pregnancy rate; similar to our study.[11] On the other hand, in the prospective study of Jun *et al*. in which they performed IVF-ICSI on their patients in 2006, the clinical pregnancy rate per transfer was significantly higher in the Asn/Asn group. In the same study, the lowest clinical pregnancy rate was observed in the Ser/Ser group.[12] In 2006, Klinkert *et al*. enrolled only patients who underwent conventional IVF in their prospective study. They investigated pregnancy rates per started cycle, pregnancy rates per transfer and implantation rates per embryo transferred. In each of these three parameters, it was found that women with the Ser/Ser genotype were significantly higher than women with the Asn/Asn genotype.[13] In a retrospective study in 2019, Lindgren *et al*. examined women undergoing conventional IVF and IVF-ICSI. In the study where they examined 5-cycle follow-up, they did not observe any statistically significant difference between the groups in the first cycle and in all other cycles in terms of live birth rate according to FSHR types.[14] These results are similar to our study. In 2019, König *et al*. analysed women who underwent conventional IVF and IVF-ICSI.[15] According to FSHR polymorphism types, while the Asn/Asn group was the lowest in terms of ongoing pregnancy per started cycle, ongoing pregnancy per embryo transfer and live birth per embryo transfer, the Asn/Ser group was the highest. In our study, the live birth rates in the Asn/Asn group were numerically lower, similar to the two studies,[13,15] but unlike these studies, the results are not statistically significant. There may be various reasons for obtaining different results in many studies conducted so far. These differences may arise from the fact that the studies are conducted in different ethnic groups, the application of different stimulation protocols, or the study designs are different.

In this study, the basal FSH levels were statistically similar between the groups. In the literature, although there are those who argue that the basal FSH value is higher in the Ser/Ser group,[5-7,11] recent studies[4,8,9,17] argue that the difference between groups are statistically similar. Similar to our findings, other studies have not found any difference in the AF between different groups with FSHR.[5,13,15]

We observed the peak E2 level was highest in the Asn/Ser group and the lowest in the Ser/Ser group ($P$ = 0.018). Although the peak E2 value was the lowest in the Ser/Ser group in some studies, the difference between the groups was not statistically significant.[5,12,15] In one study, peak E2 was the lowest in the Asn/Ser group, but the differences between the groups were not significant.[7] In another study, the peak E2 value was highest in the Ser/Ser group, but the result was still not statistically significant.[13] Some studies,[5,10] were similar to ours, where the peak E2 level was at the highest in the Asn/Ser group and the lowest in the Ser/Ser group, and the results were statistically significant. In addition, for the first time in the literature, we examined the E2 level before OPU, which was significantly higher in the Asn/Ser group ($P$ = 0.014). Similarly, the number of oocytes collected on the day of OPU is higher in the Asn/Ser group ($P$ = 0.009).

We divided the patients into three groups; poor, normal and hyper-responders according to their oocyte count. When evaluated depending on the genotype distribution, the number of patients with poor responses was significantly higher in the Ser/Ser group ($P$ = 0.011). There are studies in the literature that have found similar results to ours,[4,10] as well as studies arguing that all groups are similar in terms of poor response.[11,13]

Our limitations are; our study is a retrospective study and with the limited number of patients. The stimulation protocols we used were not uniform. Polymorphisms may vary by race/ethnicity and geographic location, and we were only able to present local data. Our strengths are that our patient selection criteria are very strict, and we have minimised the difference between groups by including only ICSI cycles.

As a result, we can say that; although the Ser/Ser polymorphism is characterised by a poor ovarian response, polymorphisms in the FSHR gene do not seem to affect the results of pregnancy per started cycle, ongoing pregnancy per started cycle, ongoing pregnancy per embryo transfer and live birth per embryo transfer. The results, however, pave the way for new studies, randomised controlled prospective and multi-centre studies are needed utilising FSHR polymorphism as a confounder for the ovarian response.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

### Data availability statement

The data supporting this study is available through the corresponding author upon reasonable request.

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