Original Paper

Ala307Thr and Asn680Ser Polymorphisms of *FSHR* Gene in Human Reproduction Outcomes

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Key Words

Assisted reproduction treatment • Controlled ovarian hyper stimulation • Infertility • FSH receptor • Polymorphisms

Abstract

Background/Aims: It is known that some markers of ovarian stimulation can help to personalize the treatment, adjusting the dose of exogenous rFSH, thus preventing excessive wear of the patient. We aimed to evaluate Ala307Thr and Asn680Ser genotypes of the *FSHR* gene in infertile women and correlate the findings with the results of ovarian response and assisted reproduction outcomes. **Methods:** Cross-sectional study covering 149 infertile women submitted to assisted reproduction treatment. Genotyping of *FSHR* variants were performed using TaqMan methodology by real time PCR. FSH and estradiol were measured by ELFA. The data was analyzed statistically. **Results:** The frequencies of the *FSHR* Ala307Thr and Asn680Ser genotypes considering the ovarian hyper stimulation response also did not differ statistically. Considering assisted reproduction outcomes, we observed that the polymorphism Ala307Thr have a statistical difference for the number of MII oocytes and embryos ($p=0.051$ and $p=0.037$, respectively), which the genotype Ala/Ala showed more embryos. The polymorphisms did not determine the FSH and estradiol serum levels and the ovarian response in the assisted reproduction treatment. **Conclusions:** The polymorphisms Ala307Thr and Asn680Ser did not determine the FSH and estradiol serum levels and the ovarian response in the assisted reproduction treatment. However, we observed that the Ala307Thr may influence the number of embryos produced.

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C. Martins Trevisan and C. Peluso have the same importance in the manuscript production.

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Introduction

Follicle stimulating hormone (FSH) is a glycoprotein secreted by the anterior pituitary [1]. It is essential for women and plays a role in the follicle development, oocyte maturation, steroidogenesis regulation, proliferation of granulosa cells and induces synthesis of the androgen-converting enzyme aromatase [2-5].

The FSH acts via FSH receptor (FSHR), which is a trans-membrane glycoprotein, and it is expressed on granulosa cells in the ovary [6]. This receptor is synthesized by a single-copy gene, located in the region 2p21-16 [7]. This gene is 192 kb in size, consists of 10 exons and 9 introns [8, 9]. Most of the extracellular domain is encoded by 9 exons; the C-terminal part of the extracellular domain, the transmembrane domain, and the intracellular domain are encoded by the large exon 10 (1234 bp) [10]. In recent times, naturally mutations in the *FSH* and *FSHR* genes have been reported, and until now there are more than 900 SNPs (Single nucleotide polymorphism) [11], two more commonly studied in *FSHR*, position 307 that changes an alanine to threonine (Ala307Thr) and position 680 that switches an asparagine to serine (Asn680Ser) [12].

In vitro fertilization (IVF) is a complex multistep process that comprises collection of oocyte-containing follicles after controlled ovarian hyper stimulation (COH) with rFSH (recombinant Follicle-stimulation Hormone), oocyte fertilization, embryo development, embryo transfer to the uterus, and implantation. All these steps are critical for successful IVF. However, the initial critical step of this complex procedure is the COH, which aim is to safely obtain a high number of mature oocytes, as well as to allow the selection of the most viable embryo for transfer [13, 14].

The gonadotropins are used for COH in order to increase the number of follicles and to predict if the ovarian response is difficult, beyond that an important interindividual variability is observed. Poor ovarian response results in cycle cancellation [15], ovarian hyper stimulation syndrome, likewise, may result in cycle cancellation due to the risk of ovarian enlargement and abdominal fluid overflow [16]. Because of that, there has been increasing interest in identify and the relative performance in ovarian reserve tests, prior embarking on COH [17].

Several parameters have been postulated as predictors of ovarian response. Since the ovarian function cannot be measured directly, the use of serum markers [FSH, inhibin B, 17- β -estradiol and anti-Müllerian hormone (AMH)] and/or ultrasound variables [ovarian volume, measurement of antral follicles, ovarian stromal blood flow] have been proven to be useful, although limited [17]. For some authors, these markers do not reflect the complex follicular dynamics, and none of them shows strong correspondence with the population of primordial follicles that remain in the gonad [18]. However, among these markers predictors of ovarian reserve the AMH and antral follicle counting (AFC) seems to be the best biomarkers. According to Peluso et al. [19], AMH and AFC show strong association with primordial follicle pool, serving as good biomarker of excessive controlled ovarian hyper stimulation response and they are useful to individualized the appropriate dose of rFSH, yet, they do not predict pregnancy rates and performing both together, it does not increase the predictive power of controlled hyper stimulation response and assisted reproduction outcomes.

Nevertheless, the FSH level on the day 3 of menstrual cycle remains, the most widely used biomarker due to its low cost, although, the genetic background of individuals seems to determine the response of patients to rFSH stimulation better than the stimulation design [20]. The response to FSH stimulation is determined by the number of follicles and their sizes, if there is a suspicion of OHSS, the dosage of estradiol is performed. The adequate response is determined by the presence of at least 4 follicles with at least 14 millimetres in size.

Consequently, the variants of *FSHR* were explored and they may be involved in the role of FSH receptor in mediated signal transduction and with ovarian response in infertile women submitted to ovarian stimulation [21, 22]. Perez Mayorga et al. [20] was the first

group that observed differences in Asn680Ser genotypes with dose of rFSH in COH. Other groups observed that the *FSHR* variants influence the basal FSH and estradiol levels with conflicting results [23-27], for this reason, the *FSHR* gene and others gene variations may play a role in modulating receptor sensitivity and intracellular second messenger cascades to exogenous hCG and other gonadotrophins [24, 28], thus the *FSHR* SNPs may cause changes in the phosphorylation and glycosylation molecular mechanisms [29, 30].

Based on this information, we believe that the polymorphisms of *FSHR* may be helpful to explain the assisted reproduction technique (ART), outcomes and ovarian responses in COH. Therefore, we aimed to evaluate the polymorphisms Ala307Thr and Asn680Ser of the *FSHR* gene in Brazilian infertile.

Material and Methods

Subjects

This is a prospective cross sectional study that included 149 infertile women, (32.4 ± 3.5 years old) they were submitted to the first high complexity assisted reproduction treatment from Human Reproduction and Genetics Center of the Faculdade de Medicina do ABC, Santo André, Brazil, between September 2011 and September 2013. Only women with infertility caused by male factor ($n=93$) or tubal factor ($n=56$) were included in this study. All patients were younger than 38 years old ($\leq 38y$), with normal serum levels of basal FSH [≤ 10.0 IU/ml], TSH [<4 mIU/L] and prolactin [<25 ng/ml], presence of both ovaries, without any morphological abnormalities, normal ovulatory cycles (25-35 days), body mass index (BMI) ≤ 30 , no history of poor response and no evidences of endocrine diseases, such as polycystic ovary syndrome. It was excluded from the study, patients with moderate/severe endometriosis (stage III and IV), and previous history of ovarian surgery or underwent chemo/radiotherapy, low-complexity protocols, and also cases with severe male factor that underwent invasive procedures for recovery sperm.

Anatomic tubal abnormalities preventing the proper functioning of the tubes, such as tubal obstruction, functional changes caused by pelvic inflammatory disease, endometriosis, or previous tubal surgery were considered tube peritoneal factors. These abnormalities were diagnosed by hysterosalpingography and/or laparoscopy.

Male factor was classified when a patient's partner presented an initial concentration of less than 15 million sperm/ml, 5 million/ml rapid progressive after sperm processing, or asthenospermia [less than 40% of motile spermatozoa considering fast progressive or nonprogressive or less than 32% if we consider only the rapid progressive sperms], according to the World Health Organization (WHO, 2010) [31].

Clinical data and peripheral blood samples were collected only after explaining the study aims and obtaining a signed informed acquiescence form, as approved by the local Research Ethics Committee.

Ovarian Stimulation

Ovulation was induced by rFSH an initial daily use of 100 IU which was administered for 10 days, starting on the second day of menstruation. From the 6th to the 10th day, the GnRH antagonist (Orgalutran) was also administered. Between day 10th and 11st, when the follicles reached a diameter of approximately 17 mm, as determined by transvaginal ultrasound, the patients were given human chorionic gonadotropin [hCG], we used the referred size because the follicle has a higher chance of getting a mature oocyte (metaphase II oocytes - MII) after the hCG administration, and on the 13rd day the oocyte retrieval was performed [32]. This protocol is variable, when the bigger follicle achieves at least 14mm, the GnRH antagonist administration happens and the oocyte retrieval is performed 36 hours after hCG dispensation. This is fulfilled when the follicles have approximately 17 mm, and may be occur from the 6th to the 12th day of the medication [32].

As ovarian response, we considered: i) ovarian Hyper stimulation syndrome (OHSS), featured by multiple ovarian follicles (≥ 20 follicles) together with possible clinical symptoms, such as ascite, hematological changes (hemo concentration), pleural effusion, liver and/or coagulation abnormalities, according to the classification proposed by Golan et al. [33], besides ≥ 4000 IU of serum estradiol; ii) Hyper response, when after 6 days of ovarian stimulation with gonadotropins, there was the development of $\geq 12 \leq 19$ follicles, without clinical symptoms of OHSS; iii) Poor response, when after 6 days of ovarian stimulation with gonadotropins only up to 3 follicles smaller than 14 mm developed; and iv) Satisfactory response, when after 6 days of ovarian stimulation with gonadotropins 4 to 12 follicles larger than 14 mm developed.

Embryo transfer

Embryo transfer in a maximum of four embryos, as indicated by the Brazilian Federal Council of Medicine, was performed on the third day after fertilization. The luteal phase support was made by vaginal progesterone at a dose of 600 mg/day, starting on the ovarian puncture day.

Clinical Pregnancy

The pregnancy is confirmed by serum beta hCG fraction (β hCG) on the 12nd day after embryo transfer. The clinical pregnancy is considered, when it is identified intrauterine gestational sac by transvaginal ultrasound from the fourth or fifth week of gestation.

Hormonal Measurements

Basal FSH and estradiol levels were obtained and measured on day 3 of the menstrual cycle by radioimmunoassay using commercial RIA kits (ELFA, Enzyme Linked Fluorescent Assay, Mini-Vidas FSH and Mini-Vidas ESTRADIOL II -BioMerieux, Hazelwood, Missouri).

Genotyping

Five millilitre of peripheral blood was collected in an EDTA-containing tube and genomic DNA was extracted from lymphocytes according to salting out method (Lahiri and Nunberger) [34]. *FSHR* polymorphisms detection (919G>A/Ala307Thr/rs6165 and 2039A>G/Asn680Ser/rs6166) was performed using TaqMan system by real time polymerase chain reaction (PCR), with commercially available primers and probes (rs6165- Context Sequence [VIC/FAM] GCCAGAGAG GATCTCTGACCCCTAG[C/T]CTGAGTCATATAATCAACTTCTTGC and rs6166 - Context Sequence [VIC/FAM] AGGGACAAGTATGTAAGTGGAACCA[C/T]TGGTGACTCTGGGAGCTGAAGAGCA) available by Life Technologies (Foster City, CA, USA). Assays were performed with TaqMan Genotyping Master Mix with 50 to 100 ng of DNA per reaction. PCR conditions were 40 denaturation cycles of 15 seconds at 95°C and 1 minute of annealing/extension at 60°C.

Statistical Analysis

Statistical analysis was realized using SPSSv18.0 (Statistical Package for Social Sciences version 18.0). The Chi-squared was used to verify the Hardy-Weinberg equilibrium of *FSHR* polymorphisms, and also to certify the associations between polymorphisms and the variables ovarian stimulation and pregnancy, as well as the Fisher test. The Mann-Whitney test or T test was used to examine the polymorphisms effects as dominant and recessive genetics models to the variables estradiol (E2), FSH, follicles visualized at ultrasonography (USG), oocytes retrieved, MII, injected oocytes, embryos, embryo transfer and frozen embryo. The statistical level considered was $p < 0.05$ or 5%.

Results

Among the 149 patients, the mean serum FSH concentration was 6.18 ± 1.8 mIU/mL, and the estradiol serum level was 43.0 ± 11.1 mIU/mL. The association of serum FSH and serum estradiol with Ala307Thr polymorphism were not statistically significant, $p = 0.402$; $p = 0.381$, respectively (Table 1). The same was observed for Asn680Ser $p = 0.607$; $p = 0.957$, respectively (Table 2).

The frequencies of the *FSHR* genotypes at position 307 were: 24.2% (36/149) Ala/Ala; 44.9% (67/149) Ala/Thr and 30.9% (46/149) Thr/Thr, and at position 680 were: 36.9% (55/149) Asn/Asn; 42.3% (63/149); and 20.8% (31/149).

When we analyzed the ovarian stimulation, we noticed that 33.6% (50/149) was poor responder; 57% (85/149) had an expected response with 4–12 follicles; 4.7% (7/149) were hyper responder and 4.7% (7/149) suffered the ovarian hyper stimulation syndrome. Comparing with the polymorphisms we did not watched a statistically significant difference for any of these groups, for Ala307Thr and Asn680Ser, $p = 0.460$ and $p = 0.610$, respectively.

When we analyzed the assisted reproduction technique (ART) outcomes, we detected that the polymorphism Ala307Thr has a statistical difference for the number of embryos,

Table 1. Analyse of serum basal FSH, estradiol levels and ART outcomes according to Ala307Thr genotypes. N – number of individuals; SD – standard deviation; *Kruskal-Wallis Test

Ala307Thr	Ala/Ala				Ala/Thr				Thr/Thr				p*
	n	Mean	SD	Median	n	Mean	SD	Median	n	Mean	SD	Median	
FSH	36	6.41	1.96	6.24	66	6.49	1.73	6.51	45	6.0	2.0	5.72	0.402
E2	35	43.7	10.96	41.79	66	44.72	11.89	42.93	46	46.42	10.23	44.35	0.381
USG follicles	36	6.72	5.11	5.5	67	5.84	4.60	5	46	7.93	6.87	6	0.361
Retrieved Oocytes	36	5.92	4.43	5	67	4.87	4.11	4	46	7.24	6.17	5.5	0.092
MII	31	6.29	4.65	6	51	4.73	4.0	3	41	5.73	4.0	5	0.051
Injected Oocytes	30	6.13	4.74	5	49	4.5	3.6	3	40	5.35	4.24	5	0.128
Embryos	27	3.3	1.8	3	49	2.53	2.15	2	39	2.59	1.69	2	0.037
Transferred Embryo	20	1.9	0.44	2	46	1.7	0.62	2	34	1.62	0.69	2	0.187
Frozen Embryo	24	0.79	2	0	43	0.53	1.7	0	41	1.66	3.39	0	0.125

Table 2. Analyse of serum basal FSH, estradiol levels and ART outcomes according to Asn680Ser genotypes. N – number of individuals; SD – standard deviation; *Kruskal-Wallis Test

Asn680Ser	Asn/Asn				Asn/Ser				Ser/Ser				p*
	n	Mean	SD	Median	n	Mean	SD	Median	n	Mean	SD	Median	
FSH	53	6.12	1.93	6.16	63	6.47	1.76	6.32	31	6.37	2.04	6.18	0.607
E2	55	45.24	11.43	43.31	62	44.92	11.68	43.96	30	44.79	9.76	41.97	0.957
USG follicles	55	7.27	6.54	5	63	5.98	4.46	5	31	7.13	5.66	6	0.738
Retrieved Oocytes	55	6.58	5.92	5	63	5.02	3.90	4	31	6.26	5.0	5	0.428
MII	48	5.6	4.10	5	49	5.0	3.86	4	26	6.04	5.09	5.5	0.487
Injected Oocytes	47	5.34	4.16	5	47	4.74	3.52	4	25	5.96	5.18	5	0.552
Embryos	46	2.85	2.25	2	46	2.39	1.48	2	23	3.17	2.05	3	0.201
Transferred Embryo	41	1.66	0.65	2	42	1.71	0.63	2	17	1.82	0.52	2	0.603
Frozen Embryo	44	1.61	3.30	0	44	0.50	1.66	0	20	0.85	2.15	0	0.099

p=0.037, which the genotype Ala/Ala showed more embryos than Ala/Thr (Table 1). This result was endorsed in a dominant model, where the genotype Ala/Ala showed more embryos than Ala/Thr and Thr/Thr, p=0.012. The variant 307 also demonstrated a statistical trend with MII oocytes. The genotype Ala/Ala displayed more MII oocytes than Ala/Thr and Thr/Thr, p=0.051.

About the others ART outcomes, such as follicles visualized on ultrasound, retrieved oocytes, injected oocytes, embryos transferred and frozen embryos, we compared with the polymorphism Ala307Thr and we did not apprehended a statistically significant difference for divergent genotypes (Table 1), the same was observed with Asn680Ser (Table 2). The clinical gestation association with Ala307Thr and Asn680Ser was not statistically significant, p= 0.294 and p= 0.956, respectively.

Discussion

Allelic variants of *FSHR* determine different *FSHR* sensitivity's [20, 24, 26]. Because of that we expected to find that *FSHR*'s variants influence in the reproduction outcomes, in our results we observed that the number of MII oocytes and embryos are higher in Ala307Ala women. But the other clinical parameters we did not detected differences in Ala307Thr and Asn680Ser genotypes.

In 2013, a study with 450 Chinese infertile women showed that the polymorphisms Ala307Thr and Asp680Ser were associated with the ovarian response to FSH [25]. In this study the ovarian response was classified in poor response (less than five oocytes), normal response (between five and 14) and high response (more than 14 oocytes). Stimulation was induced using gonadotrophin hormone agonist and an empirical dose of Urofollitropin, depending on the serum estradiol levels. The genotypes Ala/Ala at the position 307 and Ser/Ser at the position 680 revealed higher level of basal FSH and higher rates of poor response,

Table 3. Brief summary of the literature about Ala307Thr and Asn680Ser in assisted reproduction treatment (ART) - analyses with ovarian response and ART outcomes

Study	Ethnic	Clinical Parameters	Findings	Conclusion
Perez et al. [20]	161 infertile German women	Ser/Ser needed the lowest estradiol levels and basal FSH level were significantly different among the genotypes.	The dose of FSH required for successful stimulation was significantly different among the three genotypes.	Ovarian response to FHS stimulation depends on <i>FSHR</i> genotype.
Jun et al. [23]	263 infertile Korean women	Basal FSH levels were significantly higher for the Ser/Ser variant. Whereas, estradiol levels were not significantly different among the genotypes.	The variant Ser/Ser showed fewer oocytes. Clinical pregnancy rate was significantly higher in the Asn/Asn group.	In an Asian population, the polymorphism Asn680Ser may be associated with a different ovarian response to controlled ovarian Hyperstimulation.
Achrekar et al. [35]	50 infertile Indian women and 100 controls	Thr/Thr required maximum amount of the exogenous FSH. The variant Ala/Ala had significantly higher levels of estradiol. For the position 680, no differences were observed.	For the polymorphism 307, 80% of the patients that suffered OHSS had homozygous or heterozygous Ala allele.	The ovarian response to FSH stimulation may depend on FSH receptor genotype.
Yan et al. [25]	450 Chinese women	Basal FSH levels were higher for the variant Ala/Ala at 307 position, and for variant Ser/Ser at 680 position.	The variants Ser/Ser and Ala/Ala had significantly higher rates of poor response.	The genotypes Ala/Ala and Ser/Ser, require longer stimulation.
Lledo et al. [42]	145 Spanish oocyte donors.	The variant Ser/Ser showed fewer AFC and needed higher doses of rFSH to COH.	In a population of fertile egg donors, the <i>FSHR</i> gene polymorphism at position 680 is associated with different ovarian responses to COH.	The genotype of the <i>FSHR</i> gene is an important factor to determine the prognosis of the COH cycles on fertile women with normal ovulation.
Present Study	149 Infertile Brazilian women	Basal FSH and Estradiol levels were not correlated with genotypes of polymorphism at position 307, neither for the position 680	The variant Ala/Ala had more embryos than the Ala/Thr and Thr/Thr genotypes.	In Brazilian population <i>FSHR</i> polymorphism do not seem to be associated with ovarian response and assisted reproductive outcomes.

however, there was no genotypes association and OHSS, neither at position 307 or 680 [25].

For position 307 the genotype Ala/Ala in Indian women, seems to need a low amount of FSH for ovarian stimulation, moreover, this genotype had an increased risk of developing the ovarian hyper stimulation syndrome (OHSS) [35]. Another study, in United Kingdom, with 421 infertility women that investigated the association of Ala307Thr and Asn680Ser polymorphisms with markers of ovarian reserve (AMH, antral follicle count (AFC), and basal FSH) did not observed differences between the genotypes [36].

For position 680, Jun et al. [23] demonstrated women with Ser/Ser variant had significantly higher basal FSH levels, and required higher doses of exogenous FSH for stimulation. In other studies with German women, this same Ser/Ser variant showed the lowest estradiol levels [20, 37], the same was observed in a study with human granulosa cells (CG) [38].

The studies about *FSHR* are controversial, and in our study we did not find a statistically significant difference with ovarian stimulation and different genotypes (Table 3). Because of that, we believed that *FSHR* variants may interfere in the FSH sensitivity, however, differently in each population. Nevertheless, we observed that the number of oocytes MII and embryos is different in genotypes of Ala307Thr.

These differences may be explained by the distribution of 307 and 680 *FSHR* SNPs, which have a great variability, when they are compared in different populations and even in populations that are considered to be similar of the genetic background, they show different frequencies [39]. About the Asn680Ser, Jun et al. [23] it was realized that women who are Ser680Ser, showed lower estradiol levels and fewer captured oocytes than other genotypes. Similarly, Greb et al. [40] observed that Ser680Ser had higher FSH levels, and they suggest that higher FSH levels are necessary to produce the same estradiol concentrations in Ser680Ser.

Although the literature shows such correlations with hormonal measurements and ART's outcomes, we did not find the same results. We must say that, this is possible due to the high genetic variability of the Brazilian natives and the other studies were conducted in European and Asian populations (Table 3).

La Marca [29] reports the association between *FSHR* SNPs and serum day 3 FSH concentration, probably, depends on the ethnic group and in any event is not so strong as previously believed, since several studies have failed to demonstrate this association.

Regarding pregnancy rates, we did not detect differences according to *FSHR* Ala307Thr and Asn680Ser genotypes. Other groups also did not find any correlation between *FSHR* variants and pregnancy [41-45]. However, Jun et al. [23] showed that patients with Asn/Asn genotype had the clinical pregnancy rate significantly higher than the others. Another study achieved that patients with Ser/Ser polymorphism had implantation and pregnancy rates that were three times higher compared with patients with polymorphism Asn/Asn [46]. These results demonstrated that the clinical pregnancy has controversial results, as well as the reproductive outcomes.

This present study is important, because we helped to identify the effect of *FSHR* polymorphisms in Brazilian population. And we observed that Brazilians do not have different ovarian responses when compared with Ala307Thr and Asn680Ser genotypes, which were different in other populations. Therefore, it is necessary to investigate others forms to try to predict the ovarian response in reproductive treatments.

A major limitation of our study is the relatively low number of patients. Besides, only two SNPs were genotyped for *FSHR* gene, and these markers may not provide full coverage for genetic test, although these markers are most commonly studied.

However, the small number of studied patients is due to selection criteria, once all the patients included in this study had only women with infertility caused by male factor or tubal factor; they were younger than 38 years old, with normal serum levels of basal FSH, TSH and prolactin, presence of both ovaries without any morphological abnormalities, normal ovulatory cycles, BMI ≤ 30 , no previous history of poor response and no evidences of endocrine diseases. Moreover, we excluded from the study patients with moderate/severe endometriosis (stage III and IV) and cases with severe male factor that underwent invasive procedures for recovery sperm.

Conclusion

Concisely, in Brazilian studied population, the polymorphisms Ala307Thr and Asn680Ser did not determine the FSH and estradiol serum levels and the ovarian response in the assisted reproduction treatment. However, we observed that the Ala307Thr may influence the number of MII oocytes and embryos produced.

Disclosure Statement

The authors declare no conflict of interest.

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