

Luteal phase stimulation in double ovarian stimulation cycles is not affected by the follicle-stimulating hormone (FSH) receptor genotype: double ovarian stimulation is beneficial independently of the genotype at position 680 of the follicle-stimulating hormone receptor

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Objectives To determine whether follicle-stimulating hormone receptor (FSHR) genotype influences the outcome of ovarian stimulation treatment in luteal phase.

Methods A total of 299 patients were included in a retrospective study between July 2017 and December 2021. These patients carried out a double stimulation protocol and the variant Asn680Ser (rs6166; c.2039A>G) of FSH receptor was genotyped either as part of the pre-treatment fertility tests or for the current study. Patients undergoing a double stimulation treatment who could not be genotyped were excluded from this analysis.

Results The results obtained from ovarian stimulation in luteal phase were better than those obtained in conventional follicular phase. Statistically significant differences ($P < 0.001$) were found in the number of retrieved oocytes (5.47 vs. 4.18), retrieved MII (4.52 vs. 3.29) and fertilised oocytes (3.81 vs. 2.20). Furthermore, these differences remained regardless of the FSH receptor genotype for the 680 position in all groups ($P < 0.05$). In addition, stimulation in luteal phase lasts longer and requires more gonadotropins than in follicular phase. This is especially noteworthy in patients with Ser/

Ser genotype, who required a slightly higher dose of gonadotropins compared to other genotypes in luteal phase, as previously observed in the follicular phase for this genotype. No significant differences in age, anti-Müllerian hormone levels, antral follicle count, BMI and type of trigger used in luteal phase were observed among groups of patients with different FSH receptor genotypes.

Conclusion All patients undergoing IVF seem to benefit from luteal phase ovarian stimulation, regardless of their FSHR genotype. *Pharmacogenetics and Genomics XXX: XXXX-XXXX* Copyright © 2023 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

In recent years, an increasingly higher number of patients were drawn to assisted reproduction techniques in order to fulfil their desire to become parents. The increment in sterility rates, which are close to 15% of the theoretical couples population worldwide [1], is caused by biological changes resulting from modifications in social and living habits, advanced maternal age and the social acceptance of new family models, among others. These facts make many women require oocyte donation to become mothers due to their low ovarian reserve, which limits the stimulation performance. In these cases, a tailored treatment can make the difference between achieving pregnancy with their own oocytes or not.

To solve this problem, several approaches have been used, that is, double ovarian stimulation and the application of

pharmacogenetics, especially regarding the follicle-stimulating hormone receptor (FSHR). This receptor, which is the target of the follicle-stimulating hormone and mediates its action in the organism, belongs to the G protein-coupled receptors superfamily, characterised by a transmembrane complex consisting of seven hydrophobic helices with intra- and extracellular domains [2–4] and is expressed in the granulosa cells of the antral follicles. The gene encoding FHSR, which is located on chromosome 2p21, consists of 10 exons and spans 54kb. More than 1000 SNPs have been located within the gene encoding the FSHR, but only a few belong to the coding regions or exons. Of these, the one that most determines the response to ovarian stimulation is the rs6166 which is also known as c.2039A>G, resulting in Asn680Ser amino acid exchange [5–7], which is located in the protein intracellular domain and where asparagine can be substituted

by serine. The rs6166 polymorphism is linkage disequilibrium with rs6165 (Thr307Ala) resulting in the most frequent allelic combinations of Thr307-Asn680 and Ala307-Ser680. In order to simplify, most studies focus almost exclusively on polymorphisms at codon 680 [8].

In previous studies, this polymorphism has been shown to influence the ovarian response to FSH stimulation in patients undergoing IVF treatments. A preliminary study [8], showed that the amount of FSH required to obtain an optimal ovarian response is lower in patients with the Asn/Asn genotype, compared to those with the Ser/Ser and Asn/Ser genotypes, who have lower sensitivity to FSH. Other studies [9,10] evaluated the influence of the different FSH receptor genetic variants, as well as its association with fertilisation rates and oocytes retrieved after ovarian stimulation. These findings made it possible to carry out a pharmacogenetic intervention, especially in patients with low ovarian reserve, in order to tailor the ovarian stimulation protocols based on each patient's genotype, which has led to an improvement in the IVF cycles results.

On the other hand, one of the recently implemented strategies, with the aim of increasing the number of oocytes retrieved in IVF treatment, is double ovarian stimulation. This ovarian stimulation protocol consists of carrying out two stimulations and two ovarian punctures in a single menstrual cycle. The first stimulation is carried out in the follicular phase and, after treatment with gonadotropins, the first follicular puncture is performed, and the recovered mature oocytes are vitrified or fertilised. Between 2 and 5 days after the previous puncture, once the luteal phase has been reached, the second ovarian stimulation begins; after another ovarian stimulation, the second follicular puncture is carried out.

This stimulation protocol is indicated for those patients with low ovarian response in whom, after a first stimulation in the follicular phase, it is not possible to retrieve an optimal number of oocytes [11–13]. It is also indicated for patients with other poor prognostic factors, such as age over 38 years old, previous failed IVF treatments, or for patients who have little time to carry out treatment, as is the case of cancer patients before starting chemotherapy [14].

Different investigations [15–17] have shown that, the number of oocytes collected and mature oocytes in the ovarian stimulation in luteal phase is higher. Also, a higher rate of blastocyst formation was observed. This protocol could offer an advantage for patients with poor prognosis and advanced reproductive age because the oocytes collected after both stimulations would accumulate, optimising the chances of achieving pregnancy. Furthermore, a recent randomised study in 80 patients [18] has shown non-inferiority results in double stimulation patients compared to women who underwent two

ovarian stimulations in consecutive cycles. It has not been studied yet whether the possible difference in outcomes occurs equally in patients with different genotypes for FSH receptor.

We must bear in mind that protein folding is influenced by factors that can fluctuate in the different phases of menstrual cycle, such as cellular and intercellular medium polarity and pH [19]. For this reason, the objective of this study objective is to discern if the follicle-stimulating hormone receptor genotype, influence the ovarian stimulation treatment results in luteal phase in order to know if a tailored treatment would be beneficial. In luteal phase, the physiological and hormonal environment is different from that of the follicular phase [20]. To our knowledge, all studies carried until now, analysed the behaviour of different FSH receptor genotypes in ovarian stimulation in follicular phase, and none of them do it in the luteal phase.

Materials and methods

Study population

This retrospective study included patients from a private fertility clinic who had been treated with double ovarian stimulation protocol between July 2017 and December 2021. The data of 299 double stimulation cycles were traced in the clinical database, corresponding to 272 patients with poor prognostic factors (such as low ovarian reserve or over 38 years old) or in patients who have little time to carry out treatment (cancer patients or elective vitrification). They either have FSH receptor genotyped as part of the fertility tests done before treatment or, their DNA is available at the centre and could be genotyped for this study, prior signature of informed consent. Patients who underwent the double stimulation protocol and whose DNA was not available for FSH receptor genotyping, and those patients in whom any of the ovarian stimulation cycles were cancelled were excluded from the study. Also, other clinical variables were collected such as age, anti-Müllerian hormone levels, antral follicle count and BMI.

Ovarian stimulation

The double ovarian stimulation protocol was personalised by a specialised gynaecologist according to the patient's clinical characteristics (age, hormone levels, antral follicular count, and results obtained in previous cycles). Both stimulations were performed with recombinant follicle stimulation hormone (rFSH) or highly purified FSH (HP-FSH), and human menopausal gonadotropin (hMG). The gonadotropin type was the same for both ovarian stimulations in the same patient, as well as the initial dose of gonadotropins, extending treatment until the oocytes maturation.

After basal assessment of ovaries, follicular phase stimulation was initiated between the second and fourth day

of menstrual cycle. Daily administration of a gonadotrophin-releasing hormone antagonist (cetorelix 0.25 mg or ganirelix 0.25 mg) was started when the leading follicle had a diameter ≥ 13 –14 mm until the ovulation trigger day. As soon as at least two follicles had reached 17–18 mm in diameter, ovulation was triggered with a subcutaneous bolus of triptorelin at a dose of 0.2 mg and oocyte retrieval was performed 36 h later.

After the first oocyte retrieval, luteal phase stimulation was initiated. Prevention of LH surge during luteal phase was performed with 200 mg oral micronized progesterone daily, a gonadotrophin-releasing hormone antagonist or no medication. When at least two follicles had reached a diameter of 17–18 mm, ovulation was triggered with a subcutaneous bolus of GnRH agonist (triptorelin 0.2 mg), a bolus of human chorionic gonadotrophin (hCG) 10 000 IU or both, depending on the gynaecologist's criteria. Oocyte retrieval was performed 36 h later.

Oocyte retrieval, intracytoplasmic sperm injection and blastocyst culture procedures were performed following established standard protocols.

FSH receptor genotyping

In order to genotype FSH receptor, DNA was extracted from the patient's biological sample, either from peripheral blood or from buccal swab. For the DNA purification from blood-EDTA, the commercial MagMAX DNA Multi-Sample Ultra 2.0 kit (Thermo Fisher Scientific) was employed using the KingFisher Duo Prime system (Thermo Fisher Scientific). In case of buccal swabs, the kit used was QIA amp DNA Mini and Blood (QIAGEN).

Once the DNA was extracted, its concentration was quantified by fluorimetry with Invitrogen Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and Invitrogen Qubit 3 Fluorometer (Thermo Fisher Scientific) following manufacturer's instructions.

Next, real-time PCR for allelic discrimination of rs6166 was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems. Ref: 4376600). For this, the enzyme TaqPath ProAmp Master Mixes (Thermo Fisher Scientific. Ref: A30865) and specific probes for each of the alleles were used. Analysis was carried out in accordance with the manufacturer's instructions.

Oocyte vitrification program

Mature oocytes were vitrified following the Cryotop protocol with Kitazato solutions. Oocytes were equilibrated in a solution containing 7.5% (v/v) ethylene glycol (EG), 7.5% (v/v) dimethylsulfoxide (DMSO). They were then, transferred to vitrification solution (VS) containing 15% (v/v) EG, 15% (v/v) DMSO, and 0.5 M trehalose, washed thoroughly to remove equilibration solution, and loaded in the Cryotop tip before plunging in liquid nitrogen, no more than 50–60 s.

For oocyte devitrification, the cryotop tip was submerged as fast as possible in thawing solution (TS, 1M trehalose) at 37 °C. Oocytes were recovered from TS in one minute, and transferred to dilution solution (DS, 0.5M trehalose, room temperature) for three minutes, followed by 5 min in washing solution (WS, no osmotic agents, room temperature).

Oocytes were fertilised with intracytoplasmic sperm microinjection after 2–3 h post-warming.

Statistical analysis

For evaluation of normal distributions, the Shapiro-Wilk's test was performed. As all numerical variables were non-normally distributed, the comparison between means was carried out using non-parametrical tests. First, a comparative analysis between genotypes of the patients' descriptive variables, included in the study, was carried out using Kruskal-Wallis with Holm correction test. Afterward, Pearson's Chi-squared test was used to evaluate differences in the medication used to trigger ovulation in luteal phase.

Finally, the results of follicular phase stimulation and luteal phase stimulation in the same patients were compared using the Mann-Whitney-Wilcoxon test, both globally and according to their genotype. No corrections have been made for age, AMH, AFC or BMI since the comparison was made in a paired manner, that is, the results of ovarian stimulation in follicular phase vs. luteal phase of the same patient in the same menstrual cycle were compared.

To perform the data statistical analysis, R Statistical Software program, version 4.0.3., and Software Statistical Product and Service Solutions, version 20.0 (SPSS, Chicago, IL, USA) were used.

Institutional review board

The design of this study was approved by the Institutional Review Board (IBB21-2022).

Results

Study population

Of the 299 patients participating in the study, 184 (62%) were ≥ 38 years of age; 68 (23%) had low reserve under the age of 38; 24 (8%) had infertility of unknown origin; 16 (5%) for organisational reasons at the patient's request and 7 (2%) for elective social vitrification.

The patients in the study had an advanced reproductive mean age, over 38 years old in all genotypes (38.42 ± 3.08 for Ser/Ser, 38.56 ± 3.46 for Asn/Ser and 38.62 ± 3.26 for Asn/Asn). The ovarian reserve was low or suboptimal whether we take into account the levels of anti-Müllerian hormone in pmol/L (5.93 ± 5.28 for Ser/Ser, 6.31 ± 5.04 for Asn/Ser and 6.95 ± 6.23 for Asn/Asn) or the antral follicle count (7.59 ± 3.91 for Ser/Ser, 6.83 ± 4.16 for Asn/

Ser and 7.45 ± 5.32 for Asn/Asn). This is because both advanced reproductive age and low ovarian reserve are indications for a double ovarian stimulation treatment. Regarding the BMI, the patients have normal weight for the three groups (22.87 ± 2.81 for Ser/Ser, 22.07 ± 3.35 for Asn/Ser and 22.62 ± 3.65 for Asn/Asn).

Twenty percent of the cycles ($n = 59$) had been performed in patients with Serine/Serine genotype; 48% ($n = 144$) in Serine/Asparagine (SN) genotype; and 32% ($n = 96$) in Asparagine/Asparagine (Asn/Asn) genotype. Although the percentage of patients with Ser/Ser genotype is lower, it corresponds to this genotype prevalence in Caucasians [21].

Figure 1 shows that no significant differences between genotype groups in terms of age ($P = 0.737$), anti-Müllerian hormone levels ($P = 0.641$), antral follicle count ($P = 0.384$) and BMI ($P = 0.301$) were found.

On the other hand, Table 1 shows that the trigger type used in luteal phase was comparable between groups of patients with different FSH receptor genotypes, so this factor does not either have an influence in the comparisons between different groups.

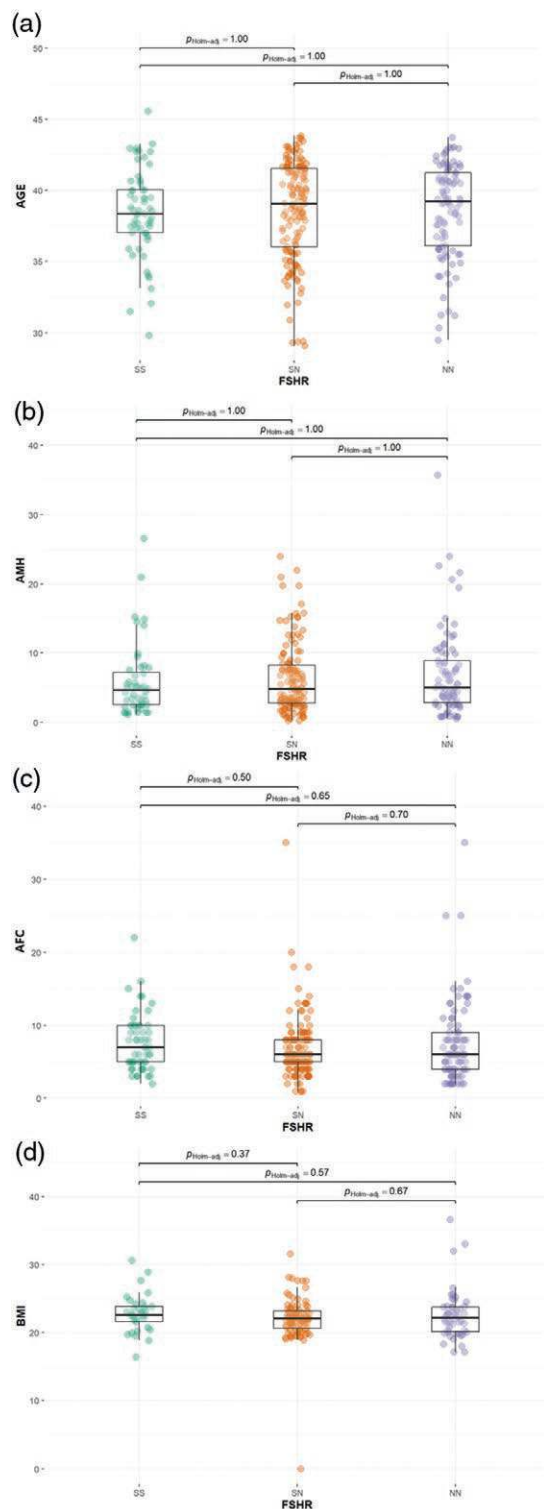
Comparison of ovarian stimulation performance in follicular phase and luteal phase

Table 2 compares the performance of ovarian stimulation in follicular phase and in luteal phase, whether we consider the patients' genotype or not. In global analysis, the performance of ovarian stimulation in luteal phase was better than in follicular phase in terms of total number of retrieved oocytes, MII oocytes and fertilised oocytes ($P < 0.001$).

By dividing patients into groups according to their genotype for position 680 of the FSH receptor, the differences between ovarian stimulation in follicular and luteal phase in the same patient and same menstrual cycle are maintained for all genotypes with respect to the parameters mentioned above (retrieved oocytes, MII oocytes and fertilised oocytes; $P < 0.05$). For the Ser/Ser and Asn/Asn genotypes, the increase in the total number of oocytes recovered in luteal phase with respect to follicular phase is around 40% (Ser/Ser genotype, 3.80 vs. 5.31; Asn/Asn genotype, 3.68 vs. 5.03; $P < 0.001$), whilst in the group of patients with Asn/Ser genotype, the increase is 20% (4.01 vs. 4.81; $P = 0.005$). Also, the number of follicles with a diameter higher than 15 mm is higher in the luteal phase compared to follicular phase in the same menstrual cycle for the same patient, with differences of about 1 follicle in all genotypes ($P < 0.05$).

In addition, ovarian stimulation in luteal phase is longer than in follicular phase and the gonadotropins intake is greater, whether we analyse the study patients in global ($P < 0.001$) or dividing them according to their genotype for position 680 of the FSHR ($P < 0.05$).

Fig. 1



Comparative analysis between the different genotypes for the 680 position of the FSH receptor (FSHR) for the descriptive variables of the patients included in the study. No statistically significant differences were found in age (a), anti-Müllerian hormone levels (b), antral follicle count (c) and BMI (d). NN, Asparagine/Asparagine; SN, Serine/Asparagine; SS, Serine/Serine. Statistical test: Kruskal–Wallis with Holm correction.

Table 1 Medication used to trigger ovulation in luteal phase

Type of trigger	N	SS (N = 59) ^a	SN (N = 144) ^a	NN (N = 96) ^a	P-value ^b
None	299	5/59 (8.5%)	15/144 (10.4%)	11/96 (11.5%)	0.935
Triptorelin 0.2 mg		5/59 (8.5%)	17/144 (11.8%)	10/96 (10.4%)	
hCG 10,000 IU		18/59 (30.5%)	50/144 (34.7%)	32/96 (33.3%)	
Both		31/59 (52.5%)	62/144 (43.1%)	43/96 (44.8%)	

NN, Asparagine/Asparagine; SN, Serine/Asparagine; SS, Serine/Serine.

^an/N (%).

^bPearson's Chi-squared test.

Finally, we observed that the increase in the gonadotropins dose in the luteal phase with respect to follicular phase seems greater in the group of patients with Ser/Ser genotype (396 IU more in ovarian stimulation in luteal phase compared to follicular phase) than in patients with Asn/Ser genotype (278 UI) and Asn/Asn genotype (236 IU), although the difference does not reach statistical significance.

Discussion

To our knowledge, this is the first study comparing results of ovarian stimulation carried out in follicular phase to the one in luteal phase taking into account the genotype for the 680 position of the FSH receptor. These results make the double ovarian stimulation strategy a very good option for patients with any genotype for FSH receptor, as it allows them to obtain a better performance in treatment and retrieve more oocytes in less time; also allows to initiate ovarian stimulation on different days of the cycle obtaining good results, as noted above [22–25]. The three genotype groups are comparable in terms of age, anti-Müllerian hormone levels, antral follicle count, BMI, and the trigger type used in luteal phase.

It is important to highlight that, although studies have shown greater performance of ovarian stimulation in luteal phase compared to follicular phase, and a recent randomised study [18] has shown non-inferiority results in double stimulation patients compared to women who underwent two ovarian stimulations in consecutive cycles, it is not yet known what causes the improvement in luteal phase performance. It could be due to the gonadotropin's impregnation in the previous treatment in follicular phase, by the needle mechanical action in the ovarian puncture in follicular phase or because the hormonal environment of the luteal phase modifies the FSH receptor structure, making it more or less affined to the G_s subunit, which induces adenylyl cyclase activity and activates the FSH receptor signalling pathway [26,27], among other theories. In accordance with the literature already published, we need caution and time to collect more data and to select the right population and protocol which could benefit from this strategy.

If we analyse the results differentiating patients by genotypes, in case of Ser/Ser patients, they have a higher

gonadotropin consumption compared to the other genotypes in luteal phase, the same as in studies in follicular phase, because they have a higher FSH threshold in controlled ovarian stimulation protocols [28]. Despite having a higher FSH threshold, when treating this group of patients with a higher dose of gonadotropins and, in addition, with a pharmacogenetic approach [29], we achieved a higher yield in ovarian stimulation for this group, although they present a greater 'resistance' to FSH [30].

On the other hand, patients with Asn/Ser and Asn/Asn genotypes also benefit from this protocol. They presented the best results in previous studies [8,29] and, with double ovarian stimulation, a greater number of oocytes can be recovered in less time. These results are in agreement with a comparative study [21] indicating that the asparagine in position 680 of the FSHR is more common in humans than in other less evolved mammalian species, so this allele is postulated as an evolutionary advantage. An *in vitro* study [31] showed that intracellular cAMP production is faster in granulosa cells homozygous or heterozygous for asparagine with respect to homozygous serine/serine cells, in addition to producing greater amount of progesterone [32]. cAMP is produced when FSHR, once bound to FSH, binds to the G_s subunit. This increase in cAMP levels activates protein kinase A, which triggers the FSHR molecular signalling pathway [26,27].

It should be noted that the Asn/Ser genotype is the one obtaining the least benefit from ovarian stimulation in luteal phase with respect to stimulation in follicular phase. This may be due to the fact that this genotype optimisation at the dose level, and type of gonadotropin has been less in this study than in patients with Ser/Ser genotype, but the results are also positive for this group of patients.

As limitations of the study, we mention its retrospective design, the number of patients included in it, especially in the group of patients with the Ser/Ser genotype due to its lower prevalence in the population [21], the advanced reproductive age of the patients and the influence of other parameters that have not been analysed. Future prospective studies with a larger sample size and a more homogeneous sample distribution among the genotype

Table 2 Comparison of ovarian stimulation performance in follicular phase and luteal phase globally and according to the genotype for the FSH receptor

Variable	Global (N = 299)			SS (N = 59)			SN (N = 144)			NN (N = 96)		
	FP	LP	P-value	FP	LP	P-value	FP	LP	P-value	FP	LP	P-value
FSH dose (UI)	2,737.77 (818, 11)	3,110.47 (845, 47)	<0.001 ^a	2,944.49 (1,115, 01)	3,340.25 (921, 99)	0.002 ^a	2,824.13 (764, 18)	3,102.43 (834, 16)	<0.001 ^a	2,829.82 (829, 78)	3,065.62 (916, 73)	0.028 ^a
Days of stimulation	9.54 (2, 02)	10.70 (2, 37)	<0.001 ^a	9.29 (2, 19)	10.97 (2, 22)	<0.001 ^a	9.74 (2, 17)	10.67 (2, 50)	<0.001 ^a	9.80 (2, 21)	10.28 (2, 55)	0.114
N° of follicles ≥15 mm	4.96 (3, 11)	5.99 (3, 92)	<0.001 ^a	4.64 (2, 50)	5.76 (4, 09)	0.012 ^a	4.38 (2, 76)	5.17 (3, 42)	0.002 ^a	4.59 (3, 25)	5.55 (3, 77)	0.005 ^a
Oocytes retrieved	4.18 (3, 05)	5.47 (3, 71)	<0.001 ^a	3.80 (2, 97)	5.31 (3, 48)	<0.001 ^a	4.01 (2, 63)	4.81 (3, 09)	0.005 ^a	3.68 (3, 06)	5.03 (3, 72)	<0.001 ^a
MII oocytes	3.29 (2, 50)	4.52 (3, 31)	<0.001 ^a	2.88 (2, 44)	4.46 (3, 26)	<0.001 ^a	3.24 (2, 32)	3.89 (2, 82)	0.017 ^a	2.98 (2, 41)	4.11 (3, 25)	<0.001 ^a
Fertilised oocytes	2.20 (1, 98)	3.81 (2, 75)	<0.001 ^a	2.08 (1, 76)	4.03 (2, 65)	<0.001 ^a	2.14 (1, 85)	3.12 (2, 35)	<0.001 ^a	2.10 (1, 79)	3.69 (2, 85)	<0.001 ^a

Statistical test: Mann-Whitney-Wilcoxon.
 FP, follicular phase; LP, luteal phase; NN, Asparagine/Asparagine; SN, Serine/Asparagine; SS, Serine/Serine.
^aDenotes a statistically significant difference (P < 0.05).

groups could contribute to verify the results obtained in this study.

Regarding the hypothesis about why a greater performance of ovarian stimulation in luteal phase is obtained compared to follicular phase, we could suggest that the luteal phase hormonal environment could modify the FSH receptor structure by changing its environment [19] and modifying its affinity to the G_s subunit. Possibly, the LH peak, which begins the luteal phase, produces small variations in the receptor conformation.

Therefore, to elucidate this question, it is necessary to carry out a prospective study in which the same patients are stimulated in the follicular and luteal phase in different menstrual cycles, not consecutively. Also, these patients should be randomised by genotype in order to study possible differences between them. This way, stimulation in luteal phase would not be preceded by stimulation in follicular phase and it could be discerned whether a better performance in this phase in some genotypes is related to the differences in the physiological environment at the different cycle stages or is caused by activating the ovary in the previous stimulation.

In addition, in order to compare the clinical results obtained in both phases, it would be necessary for the oocytes to be fertilised in fresh and frozen once they reach blastocyst stage, which have a higher survival rate to vitrification, especially when maternal age is advanced [33], as well as to carry out the chromosomal analysis of the blastocysts obtained. This knowledge could shed light on the molecular mechanisms that modulate FSH signalling and be involved in the development of new drugs adapted to this polymorphism to be used in assisted reproduction.

Finally, we must not lose sight to the fact that, to date, the mechanism and safety of double stimulation in the same cycle have been little addressed. Recent studies [34,35] propose that, while in a single ovarian stimulation, FSH acts on cells about to differentiate into mature oocytes, prolonged exposure to FSH produced by double stimulation would act also on less differentiated ovarian stem cells. Also, recent evidence [36] suggests that collection of more immature oocytes in luteal phase may increase the risk of ovarian cancer, as a greater rate of borderline ovarian tumours was seen in patients undergoing ovarian stimulation compared to the general population. These studies that question the procedure's safety are subsequent to the recruitment of patients for the study.

This fact could question the protocol safety, so it is necessary to delve into this matter, since it is an increasingly common medical practice in most assisted reproduction centres.

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Conflicts of interest

There are no conflicts of interest.

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