The relevance of the individual screening for genetic variants in predicting ovarian response

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Objective To investigate if polymorphisms of some genes involved in folliculogenesis predict ovarian response.

Methods This prospective randomized study includes 124 egg donors genotyped for six SNPs ESR1 (rs2234693), AMHR2 (rs2002555), GDF-9 (rs10491279 and rs254286), AMH (rs10407022) and LHCBR (rs229327) genes and four STRs in ESR1 rs3138774), SHBG (rs6761), CYP19A1 (rs60271534) and AR genes (CAG repeats in exon 1). All donors followed standard ovarian stimulation protocol using a daily dose of 225 UI. The genotypes obtained were compared with the ovarian stimulation outcome.

Results Regarding the number of retrieved oocytes, we found statistical differences for the ESR1 SNP and STR (19.3 ± 8.9 for TT vs 15.3 ± 6.2 for CC/CT, $P=0.027$; 19.1 ± 8.3 for <17repeats vs 14.7 ± 6.2 for >17repeats, $P=0.020$). Moreover, women carrying TT in the ESR1 at position c.-397T>C with ESR1 (TA)n=17 retrieved the highest number of oocytes (20.4 ± 9.3) ($P=0.001$). Concerning AMHR2, we observed an association with the length of stimulation (9.1 ± 1.4 d for AA vs 9.7 ± 1.3 d for AG/GG, $P=0.021$) and gonadotropin received (2050 ± 319 for AA vs 2188 ± 299 for AG/GG, $P=0.017$). No significant differences were observed for the other polymorphisms ($P>0.05$).

Conclusion The polymorphisms in ESR1 and AMHR2 genes showed a clear association with the number of retrieved oocytes and the stimulation data, respectively. Our results suggest that polymorphisms in the genes for key reproductive hormones receptors could be used to predict the ovarian response and to personalize the stimulation prior the treatment. *Pharmacogenetics and Genomics* 29: 216–223 Copyright © 2019 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Despite the rapid evolution of ART techniques, success remains an issue as it is dependent on many factors. One of those factors is the number of oocytes obtained after controlled ovarian stimulation (COS) [1]. Many different factors can influence the response to ovarian stimulation and these can be different for each individual. To improve IVF success, personalized therapies have been the new focus of COS. Pharmacogenetics is the study of how the genetic profile of an individual determines the response to a pharmacological treatment [2]. The presence of individual variability of response to drugs is well known, and although many nongenetic factors can have an influence, recent research suggests that genetic factors are likely to be the main responsible for the variability [2]. The key genetic factor is sequence variants, or single nucleotide polymorphisms (SNPs), present in relevant genes such as those encoding drug targets [3]. Around 19 million SNPs have been identified in the human genome, and many have already been associated with alteration of drug effects. Therefore, the challenge of pharmacogenetics is to establish the relation between SNP and drug response to develop a diagnostic test that predicts the reaction to a drug, improving the treatment and be more cost-effective optimizing the financial burden [4]. Therefore, pharmacogenetics in reproductive medicine could be used to select the best treatment for ovarian stimulation and to obtain the best outcome.

The influence that genetics may have on ovarian response to stimulation has been studied in multiple occasions. Most of the research has focussed on the receptors of the main gonadotropin used in COS: follicle stimulating hormone receptor (FSHR) [5]. Most investigation has been focussed on p.N680S in the *FSHR* gene, revealing an association between the polymorphism and the response to ovarian stimulation [6]. This variant was the first biomarker for ovarian response, initiating the first step into a personalized treatment protocol [7]. Despite genotyping for *FSHR* is useful, it cannot provide enough predictive information on its own, and therefore, other markers should also be taken into consideration. Polymorphisms in the oestrogen receptor gene (*ESR1*) have also been

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studied, and they have proven to be linked to premature ovarian failure. Therefore, they could be used to predict ovarian response [8]. The CAG repeat lengths present on the human androgen receptor (AR) and their influence on ovarian response have also been studied; however, these were found to be associated with ovarian reserve, but not ovarian response [9]. Further research has also focussed on other genes involved in folliculogenesis, such as *BMP15* [10], *GDF-9* [11] and antimullerian hormone (*AMH*) [12]. Despite the evidence of polymorphisms findings in these genes being associated with COS, their efficacy still needs further evaluation before including them in clinical tests [13].

The aim of this study was to evaluate 10 genetic variants located on genes involved in the ovarian function and their relationship with ovarian stimulation. These variants are located in the following genes: oestrogen receptor 1 (*ESR1*), *AMH*, AMH receptor 2 (*AMHR2*), luteinizing hormone choriogonadotropin receptor (*LHCGR*), growth differentiation factor-9 (*GDF9*), sex hormone binding globulin (*SHBG*), oestrogen synthetase or aromatase (*CYP19A1*) and *AR*.

Methods

Study population

Egg donors are the most adequate model to evaluate ovarian stimulation and embryo implantation potential, because donors are similarly aged young females with standard ovarian function, thus obtaining similar oocyte and embryo quality. Selection of donors is performed following the clinic's strict criteria, as well as the legal requirements of the Spanish law (Spanish law 14/2006) and the European Society of Human Reproduction and Embryology and American Society of Reproductive Medicine guidelines for oocyte donors. This includes performing chromosomal and genetic evaluation, karyotyping, as well as an extensive gynaecological examination and testing for infectious diseases. In addition to this, the clinic also performs an expanded carrier genetic screening for severe autosomal recessive and X-linked diseases, the gene panel contains 555 genes. Moreover, due to the fact that the FSHR variant p.N680S is associated with ovarian response [6], donors were genotyped for the FSHR variant p.N680S and the result was used as a confounder factor when statistical analysis was performed.

In this study, we included 124 Caucasian women who attended Instituto Bernabeu to undergo oocyte donation. Informed consent for collecting peripheral blood to be used for molecular analysis was obtained from all women participating in the study. The study was approved by the Ethical committee (2015-003779-31).

Single nucleotide polymorphism genotyping

DNA from all subjects was extracted from peripheral blood lymphocytes and isolated using Wizard DNA Purification Kit (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions.

SNP genotyping was determined using the predesigned TaqMan allelic discrimination assays on six different variants present in *ESR1* (rs2234693), *AMHR2* (rs2002555), *GDF-9* (rs10491279 and rs254286), *AMH* (rs10407022) and *LHCBR* (rs229327) genes (Life Technologies Corporation, Pleasanton, California, USA). The amplification was performed using StepOne Real-Time PCR System from Applied Biosystems (Carlsbad, California, USA), following the manufacturer's instructions. Results were analysed using the StepOne Software version 2.2.

STR genotyping

A variety of STRs located in *ESR1* (rs3138774), *SHBG* (rs6761), *CYP19A* (rs60271534) and *AR* (CAG repeats in exon 1) were analysed. Their size was determined through PCR amplification using primers flanking the polymorphism repeats carrying a 6-FAM modification at 5′.

The PCR conditions for the amplifications were different between each STR previously published. To amplify *ESR1* STR, 45 cycles were performed with an initial denaturation of 10 minutes at 94°C, followed by denaturation for 30 s at 94°C, annealing for 45 s at 55°C, elongation for 1 minute at 72°C and a final extension of 7 minutes at 72°C [14]. For *CYP19A1*, 27 cycles were carried out with an initial denaturation that lasted 5 minutes at 94°C, followed by denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C, extension for 1 minute at 72°C and a final extension of 8 minutes at 72°C [15]. In the case of *SHBG*, 40 cycles were performed, and the initial denaturation lasted 12 minutes at 95°C, followed by denaturation for 15 s at 94°C, annealing of 60°C at 15 s, extension of 30 s at 72°C and a final extension of 12 minutes at 72°C [16]. Finally, for *AR*, 35 cycles were carried out, with initial denaturation of 5 minutes at 95°C, denaturation for 45 s at 94°C, annealing for 45 at 55°C, extension for 45 at 72°C and a final extension of 10 minutes at 72°C [17]. PCR was followed by capillary electrophoresis to determine the size of each product. This was performed using SeqStudio Genetic Analyzer from Applied Biosystems (Thermo Fisher Scientific).

Ovarian stimulation and oocyte retrieval

A standard ovarian stimulation protocol was followed by all women included in the study. To avoid confusion factors, all donors included in the study receipt the same dose of FSH. They were administered a daily dose of 225 UI of FSH (Fostipur; Angelini International, Barcelona, Spain or Bemfola; FINOX AG, Burgdorf, Switzerland). It is a main strength of our study because using the same protocol avoids any biases and it is of great importance when comparing the ovarian response outcome. A GnRh antagonist 25 mg of cetrorelix (Cetrotide; Merck-Serono, Paris, France) was also administered daily when the leading

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follicle reached 14 mm. The triggering of the LH surge was performed using 0.4 mg of subcutaneous triptorelin (Decapeptyl; Ipsen Pharma, Paris, France). Oocyte pickup was performed 36 hours later through ultrasound guided transvaginal puncture under sedation. The IVF procedure, including sperm and oocyte preparation, embryo culture and transfer, were conducted by following the IVF laboratory guidelines.

Statistical analysis

The genotypes obtained were compared with the ovarian stimulation, with primary end-points being length of stimulation, gonadotropin dosage and number of retrieved oocytes. For STR repeats length, we considered the biallelic mean, as previous reports, and a cutoff for each STR was calculated according to the median [9]. The data were analysed using a statistical package for the social science software (version 20.0; SPSS Inc., Chicago, Illiniois, USA). The values were given as averages ± SD, and the main end-points included stimulation length, gonadotropin consumption and number of oocytes retrieved from the donors. Categorical values were tested using Pearson's w2-test, while for continuous variables (donor characteristics), an analysis evaluating the variations between groups was performed. Adjustment for cofounding factors was performed for the ovarian stimulation data analysis, using linear regression and adjusting for age, smoking, BMI and AMH, because these factors are known to alter ovarian response. Genotypes were included in the study as dummy variables, and *P* values were considered significant when lower than 0.05.

Results

Clinical characteristics

Clinical and biological characteristics of patients were measured, such as the age, tobacco consumption, the antral follicle count (AFC), BMI and the blood levels of FSH and AMH (Table 1). The mean age of the oocyte donors included in the study was 23.9 ± 3.5 years. The average BMI was 21.9 ± 2.5 kg/m^2. The mean AMH and FSH level was 45.44 ± 23.5 pmol/L and 4.7 ± 2.5 mUI/ml, respectively, and the mean number of AFC was 14.2 ± 2.8. Regarding the ovarian stimulation data, the mean length of stimulation was 9.3 ± 1.4 days. The mean number of gonadotropins consumed was 2087.3 ± 318.5 IU and the average of the number of oocytes retrieved 16.6 ± 7.3.

Single nucleotide polymorphism and STR genotyping

A total of 124 women were genotyped for the SNPs located in the *ESR1*, *AMH*, *AMHR2*, *LHCGR*, *GDF9* genes and STRs in *ESR1*, *SHBG*, *CYP19A1* and *AR* genes. Regarding to SNPS, the number of women having each allele is summarized in Table 2, along with their according frequency. Genotype frequencies observed during this study followed the Hardy–Weinberg equilibrium. Also, our genotype distribution agrees with general population data (openSNP database). As for STRs, the length of the repeats of each allele was measured for each patient, and the biallelic mean was calculated for each STRs. A general description of the distribution of biallelic mean on the number of repetition on both alleles can be observed in Fig. 1.

Table 1 Main characteristics of study participants and analysed parameters

Total *n*=124	Average ± SD
Age (y)	23.9 ± 3.5
Smoker (%)	46
AFC	14.2 ± 2.8
BMI (kg/m^2)	21.9 ± 2.5
FSH (mUI/ml)	4.7 ± 2.5
AMH (pmol/L)	45.4 ± 23.5
Length of stimulation (days)	9.2 ± 1.4
Gonadotropin consumption (IU)	2087.3 ± 318.5
Number of oocytes retrieved	16.6 ± 7.3

AFC, antral follicle count; AMH, antimullerian hormone; FSH, follicle stimulating hormone.

Table 2 Single nucleotide polymorphisms genotype distribution among patients

Gene/SNP ID	Genotype/allele	*n*	Frequency (%)	General population Frequency (%)[a*]
ESR1 rs2234693				
	TT	40	32.2	31
	TC	58	46.8	49
	CC	26	21.0	19
AMH rs10407022				
	TT	84	69.4	65
	GT	36	29.8	31
	GG	1	0.8	3
AMHR2 rs2002555				
	AA	91	73.4	70
	AG	27	21.8	27
	GG	6	4.8	3
LHCGR rs2293275				
	GG	57	46.0	37
	AG	51	41.1	47
	AA	16	12.9	16
GDF-9 rs10491279				
	AA	46	37.1	52
	AG	57	46.0	34
	GG	21	16.9	14
GDF-9 rs254286				
	CC	87	70.1	50
	CT	33	26.6	47
	TT	4	3.3	3

AMH, antimullerian hormone; AMHR2, antimullerian hormone receptor 2; ESR1, oestrogen receptor gene 1; GDF9, growth differentiation factor-9; LHCGR, luteinizing hormone choriogonadotropin receptor.
[a]openSNP database.

Gene polymorphisms and ovarian response

The results obtained from above were compared with the ovarian stimulation data of each patient. The length of stimulation, the amount of gonadotropin consumed by the patient and the number of oocytes that were retrieved were taken into consideration for the analysis. As it can be observed in Table 3, there is no significant variation between genotype groups in their length of stimulation nor in the doses of gonadotropins that were consumed, nor the number of retrieved oocytes for variants in *AMH*, *LHCGR*, *GDF-9*, *SHBG*, *CYP19A1* and *AR* genes.

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Table 3 Associations between selected single nucleotide polymorphisms and STRs and ovarian response

Gene/SNP ID			*P* value
ESR1 rs2234693 dominant model			
Genotype	TT	CC/CT	
n	40	84	
Length of stimulation (days)	9.2 ± 1.4	9.3 ± 1.4	0.801
Gonadotropin consumption (IU)	2075 ± 312	2093 ± 323	0.743
Number of retrieved oocytes	19.3 ± 8.9	15.3 ± 6.2	0.027[a]
AMH rs10407022 dominant model			
Genotype	TT	GG/GT	
n	84	37	
Length of stimulation (days)	9.5 ± 1.4	9.0 ± 1.4	0.107
Gonadotropin consumption (IU)	2122 ± 324	2025 ± 305	0.122
Number of retrieved oocytes	16.8 ± 7.3	15.8 ± 7.7	0.269
AMHR2 rs2002555 dominant model			
Genotype	AA	AG/GG	
n	91	33	
Length of stimulation (days)	9.1 ± 1.4	9.7 ± 1.3	0.021[a]
Gonadotropin consumption (IU)	2051 ± 319	2189 ± 299	0.017[a]
Number of retrieved oocytes	16.7 ± 7.7	16.6 ± 6.4	0.825
LHCGR rs2293275 dominant model			
Genotype	GG	AG/GG	
n	57	67	
Length of stimulation (days)	9.4 ± 1.4	9.2 ± 1.5	0.663
Gonadotropin consumption (IU)	2100±308	2076 ± 329	0.684
Number of retrieved oocytes	16.5 ± 7.5	16.6 ± 7.3	0.930
GDF-9 rs10491279 recessive model			
Genotype	AA/AG	GG	
n	103	33	
Length of stimulation (days)	9.4 ± 1.4	9.0 ± 1.4	0.769
Gonadotropin consumption (IU)	2102 ± 320	2014 ± 306	0.800
Number of retrieved oocytes	16.0 ± 6.5	19.3 ± 10.5	0.459
GDF-9 rs254286 dominant model			
Genotype	CC	CT/TT	
n	87	37	
Length of stimulation (days)	9.4 ± 1.4	9.1 ± 1.4	0.286
Gonadotropin consumption (IU)	2106 ± 323	2043 ± 306	0.317
Number of retrieved oocytes	16.7 ± 7.5	15.7 ± 6.8	0.379
ESR1 rs3138774			
Genotype	<17	≥17	
n	49	33	
Length of stimulation (days)	9.1 ± 1.5	9.0 ± 1.4	0.410
Gonadotropin consumption (IU)	2042 ± 328	2125 ± 317	0.445
Number of retrieved oocytes	19.1 ± 8.3	14.7 ± 6.2	0.020[a]
SHBG rs6761			
Genotype	<7	≥7	
n	66	58	
Length of stimulation (days)	9.1 ± 1.5	9.5 ± 1.3	0.171
Gonadotropin consumption (IU)	2043 ± 335	2138 ± 293	0.148
Number of retrieved oocytes	16.7 ± 7.5	16.5 ± 7.3	0.976
CYP19A1			
Genotype	<9	≥9	
n	109	13	
Length of stimulation (days)	9.3 ± 1.5	9.1 ± 1.0	0.547
Gonadotropin consumption (IU)	2096 ± 329	2042 ± 233	0.570
Number of retrieved oocytes	16.5 ± 7.5	16.3 ± 6.8	0.928
AR			
Genotype	<24	≥24	
n	46	72	
Length of stimulation (days)	9.5 ± 1.2	9.2 ± 1.6	0.401
Gonadotropin consumption (IU)	2128 ± 280	2070 ± 349	0.613
Number of retrieved oocytes	15.3 ± 5.2	17.5 ± 8.4	0.261

AMH, antimullerian hormone; AMHR2, antimullerian hormone receptor 2; AR, androgen receptor; CYP19A1, oestrogen synthetase or aromatase; ESR1, oestrogen receptor gene 1; GDF9, growth differentiation factor-9; LHCGR, luteinizing hormone choriogonadotropin receptor; SHBG, sex hormone binding globulin; SNP, single nucleotide polymorphism.

The rs2234693 polymorphism in the *ESR1* gene causes a c.-397 T>C change and is located upstream of the gene, in the promoter region. According to Table 3, there is no significant variation between genotype groups in their length of stimulation, nor in the doses of gonadotropins that were consumed. However, we found statistical differences in the number of oocytes retrieved when we compared genotypes according to the dominant model for the *ESR1* SNP. Patients having the TT genotype retrieved a higher number of oocytes than the TC and CC genotypes (19.3 ± 8.9 for TT vs 15.3 ± 6.2 for CC/CT, $P = 0.027$).

Concerning AMHR2, the variant present in this gene that was analysed in this study corresponds to rs2002555, a variation causing an A>G change in the promoter sequence of the gene (position c.-482). Table 3 shows statistical significant differences in the length of stimulation (9.1 + 1.4 for AA vs 9.7 ± 1.3 for AG/GG, $P = 0.021$) and gonadotropin received during stimulation (2050 ± 319 for AA vs 2188 ± 299 for AG/GG, $P = 0.017$) between genotypes when the results were analysed according to the dominant model. Patients having the AG/GG genotype required higher units of gonadotropins and more days of stimulation than the AA genotypes. However, no significant differences were seen in the number of retrieved oocytes between genotypes.

The microsatellite analysed in *ESR1* corresponds to the rs3138774 genetic variant, which is a series of TA repetitions (TA)n present in the alpha promoter of the gene. The biallelic mean was analysed and categorized into two groups; the first one including the number of repeats being lower than 17 and the second including those being equal or higher than 17. The breakpoint chosen was 17 as it was observed to be the median. The ovarian stimulation data of both groups were compared and no significance differences were reported in the length of stimulation nor the gonadotropins consumption. However, a significant difference was obtained in the number of retrieved oocytes (19.1 ± 8.3 for <17 repeats vs 14.7 ± 6.2 for ≥17 repeats, $P = 0.020$). Patients having the less than 17 (TA)n repeats in the ESR1 retrieve a higher number of oocytes than patients carrying equal or higher than 17.

Due to the fact that the genotype in the *ESR1* SNP and STR was associated with the number of retrieved oocytes, another analysis was performed which combined data obtained for the ESR1 SNP and STR, to see if a particular combination of SNP genotype and STR repeat length is associated with a better or worse ovarian response to stimulation. As previous findings, no statistical significance was observed for the length of stimulation nor for the gonadotropins consumed. Nevertheless, a statistical significance was obtained for the number of oocytes retrieved (Table 4), in which the group having 17 repeats or more and CC/CT genotype retrieve the least number of oocytes (14.05 ± 5.6) comparing with other groups ($P = 0.001$).

Discussion

Oocyte donation is the best model to evaluate the determinants of stimulation and embryo implantation potential because donors are young women of similar age with

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Table 4 Associations between oestrogen receptor gene single nucleotide polymorphism s and STRs and ovarian response

Genotype	<17+CC/CT	<17+TT	≥17+CC/CT	≥17+TT	
N	19	30	67	7	*P* value
Length of stimulation (days)	8.8 ± 1.4	9.3 ± 1.5	9.5 ± 1.4	9.3 ± 1.3	0.328
Gonadotropin consumption (IU)	1974 ± 314	2085 ± 334	2129 ± 322	2089 ± 385	0.333
Number of retrieved oocytes	19.1 ± 6.8	19.1 ± 6.8	14.1 ± 5.6	20.4 ± 9.3	0.001[a]

Linear regression performed for statistical analysis with the cofounding factors being age, smoking, BMI and AMH.
AMH, antimullerian hormone.

normal ovarian function. The aim of this research was analysing a population consisting of 124 oocyte donors and observing whether a particular genetic profile has an influence on how they respond to an ovarian stimulation protocol. Through the analysis of several variants present in genes that are known to be involved in the ovarian cycle, and comparing those to the ovarian stimulation data, our data suggest that the polymorphisms in the *ESR1* and *AMHR2* genes showed a clear association with the number of retrieved oocytes and the stimulation data, respectively, in young normoovulatory patients. Furthermore, in our study, we used the same daily doses in all the cycles. The use of fixed dose protocols is of huge importance when you want to compare the ovarian response. The prior results regardless of the gene polymorphisms studied in our research and their relationship with ovarian stimulation are still controversial. This might be due to the heterogeneity of the studied population regarding the ovarian reserve and the varied exposures including different doses for COS. To the best of our knowledge, these data show for the first time the relationship between the polymorphisms in the *ESR1* and *AMHR2* genes and ovarian reserve stimulation using a nonconfounding model of oocyte donation. Therefore, the main strength of our study is that exactly the same dose and stimulation protocol were used so biases are avoided due to the use of different protocols and a normal ovarian reserve homogeneous population was selected; hence, biases are avoided due to heterogeneity of the studied population. Last, due to the fact that *FSHR* polymorphism is clearly related with ovarian response, donors were genotyped and the result included in the statistical analysis as a confounding factor so biases due to the effect of *FSHR* polymorphism was avoided.

An inadequate response to stimulation could affect the success of an IVF cycle. To improve the chances of successful outcome, the treatment should be tailored to the patient's characteristics. There are several factors that can predict ovarian response. Ovarian reserve is probably the most important factor in determining success rates after IVF [18]. The search for optimal biomarkers is ongoing for an accurate prognosis of the ovarian response to exogenous gonadotropins [19]. It has recently become evident that genetic factors could explain the differences among individuals in terms of response to drugs. This significant variability in ovarian response has been the focus of many pharmacogenetics studies, which have analysed the relationship between selected SNPs in candidates genes involved in the ovarian response to exogenous FSH [20]. Among them, the polymorphism N680S in the *FSHR* gene is the most studied variant and a clear evidence of their effect in the ovarian response was shown [21]. Accordingly, further studies in candidate genes should include the *FSHR* genotype to avoid any confounding consequence due to its effect. Hormonal receptor and biochemical pathway genes involved in folliculogenesis are candidate genes to pharmacogenetic approach. Our study included six different SNPs, which are located on five genes*; ESR1*, *AMHR*, *AMHR2*, *LHCBR* and *GDF-9*. Another objective was to identify the repeat length of four microsatellite regions present in four genes; *ESR1*, *SHBG*, *CYP19A1* and *AR*.

The ESR1 gene encodes a transcription factor which is activated by the binding of its ligand, oestrogen. This gene as well as its ligand play an essential role in folliculogenesis by boosting FSH activity. A variety of SNPs have been located on the gene, most of them altering the number of follicles and mature oocytes produced [22]. The (TA)n repeat has also been studied, and was found to be associated with ovarian stimulation outcome [23]. The SNP variation rs2234693 in *ESR1* causes a c.-397 T>C change upstream of the gene. It has been demonstrated that this variation forms part of a functional binding site for the B-myb transcription factor and it acts as an intragenic enhancer [24]. In the case of this SNP with the ovarian stimulation, the length and the amount of gonadotropins consumed were similar between the three genotypes. Therefore, the genotype does not seem to have an influence on how long women require to be stimulated nor on the amount of gonadotropins they consume, against the previous study showing TT genotypes were associated with a longer induction period and higher doses of medication [25]. In contrast, for the number of oocytes retrieved where patients carrying TT retrieved more oocytes than those carrying CT or CC, indicating that this genotype has an influence on the number of oocyte that women retrieve. A study performed on 109 IVF women found that women carrying the C allele presented the worst profile of ovarian stimulation [22]. A further study evaluating the IVF outcome was performed by Anagnostou *et al*. [22] in 2013 including 203 women reported that C allele was associated with worst quality embryos and smaller oestradiol levels on the Human chorionic gonadotropin day. Recently, a research in infertility

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patients revealed that C allele is more frequent in patients classified as poor responders [20]. Controversially, a prior study by de Castro *et al.* [26], presented a multilocus analysis, T allele was better represented in poor responders. In addition, another study performed on 136 infertile women demonstrated an association between the amount of medication required for COS and this SNP polymorphism, in which patients carrying TT required higher doses of FSH [26]. The discrepancy on the results among different studies may be due to a variety of factors. The studied population is different, because most studies include infertile women or poor responders. Also, the type and amount of gonadotropins administered also differ among studies, and this is an important factor that may lead to different stimulation responses. The sample size is another limitation and including more patients for the studies would provide more reliable results. Using a very large sample group with women having the same characteristics and following the exact same stimulation protocol would be ideal to obtain reliable results, but unfortunately this is technically challenging. Finally, we cannot discard other important genes involved in ovarian response as *FSHR* could explain the disagreement between studies.

For the *ESR1* microsatellite repeat, the values were categorized into two groups; the first including patients with less than 17 repeats, and the second including those with 17 repeats or more. When comparing this data to the ovarian stimulation data, patients having less than 17 repeats retrieved more oocytes but does not have any effect on the length of stimulation nor the amount of gonadotropins consumed. A previous study showed that the ESR1(TA)n biallelic mean was positive correlated with the number of follicles matured, as well as the oocytes obtained [23]. Previous studies analysing this polymorphism found that unexplained infertility seemed to be associated with shorter repeats, as the biallelic mean obtained for the infertility group was smaller than the reference [27]. Following this assumption, we would expect the combination of more repeats and CC/CT genotype (≥17+CC/CT) to retrieve the most oocytes. According to our data, the allele combination of ≥17+CC/CT was observed to be the most disadvantegous combination. Recently, a study including 150 women is also consistent with the findings of our study showing that shorter *ESR1* (TA)n microsatellite repeat polymorphism has been reported to be associated with an improved ovarian response to FSH and with successful IVF outcome [28].

Two SNPs were analysed regarding the AMH, one present in the *AMH* gene and one on its receptor (AMHR2). The AMH is a transforming growth factor β involved in tissue growth and female differentiation during embryonic stages. It also hampers follicle depletion by inhibiting follicles from transitioning. In the case of the *AMH* gene SNP, the polymorphism analysed causes T>G change in the sequence, leading to a change in the amino acid sequence (p.I49S), no association was observed between genotypes and length of stimulation, gonadotropins used nor the number of oocytes retrieved. AMH is known to interact with two receptors, AMHR1 and AMHR2, inhibiting the recruitment of primordial follicles to grow follicles, therefore leading to less follicles. AMHR2 is a serine/threoinine kinase receptor playing an essential role in sex differentiation. An SNP in the AMH receptors might therefore lead to a less bioactive complex, resulting in the recruitment of more follicles. The variant present in AMHR2 that was analysed in this study corresponds to a variation causing an A>G change in the promoter sequence of the gene (position c.-482). Our data showed that the AG and GG genotype seemed to require a longer length of stimulation and more gonadotropins, and therefore, these two were compared with the AA genotype. These results demonstrate that carrying the AA genotype may be beneficial in COS as these patients require a shorter length of stimulation as well as less gonadotropins. Karagiorga *et al.* [29] evaluated the same variant and observed that carriers of the G allele in AMHR2 had a lower number of follicles, and similar results were obtained by Yoshida *et al.* [12] and Peluso *et al.* [30], where women carrying the G allele retrieved less oocytes. This is in accordance with our findings and is believed to be caused by the abnormal functioning of the protein due to the SNP, impeding AMH performance and resulting in less follicles [29]. These results therefore indicate that *AMH* and *AMHR2* SNP genotypes may have an effect on COS outcome through changes in the AMH signalling pathway, resulting in more follicle recruitment. However, further investigation on this signalling pathway as well as a more extensive analysis of these SNPs should be performed.

In addition to *ESR1* and *AMHR2* genes SNPs, we evaluated the effect of the following variants in the ovarian response. For the GDF-9 gene, two different SNPs were analysed. The first one corresponds to rs10491279, an SNP causing a change in one of the base pairs at position 546 (c.546G>A). The codon involved in this SNP is GAG, leading to GAA and coding for glutamine. Despite the change in the base pair, the amino acid is not affected and therefore the protein remains unchanged. The second SNP analysed that was present in the GDF-9 gene was rs254286, accounting for a change in the sequence at position 447 (c.447C>T). This base pair change is present in the coding region; however, it does not cause an amino acid change due to the redundancy of the genetic code. Therefore, the amino acid remains threonine. GDF-9 is part of the transforming growth factor beta superfamily and plays an essential role in folliculogenesis, promoting oocyte maturation and embryo development. Polymorphisms in this gene have been associated with poor ovarian stimulation outcome [31,32]. In our study, no significant difference was observed between different genotypes in the length of stimulation nor in the gonadotropins consumed neither the number of retrieved oocytes. Discrepancy between results may be due to the

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studied population, because our study was conducted on donor eggs while their study was performed on infertile women undergoing IVF treatment thus a heterogeneous population.

LHCGR cell surface is the receptor for two different hormones; luteinizing hormone and chorionic gonadotropin. Consequently, this gene is involved in the maturation of follicles. Regarding the LHCBR gene, one of the most common SNPs present on this gene was analysed and corresponds to rs2293275, a variant leading to an amino acid change at position 312; serine to asparagine (p.S312R). Our results showed no association between genotypes and ovarian response. This suggests that variation in this SNP does not have any influence on COS outcome. This is in accordance with previous research studies in which no difference in the number of follicles or retrieved oocytes was observed among genotypes [21].

Besides *ESR1* gene, microsatellite repeats were analysed to the next genes. The genetic variant analysed in the SHBG is rs6761, which is a (TAAAA)n microsatellite repeat. The SHBG gene encodes a glycoprotein whose function is to bind to androgens (oestradiol and testosterone), therefore regulating the concentration of active androgens in serum. The pentanucleotide (TAAAA)n repeat polymorphism has been reported to modify the transcriptional activity of the gene, causing increase/decrease in levels of SHBG, which result in changes in the levels of free androgen in serum [33]. Our results showed no association between genotypes and ovarian response factors. In previous studies, it was observed that women carrying longer repeats had an increased number of follicles and oocytes [34]. Discrepancy between results may be due to the difference in the type of the population and analysis performed. Further research should be performed to confirm these findings and obtain more reliable results. The microsatellite analysed in CYP19A1 corresponds to rs60271534, variant containing (TTTA)n repeats. CYP19A1 gene encodes the cytochrome P450 aromatase, which is an enzyme involved in the conversion of androgens to oestrogens. Malfunction of this protein causes increased androgen levels which can result in polycystic ovary syndrome. The most commonly studied variant is an intronic tetranucleotide repeat (TTTA)n [35]. This gene encodes the cytochrome P450 aromatase, which is an enzyme involved in the production of oestrogens and androgens. Previous studies found that carriers of the shorter allele of this polymorphism had lower FSH sensitivity, fewer antral follicles, as well as a reduced size [36,37], suggesting the shorter allele to be associated with poor response to stimulation. This, however, has not been reflected on our data, just as for SHBG, no significant results were obtained for any of the ovarian stimulation factors, suggesting that the length of this repeat does not have a role on COS outcome. Moreover, at SNPs level, variants in the CYP19A1 did not affect the number of oocytes recovered nor their maturation level, suggesting that other pathways may contribute to the formation of the final oestradiol metabolite as well as its impact on the oocyte maturation [38]. Therefore, further research should be performed using a bigger sample population, different variants as well as a population of women with the same characteristics.

Finally, a highly polymorphic CAG repeat found in exon 1 and coding for glutamine was analysed in the *AR* gene. The AR mediates the action of androgens and is expressed in a variety of tissues, playing an essential role in the development of the sexual differentiation amongst others. A (CAG)n repeat expansion encoding a polyglutamine region has been studied, resulting in a downregulation of androgen-regulated genes due to changes in the transactivational activity [39]. When comparing the number of repeats to the ovarian stimulation data, no significance was obtained suggesting that there is no correlation. Several studies have been performed analysing this variant and its effect on ovarian stimulation; however, no relationship was obtained. In contrast, the length of the polymorphism has been suggested to be associated with poor responders. These individuals tend to carry longer repeats (more than 22) than the general population [40].

In conclusion in this research, genetic variants located on the genes presented showed a clear relationship with ovarian stimulation, modulating the ovarian response to gonadotropin stimulation. The polymorphisms in the ESR1 and AMHR2 genes showed a clear association with the number of retrieved oocytes and the stimulation data, respectively. This study has provided relevant information regarding SNP genotypes and their relationship with ovarian stimulation because the most important confounding factors as the same dose and stimulation protocol, FSHR genotype and profile population were controlled. Besides, our study has some limitations; a prospective randomize trail with a higher sample size should be used in future studies to corroborate the current findings and to demonstrate the benefit of the genotyping to improve the COH. In addition, research into the contribution of the effect of the polymorphism in the folliculogenesis will be of a great interest.

Our results suggest that polymorphisms in the genes for key reproductive hormones receptors on a combination of the patient's clinical characteristics, functional and hormonal biomarkers could be used to predict the ovarian response, to personalize and adjust the stimulation drugs prior the overtaken treatment, to improve efficacy and patient compliance to COS to decrease adverse drug reactions; and, last, to reduce the time to pregnancy [41].

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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