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A pharmacogenetic approach to improve low ovarian response: The role of CAG repeats length in the androgen receptor gene

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ABSTRACT

The AR (androgen receptor) polymorphism is associated with POR risk. Furthermore, the use of androgens in POR remains controversial. Our data could clarify the effectiveness of androgen pretreatment. AR genotyping could help us to identify patients at risk for POR and POR patients that will be benefited of androgen pretreatment.

Objective: The aim of this project was to investigate if the AR (androgen receptor) polymorphism could be used to identify patients at risk for POR and that will benefit from androgens pretreatment.

Study Design: To evaluate the POR risk we performed a cohort study including 231 patients (54 POR and 177 control). Moreover, we included 88 IVF-cycles performed by 44 POR-patients to assess the effect on ovarian response. All patients performed two cycles: a standard ovarian stimulation and a second one with androgen preparation. We compare the results in pair from each.

Results: POR showed the highest frequency of CAG repeats at 24 vs 22 in controls. Only 33% of POR have alleles with a repeat number below 23, compared with 50% of controls ($p < 0.05$). According to AR polymorphism ovarian response differences were shown. Patients that carried CAG repeats in *AR* gene between 22 and 24 showed an increased in the number of oocytes (2.61 in cycles without androgens vs 5.11 when they were pretreated with androgens; $p < 0.05$). For the patients that carried repeats lower than 22 and higher than 24, no differences were reported in the number of oocytes obtained in the cycle with or without androgens (2.94 vs 2.56; $p = 0.88$). Similar results were obtained for mature oocytes in patients that carry a number of CAG repeats between 22 and 24 (1.86 MII in cycles without androgens vs 4.04 MII when they were pretreated with androgens; $p < 0.05$). No differences in the number of MII oocytes were found in patients that get out of 22 and 24 repeats between the two cycles (2.31 vs 2.13; $p = 0.88$).

Conclusion: The AR polymorphism is associated with POR risk, patients with repeats greater than 22 show a higher risk. Our data suggest that AR genotype could play a role in natural ovarian aging. Furthermore, the use of androgens in POR remains controversial. Our data suggest that the AR genotype could clarify the effectiveness of the androgen pretreatment. AR genotyping could help us to identify patients at risk of POR and POR patients that could benefit from transdermal testosterone pretreatment.

Introduction

Despite the great advances in the field of assisted reproductive technologies (ART) in recent years, one of the fundamental steps to achieve success is still dependent on the number of eggs obtained after controlled ovarian stimulation (COS) [1,2].

Patients with low response to COS have been a challenge for ART and nowadays poor ovarian responders (POR) constitute 9–24% of all women undergoing COS [3]. The incidence is probably increasing because women are delaying motherhood and the average age at which women have access to ART is growing [4]. This fact becomes clinically relevant to predict the length of their fertile lifespan, which may be used during informed decision-making about timing of childbearing. The large variability in ovarian reserve in patients of the same age has prompted researchers to find a more reliable marker than chronological age to predict POR.

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Recently, new biomarkers, such as the serum concentration of anti-Müllerian hormone (AMH) and antral follicular count (AFC), have been suggested as valuable markers for predicting the ovarian reserve and the response to COS [5,6]. However, the variation of the AMH values during the life of women is not well known [7]. Thus genetics could be an option to predict the end of reproductive lifespan for a woman allowing to make decisions about family planning.

Although many factors influence the effects of medications, during recent years it has become evident that genetic factors could explain the differences between individuals in drug response. These differences are due to variants in genes encoding drug targets [8]. The challenge for pharmacogenetics is to establish the relation between gene variant and medication response, to develop diagnostic tests that can predict drug action and adjust therapy accordingly [8]. Therefore, genetics could be used to predict reproductive lifespan and to select the best treatment to obtain the best outcome.

The AR gene has been associated with ovarian failure in both animals [9] and humans [10]. The role of testosterone as an estradiol precursor in females is well understood [11]. However, the direct involvement of androgens in female reproductive physiology remains controversial [12]. Recent studies appear to support the importance of proper androgen levels to female fertility [13] and some strategies have been developed with the ultimate objective to increase ovarian response [14].

The human *AR* (androgen receptor) gene contains a highly polymorphic CAG repeat sequence within exon 1. Recently, variations of the AR-CAG tract, while still within the normal range (11–38), have been linked to an increase in the severity of different diseases [15]. Recent researches reported an association between CAG repeats in *AR* gene with premature ovarian failure patients [10,16].

The aim of this study is to investigate whether CAG polymorphism on *AR* gene have predictive value for POR and if it could be used to select patients that could benefit from the use of androgens in previous cycles. From our knowledge, nothing is known about the potential effect of CAG repeats in *AR* gene and COS in POR patients. In order to find a possible correlation between CAG on *AR* gene polymorphism and POR we proposed to evaluate the difference of biallelic mean of CAG-tract between controls and POR. To evaluate the effect of AR polymorphism in the COS where androgens were used, we compared the cycles with and without androgens as a pretreatment performed by the same patient.

Materials and methods

Study population

The selection and recruitment of donors in our clinic is carried out following strict quality criteria, including an extensive genetic evaluation. All donors met the legal requirements (Spanish Law 14/2006). Both ASRM and ESHRE guidelines for oocyte donors are followed.

POR patients were selected according to the Bologna criteria. Briefly, at least two of the following three features must be present: (a) advanced maternal age or any other risk factor for POR; (b) a previous POR; and (c) an abnormal ovarian reserve test [17]. In order to avoid natural ovarian aging we included patients aged below 39 years.

In this study, we included the results of CAG polymorphism in the *AR* gene from 231 women: 177 oocyte donors and 54 POR patients. Moreover 44 POR patients that performed 88 IVF cycles were genotyped. The results from stimulation were showed in the present research.

All the subjects included in the study gave their informed consent to collect peripheral blood samples suitable for molecular analysis. This study involved only retrospective analysis of anonymous medical records and was approved by the Instituto Bernabeu Institutional Review Board.

Genotyping

DNA was isolated from pheripheral blood according to the manufacturer instructions (Wizard® Genomic DNA Purification Kit, Promega, USA). CAG repeat of exon 1 in the *AR* gene was amplified from the genomic DNA using the TaKaRa LA Taq kit (Takara Bio Inc, Shiga, Japan) and primers flanking the CAG repeat region [18]. For genotyping 1.0 μl of PCR product analyzed on ABI 310 DNA analyzer (Thermo Fisher, Madrid, Spain) using Gene Mapper software to ascertain the size of AR alleles and the number of repeats. The number of CAG repeats was calculated in relation to a standards obtained by sequencing.

Ovarian stimulation and oocyte retrieval

Stimulation was initiated on day 3 of the menstrual cycle with 150 IU of rFSH (Gonal-F; Merck) and 150 IU of hMG (Menopur HP; Ferring). A flexible approach for antagonist cotreatment (Orgalutran, Organon; or Cetrotide, Merck) was initiated whenever the leading follicle reached 14 mm in diameter. Final oocyte maturation was triggered with a single injection of recombinant HCG (Ovitrelle; Merck). Oocyte pick-up (OPU) was performed 36 h later.

Testosterone priming

In the cycle using androgen priming, a daily dose of 10 mg of testosterone gel (Testim; Ferring) was applied transdermally onto the inner thigh daily, for 21 days, as suggested by Kim et al. [19]. Testosterone was dosed introducing the entire contents of the tube (50 mg) in a syringe and subsequent administration of 1/5 of that amount.

Statistical analysis

Values are presented as averages ± SD for continuous data and percentages for categorical variables. Data were analyzed with Statistical Package for the Social Sciences (SPSS) software (version 20.0, SPSS, Inc., Chicago, IL, USA). For repeats CAG length we considered the biallelic mean, as previous reports. The primary endpoints were total number of oocytes and MII retrieved. Continuous variables were tested using a t-student for paired samples to evaluate differences among the groups. Pearson's chi-square test was used for categorical variables. A $p < 0.05$ was considered significant.

Results

Comparative analysis of CAG repeats length genotyping between donors and POR

The clinical characteristics of the POR patients are detailed in Table 1. These data agree with the definition of POR, patients with low AFC (3.4 ± 1.8) and AMH (0.6 ± 0.5 ng/ml). According to our selection criteria the mean age was 34.2 ± 3.0 years.

In this study we included the results of the AR CAG repeat counts obtained from 231 women (177 control oocyte donors and 54 POR). The AR CAG repeat numbers were in the normal polymorphic range in all patients. The frequency of the biallelic mean of both groups are represented in Fig. 1a. In the 177 oocyte donors analyzed the biallelic mean of CAG repeats ranged from 18 to 29. The 22 CAG repeat alleles were found to be at the highest frequency. For the 54 POR patients included in the study, the

Table 1
Clinical characteristic of POR patients.

Total n = 54	Average±SD
Patient Age (years)	34.2 ± 3.0
AMH (ng/ml)	0.6 ± 0.5
AFC	3.4 ± 1.8
Retrieved oocytes in previous cycle	2.7 ± 1.3
% of previous cancelled cycle[a]	48.1

[a] Cancelled because a number of follicles lower than 3.

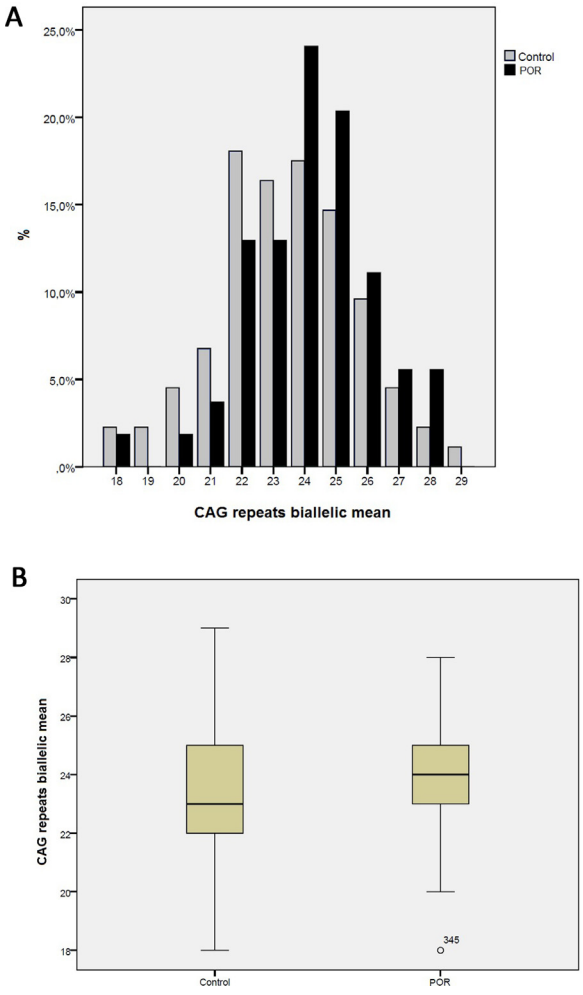

Fig. 1. CAG repeat length in *AR* gene of control and POR. (a) Biallelic mean distribution between POR and controls. (b) Biallelic comparison between groups ($p < 0.05$).

biallelic mean of CAG repeats ranged from 18 to 28. The 24 CAG repeat alleles were found to be at highest frequency. Fig. 1b compares the biallelic mean of CAG repeats between both groups, significant differences were reported between donors and POR (23.46 vs 24.15 $p < 0.05$). Only 33% of POR patients have alleles with repeat number below 23, compared with 50% of controls ($p < 0.05$).

Ovarian stimulation outcomes in POR with or without transdermal testosterone priming

In the study group 88 cycles were performed by 44 patients: one treatment without androgens pretreatment and the second one with androgens preparation. The ovarian stimulation parameters were compared in pair from each between the cycles with or without androgens used as a pretreatment by the same patient. Table 2 summarizes the ovarian stimulation parameters in the 88 COS cycles. The duration of stimulation and total dose of gonadotrophins required were similar between androgens pretreatment and no pretreatment cycles. Differences were shown in the female age (35.9±3.3y in cycles pretreated without androgens vs 36.7±3.5y in cycles pretreated with androgens for group 1 and 34.7±3.4y in cycles pretreated without androgens vs 36.1±3.7y in cycles pretreated with androgens for group 2; $p < 0.0001$) (Table 2). Patients in cycles with androgens were older than the previous cycle without androgens. This difference is in favour of better COS outcome in the first cycle where patients were younger. According to AR polymorphism and ovarian response differences were shown in oocyte and MII yield (Table 2). Patients that carried CAG repeats in *AR* gene between 22 and 24 showed an increased in the number of oocytes when they were pretreated with androgens, from 2.61 oocytes yielded in the cycle without androgens to 5.11 in the cycle with androgens ($p < 0.0001$). For the patients that carried a number of repeats lower than 22 and higher than 24 no differences were reported in the number of oocytes obtained in the cycles with or without androgens (2.94 vs 2.56; $p = 0.88$). Similar result was obtained for mature oocytes. More MII oocytes were obtained in the pre-treated cycle in patients that carry a number of CAG repeats between 22 and 24 (1.86 vs 4.04; $p < 0.0001$). No differences in the number of MII oocytes were found in patients that get out of 22 and 24 repeats between the two cycles (2.31 vs 2.13; $p = 0.88$).

Discussion

The present study showed that CAG repeat length in exon 1 of the *AR* gene in POR patients trends to be longer than that observed in women belonging to the general population. To our knowledge this is the first investigation about the involvement of *AR* gene polymorphism in POR. Moreover, our data suggest that the AR genotype could clarify the effectiveness of the androgens pretreatment. AR genotyping could help us to identify POR patients that will be actually benefited from transdermal testosterone pretreatment.

For the first time we have a consensus about definition of POR. According with the Bologna criteria we can determine a homogeneous population [20,21]. In our study we decided to consider only patients under 39 years and no known cause or risk factor for POR. It means that all our patients were previously under COS with low response and all of them had biomarkers of POR. We included in the study group only young patients trying to avoid physiologic low ovarian reserve assuming that it's no useful to predict a low fertility potential in women over 38 years [22].

Analysis of 54 cases of POR showed that the mean CAG repeat length in exon 1 of AR gene was significantly increased compared with egg donors. POR patients showed a maximum number of repeats at 24 that decreases slowly over the interval. On the other hand, the control group showed a maximum mid-range (22 repeats) with two tails along which decreases relative frequency. The relative frequency in distribution of number CAG repeats in POR group is shifted to the right, compared to controls. Only 33% of the group of patients have alleles with repeat number below 23, compared with 50% of controls ($p < 0.05$).

While this is the first study to analyse the distribution of this polymorphism in patients with POR, its relationship with premature ovarian insufficiency (POI) has been investigated with discordant results. A study reported no significant differences in allele distribution of the CAG-tract between controls and POI in Caucasian [23]. Another investigation in Japanese women found shorter CAG repeat compared to control [16]. Finally, Chatterjee et al found longer CAG repeat in exon 1 of the AR gene in Indian

Table 2
Ovarian stimulation data in relation to AR genotype in POR patients.

AR genotype (CAG repeats)	No pretreatment	22–24			<22–>24			
Cycle	Average + SD	Testosterone pretreatment Average + SD	IC 95%	p	No pretreatment Average + SD	Testosterone pretreatment Average + SD		p
N	28	28			16	16		
Age	35.9 ± 3.3	36.7 + 3.5	(−1.1, −0.4)	0.0001	34.7 ± 3.4	36.1 + 3.7	(−2.2, −0.7)	0.001
Length of stimulation (days)	10.2 ± 2.0	10.8 ± 1.4	(−1.5, 0.4)	0.264	9.0 ± 2.5	9.1 ± 1.9	(−1.7, 1,5)	0.870
Gonadotropin used (IU)	3005 ± 1014	3321 ± 901	(−763, 131)	0.158	2958 ± 1409	2465 ± 673	(−308, 1292)	0.210
No. of retrieved oocytes	2.6 ± 1.8	5.1 ± 3.1	(−3.6, −1.4)	0.0001	2.9 ± 2.7	2.5 ± 2.1	(−1.4, 2.2)	0.660
No. MII	1.9 ± 1.5	4.0 ± 2.5	(−3.1, −1.2)	0.0001	2.3 ± 2.2	2.1 ± 1.9	(−1.4, 1.8)	0.804

Test performed for statistical analysis T-student for paired samples.

patients with POI [10]. Heterogeneity of patients with POI and different ethnic backgrounds may explain the conflicting results. Moreover, in normal ovarian reserve patients, it has been shown that AFC is associated with CAG polymorphism of the *AR* gene [24].

According to the aetiology, the relation between POR and AR polymophisms could be explained by two hypotheses: the influence of the hypoandrogenicity in the ovarian response and the influence of the androgen function in accelerate follicle depletion and therefore diminishing the ovarian reserve.

AR is expressed in cell-specific human ovarian follicles at all stages of follicular development [25]. Administration of androgens in animals demonstrated initiation of follicular recruitment, stimulation of early stages of follicular growth, and increase in the number of growing follicles [26,27]. The main mechanism to explain this fact could be that the androgens enhance the follicular sensitivity by increasing FSH receptor (FSHR) levels [28]. In humans, basal serum testosterone levels correlate with ovarian response in COS [29–31] and the improvement of COS after the administration of exogenous androgens in POR patients has been reported [32], although their use is controversial [33,34]. Recently, a randomized trial in POR patients was published reporting that testosterone pretreatment failed to increase the number of retrieved oocytes in POR patients [35]. According to our results, the controversies regarding the efficacy of androgens could be explained by the genotype in the *AR* gene. Our data suggest that patients that carried CAG repeats in *AR* gene between 22 and 24 showed an increased in the number of retrieved oocytes and MII when they were pretreated with androgens. Among patients with a number of repeats that get out of 22 and 24 no differences were found between cycles. Thus, in order to show the benefit of testosterone pretreatment in POR we need to evaluate the AR genotype to avoid confounding factors concerning different response. Future trails to explore the clinical efficacy of androgens should include the AR genotype. Recent work reported that the highest AR activity was confined to the 22 repeats CAG [36]. A poliglutamine tract of about 22 would represent the baseline activation status of the AR. A higher or lower number of glutamine residues would lead to a lower activation of the androgren-regulated genes [36]. This may explain why only patients which carried a number of CAG repeats in a range near to the highest AR activity could be benefited of the use of androgens to improve the COS.

In summary, we provided evidence to show that CAG repeat length in exon 1 of the AR gene is related with POR. This discovery together with other genetic polymorphisms could help in the future to predict years in advance the likelihood of developing a low ovarian response. Moreover, to our knowledge, these data show for the first time the relation between AR gene polymorphism and the ovarian response in androgen pretreatment cycles. This investigation reveals that in POR patients the *AR* gene polymorphism could be used as a pharmacogenetic tool to identify patients that would be benefited of the use of androgens to improve their response. Furthermore, more studies will be needed to clarify the role of androgens on the ovarian response to stimulation and also on the follicular depletion.

Conflicts of interest and source of funding

None declared.

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