Single nucleotide polymorphisms in the Anti-Müllerian hormone (AMH Ile⁴⁹Ser) and Anti-Müllerian hormone type II receptor (AMHR II –482 A>G) as genetic markers in assisted reproduction technology

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Abstract

Purpose The aim of the study was to evaluate whether the presence Antimüllerian hormone (AMH) and Antimüllerian hormone type II receptor (AMHR II) single nucleotide polymorphisms (SNPs) Ile⁴⁹Ser and -482A>G respectively are related to the assisted reproduction outcome.

Methods A prospective cross-sectional observational study was conducted in order to assess the distribution of AMH and

AMHR II SNPs in two cohorts, one of healthy women ($N=100$) and the control group and the IVF/ICSI group ($N=151$) consisted of women undergoing IVF/ICSI treatment for infertility. Furthermore, a prospective longitudinal observational study was performed on the latter group to assess possible associations of these SNPs with patients' characteristics and controlled ovarian stimulation (COS) and pregnancy outcome. **Results** Among non-carriers of the AMH (Ile⁴⁹Ser) polymorphism, basal FSH levels were lower in those with more than two of previous IVF attempts and fertilization rate was statistically higher in those with peak serum E2 levels below 1500 pg/ml, whereas among non-carriers of the AMHR II (-482 A>G) polymorphism, number of follicles was higher in those with more than two previous IVF attempts and total dose of gonadotropins was lower in those with peak serum E2 levels above 1500 pg/ml.

Conclusions There was evidence that in specific subgroups of women undergoing IVF/ICSI, AMH and AMHR II SNPs may be related to patients' characteristics and controlled ovarian stimulation and pregnancy outcome and thus may provide a means for the prediction of ovarian response in specific subgroups of women entering an IVF/ICSI program.

Keywords AMH · AMHR II · SNPs · ART · Genetic markers

Capsule SNPs in AMH and AMHR II and ART.

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Introduction

Infertility, defined as the failure to achieve pregnancy after a year of unprotected intercourse, presents higher incidence over the past years [1]. Infertile couples benefit from assisted reproduction technology (ART) methods, like in vitro fertilization (IVF). However, standard stimulation protocols in IVF are associated with variable responses to exogenous

gonadotropin treatment, namely good response, poor response and the occurrence of ovarian hyperstimulation syndrome (OHSS). Despite several years of clinical experience, precise prediction of ovarian response to controlled ovarian stimulation (COS) remains difficult. Both the variability in patient characteristics and in the response to IVF treatment illustrate the need for personalized management strategies in order to reduce the rates of cancelled IVF cycles and to optimize efficacy and safety outcomes [2, 3]. Genetic variation in signaling pathways of the ovary could play an important role in ovarian response and IVF outcomes. Studies have shown that differences seen between individuals or subpopulations of patients are often due to genetic variations, known as single nucleotide polymorphisms (SNPs) in coding regions responsible for the synthesis of hormone receptors, metabolic enzymes or transport molecules, which are specific targets for pharmaceutical drugs. The most studied SNP associated with ovarian response to COS, is the follicle stimulation hormone receptor (FSHR) variant (Ans⁶⁸⁰Ser), which along with estrogen receptor alpha variant (ESR1 gene, PvuII T/C) are practically the only genetic markers applied in clinical tests [4–7].

Anti-Müllerian hormone (AMH) constitutes a dimeric glycoprotein, member of the transforming growth factor-beta (TGF- β) superfamily [8]. In women, AMH is produced exclusively in the ovary by the granulosa cells of pre-antral and small antral follicles. The gene for AMH is located on chromosome 19 and consists of 5 exons [9, 10]. The expression pattern of AMH seems to be similar in mice and human ovary. AMH affects two major regulatory steps of folliculogenesis, the inhibition of primordial follicle recruitment and the modification of the follicle stimulation hormone (FSH) sensitivity of small antral follicles in the process of cyclic selection [11, 12]. AMH exerts its biological effects through the AMH type II receptor (AMHRII), which in turn activates AMH type I receptor (AMHRI) and creates a downstream signaling pathway. The human gene for AMHRII is located on chromosome 12, consists of 11 exons and is expressed in granulosa and theca cells [13, 14].

AMH appears to be the ideal marker for ovarian function and ovarian reserve reflecting the decline of reproductive age, as its levels are strongly correlated with the number of antral follicles detected by ultrasound [15] and are indicative of the size of the primordial follicle pool [16]. Concerning the field of reproductive medicine, extremely interesting new data on the possible clinical application of AMH measurement in the prediction of quantitative and qualitative ovarian response in ART have been published in the last few years, since AMH exhibits a relatively stable expression throughout the menstrual cycle [17]. This suggests that AMH is an attractive determinant of ovarian activity.

So far, studies in women have focused on the role of serum AMH as a marker for ovarian response. Genetic variation in AMH was studied by Kevenaar and its colleagues, who demonstrated that the AMH (Ile⁴⁹Ser) and the AMHR II (–482

A>G) SNPs were associated with an increase in estradiol (E2) levels during the follicular phase of the menstrual cycle in healthy normo-ovulatory women. This study, also, suggested that both polymorphisms contribute to the individual variation in the FSH “threshold” [18]. This concept was initially introduced by Brown [19] and now constitutes the basis for gonadotropin administration in controlled ovarian stimulation (COS) in order to achieve multiple follicular growth [20, 21]. The maintenance of follicles’ growth in order to reach the pre-ovulatory stage depends on whether FSH levels surpass a distinct concentration (FSH “threshold”) or not. Thus, the follicle that exhibits the lowest individual FSH requirement will reach the pre-ovulatory stage and, finally, ovulation [20]. Based on the above, AMH and AMHR II SNPs may modify follicular sensitivity to FSH, and this may play a role in the process of follicle selection irrespective of serum AMH concentrations.

Taking this hypothesis into consideration, it is of high importance to investigate whether these SNPs are present in women entering IVF protocols and have an impact in the clinical setting through the suggested modification in the bioactivity of AMH. Only one study has been conducted regarding the role of AMH and AMHR II SNPs on ovarian stimulation outcome and concluded that none of the SNPs determines high or low response to ovarian stimulation [22].

The present study explores for the first time prospectively the distribution of the AMH (Ile⁴⁹Ser, NCBI SNP reference rs10407022) and the AMHR II (–482 A>G, NCBI SNP reference rs2002555) polymorphisms in a Greek population. Furthermore, it represents a replication effort to elucidate the involvement of the polymorphisms in IVF parameters and ovarian stimulation outcome. This observational molecular genetic study will help to elucidate whether AMH and AMHR II SNPs can be applied as genetic markers, which can be of additive value in the prediction of ovarian response to exogenous gonadotropins.

Materials and methods

Patients

A prospective cross-sectional observational study was conducted on two cohorts, one of healthy women with a history of at least one successful previous pregnancy resulting from spontaneous conception as the control group and the case group (IVF/ICSI group), which was consisted of women undergoing IVF or ICSI treatment, in order to assess the distribution of AMH and AMHR II SNPs. In fact, from January to December 2013, a total of 330 women, who attended Fertility Institute, Diagnostic and Therapeutic Centre S.A., a private Medical Centre in Athens, were assessed for eligibility, 130 in the control group and 200 in the case group, among

which 121 and 176 were found eligible and finally 100 and 151 agreed to participate in each group respectively (Fig. 1). Women assigned to the case group underwent IVF/ICSI within a 3-month period following recruitment.

Furthermore, a prospective longitudinal observational study was performed on the case group to assess possible associations of these SNPs with patients characteristics and COS and pregnancy outcome. A subgroup analysis was also conducted in these women in order to investigate potential effects of the polymorphisms in women who present a certain combination of parameters.

The IVF/ICSI group consisted of infertile women (mean age 36 ± 5 years, mean $\pm$ SD) who had at least one year of infertility before entering the study. Other inclusion criteria were common in both groups and included age ≤ 46 years old, a regular menstrual cycle of 25 to 35 days, a normal hormone profile (according to WHO guidelines) and the presence of both ovaries. Patients with polycystic ovary syndrome (as described by Rotterdam criteria) were excluded from the study. The indications for fertility treatment for the 151 patients were male factor, tubal factor, other causes of infertility, complex etiology, and unexplained infertility. None of the patients had received ovulation induction or any other hormone treatment within three months preceding the study. Patients' demographic characteristics (age, BMI, duration of infertility, IVF trials, indication for IVF) were recorded before ovarian stimulation. In addition, stimulation dose, duration of ovarian stimulation, numbers of follicles and oocytes as well as maturation and fertilization rates were determined for each patient during the treatment cycle. The study was performed in accordance with the Helsinki Declaration of 1964 (as amended in 1983 and 1989) and approved by the Institutional review board of Fertility Institute before screening of the first patient. Furthermore, written informed consent was provided before study entry.

Hormone assays

Basal (day 3) serum FSH and Luteinizing Hormone (LH) levels were measured by electrochemiluminescence immunoassay (Roche Molecular, Biochemicals, Mannheim, Germany). Estradiol levels were evaluated using a commercially available chemiluminescent Microparticle Immunoassay (CMIA) kit (Abbott Laboratories, Abbott Park, IL, USA). Serum AMH levels were evaluated for all subjects before entering IVF program. AMH concentrations were evaluated using AMH Gen II ELISA kit (Beckman Coulter, Switzerland). Both FSH and LH assays had sensitivity of 0.100 mIU/mL and specificity of 99.9 %. The Estradiol assay had sensitivity of ≤ 10 pg/mL, precision of ≤ 5 pg/mL (total SD) for concentrations in the range of the low control (target 45 pg/mL), and ≤ 7 % total CV for concentrations in the range of the medium control (target 190 pg/mL) and the high control (target 600 pg/mL). Finally, AMH assay had sensitivity of 0.57 pmol/L, specificity 100 % and reported intra- and interassay coefficients of variation of less than 5.4 % and 5.6 %, respectively, according to the products' inserts.

COS and IVF/ICSI

COS was conducted using either a GnRH-agonist or a GnRH antagonist protocol. With regards to GnRH-agonist, both short and long protocols were implemented. Long GnRH-agonist protocol was briefly as follows: on day 21 of the previous cycle, a baseline ultrasound scan was performed and buserelin acetate intranasal spray administration began at a dose of 100 mcg five times per day (omitting the 03.00 AM dose). GnRH agonist administration was maintained until hCG was given to trigger final oocyte maturation. Ovarian suppression was evaluated by ultrasound scan and serum E_2 levels (≤ 40 pg/mL)

Fig. 1 Flow diagram illustrating the number of women assessed for eligibility, meeting the inclusion criteria and asked to participate and finally accepted to participate in the study

before starting exogenous gonadotropin administration (about 15 days after the spray onset) at a dose of 200 IU/day. Gonadotropin dose was adjusted from day 6 of stimulation according to the ovarian response monitored by daily serum estradiol determination from day 5 of stimulation and ultrasonography from day 6 of stimulation. hCG was administered when the mean diameter of at least two follicles was above 18 mm and serum estrogen levels were rising.

Short GnRH-agonist protocol was briefly as follows: on day 2 of cycle a baseline ultrasound scan and if indicated serum estradiol along with progesterone determination were conducted. Buserelin acetate intranasal spray was started on cycle day 2 at a dose of 100 mcg five times per day (omitting the 03.00 AM dose) and was maintained until hCG was given to trigger final oocyte maturation. Gonadotropin administration began on day 3 at a dose of 200 IU/day. The dose of gonadotropin was adjusted according to ovarian response on a daily basis, 6 days after the onset of gonadotropin administration onwards.

Plasma E₂ levels were measured daily starting on day 5 of ovarian stimulation with gonadotropins (day 7 of cycle) until the day after hCG administration. The first ultrasound scan was performed on day 6 of stimulation (day 8 of cycle) and subsequent scans were performed every day until oocyte retrieval.

GnRH-antagonist protocol implemented was the fixed multiple dose one. In particular, on day 2 of cycle a baseline ultrasound scan and if indicated serum estradiol along with progesterone determination were performed. Gonadotropin was initiated at a dose of 200 IU the same day and a fixed daily dose of 250 mcg of GnRH antagonist was given from day 5 of stimulation (day 6 of cycle) including the day of hCG administration. The dose of gonadotropin was adjusted on a daily basis from day 6 of stimulation days onwards, based on ovarian response. Plasma E₂ levels were measured daily starting on day 5 of ovarian stimulation with gonadotropins (day 6 of cycle) until the day after hCG administration. The first ultrasound scan was performed on day 6 of stimulation (day 7 of cycle) and subsequent scans were performed every day until oocyte retrieval.

Follicular aspiration and oocyte retrieval took place 35–36 h after the administration of 10,000 IU hCG by transvaginal ultrasound-guided puncture. Oocytes were assessed for their maturity and fertilization was performed with either conventional IVF or ICSI, depending on the presence of male factor infertility and the history of previous unsuccessful fertilization attempts through conventional IVF.

All ultrasound scans were performed by the same clinician. A single experienced clinician performed all oocyte retrievals and embryo-transfers and a single embryologist conducted fertilization with IVF or ICSI for all patients.

Embryos were scored and chosen for transfer based on normal cleavage rate, absence of fragmentation, and even sized blastomeres [23]. Clinical pregnancy was defined as the presence of a gestational sac on ultrasound at six gestational weeks.

Single nucleotide polymorphism genotyping

Single Nucleotide Polymorphism Genotyping was conducted in the Molecular Biology Unit, Division of Human Reproduction, 1st Department of Obstetrics and Gynaecology, Alexandra Hospital, Athens University Medical School.

All samples were stored at -20° C after collection and subsequently were processed for genotyping.

Genomic DNA was isolated from peripheral blood leucocytes with the QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Both Control and IVF/ICSI group women were genotyped for AMH (Ile⁴⁹Ser, rs10407022) polymorphism in the AMH gene and AMHR2 (-482 A>G promoter, rs2002555) polymorphism in the non-coding region of the AMHR2 gene. Selection of SNPs was based on previous study performed on geographically contiguous populations by Kevenaar et al. (2007), the international HapMap project (<http://hapmap.ncbi.nlm.nih.gov>) and searches in dbSNP (<http://www.ncbi.nlm.nih.gov/snp>).

The genotyping of AMH Ile⁴⁹Ser and AMHR2 -482 A>G polymorphisms was performed with real-time PCR, using the Light Cycler 480 II (Roche Diagnostics, Germany).

For the AMH Ile⁴⁹Ser polymorphism, the PCR reaction was performed using as primer pairs primer S: gAggCCCTCagAgCagAggA and primer mis: CCagTgCCACagTCACagA and as detection probes Sensor wt 5'-gCTCTTgTgggCTgCCTggA - FL and Anchor AMH 5' - LC640 - gCCagTCCAagTCTTCTCggAAGATgAggC - PH.

For the AMHR2 -482 A>G promoter SNP, the PCR reaction was performed using as primer pairs primer S: gTgAggTCAAATgCTTAgaAgCCA and primer mis: TgAggCCTTCCTATgCCCA and as detection probes Anchor AMHR2 5' - CCTTTCTAgCCTAAgATCTCTTTATATTC Aggg - FL and Sensor C 5' - LC640 - CATATCTgCAAC CgCTTgg - PH (TIB MOLBIOL, Berlin, Germany).

The RT-PCR was performed in a final volume of 20 µL, containing 5 µg DNA, 4 × Master Mix, 0.25 µM of primer S, 1 µM of primer mis, 0.15 µM of each detection probe, for both SNPs.

The PCR amplification conditions were, for both SNPs, an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 59 °C for 20 s and 72 °C for 10 s. At the end of the reaction a melting curve analysis was performed.

To verify for false positive results, random samples detected with the polymorphisms were also processed for sequencing reaction.

Statistical analysis

The results of the present study were statistically analyzed using Statistics Package for Social Sciences (SPSS, Version 15), while the Sasieni algorithm (1997) and Hardy-Weinberg equilibrium were performed with the on line calculator which is available on <http://ihg.gsf.de>.

The statistical methods used for the control of statistical hypothesis were: two independent samples *t*-test, 2-proportion test (normal approximation) and parametric one way Analysis of Variance. For qualitative data used the chi-square test (Fisher exact test). Due to the deviation from normality, non-parametric Mann–Whitney *U* test was applied in order to evaluate the univariate association of demographic and biochemical factors. This test was used as a complementary statistical test due to the relatively limited number of women comprising each subgroup to double check the results obtained by the parametric test. In addition, multivariate analysis of variance was performed to adjust the results for age and BMI. It should be noted that statistical significance was set at the level of 5 % ($p < 0.05$).

Results

Distribution of gene polymorphisms in control and IVF/ICSI group

The distribution of genotypes of AMH and AMHR II SNPs for control and IVF/ICSI group is presented in Table 1.

In respect to the distribution of AMH (Ile⁴⁹Ser) polymorphism genotypes among IVF/ICSI patients, 64.2 % (97/151) were homozygous for the wild type allele (Ile/Ile), 33.1 % (50/151) were heterozygous (Ile/Ser) and 2.6 % (4/151) were homozygous for the polymorphic variant (Ser/Ser). “Ile”

corresponds to isoleucine amino acid, while “Ser” corresponds to Serine amino acid.

With regards to the prevalence of genotypes of the (–482 A>G) polymorphism of the AMHR II receptor, 69.6 % (103/148) were homozygous for the wild type allele (A/A), 29.1 % (43/148) were heterozygous (A/G) and two women (1.4 %, 2/148) were homozygous for the polymorphic allele (G/G). “A” corresponds to the more common allele, while “G” corresponds to the less common allele, usually referred as polymorphic variant.

Genotype frequencies observed during this study are in accordance with the Hardy-Weinberg equilibrium. The distribution of genotypes for both polymorphisms did not differ statistically between the control group and IVF/ICSI group, as shown in Table 1.

IVF/ICSI patient characteristics and characteristics of COS outcome

A total of 151 women undergoing IVF/ICSI were finally enrolled in this study. The mean age was 36 ± 5 years. Their indications for IVF/ICSI were as follows: tubal factor (48.2 %, $n = 68$), male factor (36.2 %, $n = 51$), other causes of infertility (7.8 %, $n = 11$), complex etiology (4.3 %, $n = 6$) and unexplained infertility (3.5 %, $n = 5$). Patients were recruited into either a GnRH-agonist or a GnRH-antagonist protocol based on their profile according to the common practice of the Center.

The basal serum FSH and LH were 9.1 ± 7.5 mIU/L and 6.7 ± 5.2 mIU/L, respectively. The mean AMH level was 10.9 ± 10.4 pmol/L and the mean PRL level was 16.7 ± 32.9 ng/ml. The mean stimulation days were 10 ± 1 , and an average of 3314 ± 1193 IU of gonadotropin was used during ovarian stimulation. The mean E2 on the day of hCG administration was 1928.9 ± 1283 pg/ml. The mean number of follicles and oocytes per patient were 7 ± 4 and 6 ± 4 , respectively. The total pregnancy rate was 17.8 % (number of pregnancies = 26).

Data regarding AMH levels were available for 109 out of 151 women entering IVF/ICSI cycle, while the detection of

Table 1 The distribution of genotypes for both AMH Ile⁴⁹Ser and AMHR II –482 A>G polymorphisms did not differ statistically between the control group and IVF/ICSI group

	AMH Ile ⁴⁹ Ser SNP				AMHR II –482 A>G SNP			
	Non-Carriers (Ile/Ile)	Carriers		<i>p</i> -value	Non-Carriers A/A	Carriers		<i>p</i> -value
		Carriers Heterozygous (Ile/Ser)	Carriers Homozygous (Ser/Ser)			Carriers Heterozygous (A/G)	Carriers Homozygous (G/G)	
Control ($n = 100$)	72 % ($n = 72$)	24 % ($n = 24$)	4 % ($n = 4$)	0.276	68 % ($n = 68$)	30 % ($n = 30$)	2 % ($n = 2$)	0.907
IVF women ($n = 151$)	64.2 % ($n = 97$)	33.1 % ($n = 50$)	2.6 % ($n = 4$)		69.6 % ($n = 103$)	29.1 % ($n = 43$)	1.4 % ($n = 2$)	

In AMH (Ile⁴⁹Ser) polymorphism, “Ile” corresponds to isoleucine amino acid, while “Ser” corresponds to Serine amino acid. In AMHR II –482 A>G polymorphism, “A” corresponds to the more common allele, while “G” corresponds to the less common allele, usually referred as polymorphic variant

AMHR II polymorphism was held in 148 out of 151 and the detection of AMH polymorphism in the total of 151 women.

Associations of gene polymorphisms with patients characteristics and COS and pregnancy outcome among the different genotypes of IVF/ICSI group

We analyzed the associations of both polymorphisms with patients characteristics and COS and pregnancy outcome among the different genotypes in the total of 151 women. For convenience of statistical analysis and due to the small number of patients being either homozygous or heterozygous for each polymorphism, we classified those with specific genotypes in one group as carriers of polymorphisms (for AMH SNP: Ile/Ser & Ser/Ser and for AMHR II SNP: A/G & G/G). Regarding the patient characteristics, no differences were found concerning age, infertility duration, IVF trials, BMI, FSH, LH, AMH and prolactin levels between non-carriers and carriers of the polymorphisms (data not shown). Comparing carriers and non-carriers of each of the two polymorphisms in the IVF/ICSI group, no statistical significant difference was found in ovulation induction parameters and controlled ovarian stimulation outcome, apart from one parameter: regarding AMH polymorphism, women carrying the polymorphism presented lower fertilization rates compared with non-carriers ($P_a=0.177/P_b=0.049$, Table 2).tgroupa

No significant differences were observed concerning pregnancy rates between carriers and non-carriers, regarding both polymorphisms. ($P=0.999$ and $P=0.484$ respectively, Table 2).

Subgroup analysis - Associations of gene polymorphisms with patient and COS parameters and pregnancy outcome among the different genotypes of IVF/ICSI group

The group of 151 women was divided into subgroups depending on individual parameters. In particular, we examined possible associations of both polymorphisms with patient and COS parameters among two age-based groups (women aged ≤ 35 and women aged > 35), two FSH-based groups (FSH ≤ 10 mIU/L and FSH > 10 mIU/L), two serum AMH-based groups (AMH < 7.8 pmol/L and AMH ≥ 7.8 pmol/L), two previous IVF attempts-based groups (IVF attempts ≤ 2 and IVF attempts > 2) and two peak E2 levels-based groups (E2 ≤ 1500 pg/ml and E2 > 1500 pg/ml).

As a result of this categorization into individual subgroups, the parameters in which we had significant differences were, for the AMH polymorphism, FSH levels in respect to IVF trials and fertilization rate in respect to E2 levels on the day of hCG administration (Table 3a), and, for the AMHR II polymorphism, number of follicles in respect to IVF trials and total gonadotropin dose in respect to E2 levels on the day of hCG administration (Table 3b). No genotype showed

Table 2 Carriers and non-carriers of each of the two polymorphisms in the IVF/ICSI group showed no statistical significant difference in ovulation induction parameters and controlled ovarian stimulation outcome, apart from one parameter: regarding AMH polymorphism, women carrying the polymorphism presented lower fertilization rates compared with non-carriers

	AMH (Ile ⁴⁹⁰ /Ser) polymorphism			AMHR II (-482 A>G) polymorphism		
	Non-Carriers (Ile/Ile) <i>n</i> =97	Carriers (Ile/Ser & Ser/Ser) <i>n</i> =54	<i>p</i> -value	Non-Carriers (A/A) <i>n</i> =103	Carriers (A/G & G/G) <i>n</i> =45	<i>p</i> -value
Stimulation days	10±1	10±1.5	0.722a 0.730 ^b	10±1.4	10±1	0.235 ^a 0.267 ^b
Peak E2 (pg/ml)	1770.3±1175.2	2185.1±1414.6	0.067 ^a 0.076 ^b	2076.8±1347.7	1651.4±1094.9	0.081 ^a 0.110 ^b
Total gonadotropin dose (IU)	3346.6±1298	3262.5±1013.4	0.734 ^a 0.972 ^b	3304.5±1299.2	3313.4±923.1	0.974 ^a 0.614 ^b
Number of follicles	7±4	7±5	0.464 ^a 0.737 ^b	8±5	6±4	0.081 ^a 0.070 ^b
Number of COCs	6±4	6±5	0.509 ^a 0.659 ^b	6±4	5±3	0.104 ^a 0.117 ^b
Maturation rate	73.72 %±16.60 %	68.97 %±19.10 %	0.199 ^a 0.224 ^b	72.72 %±17.90 %	70.31 %±17.50 %	0.557 ^a 0.381 ^b
Number of fertilized oocytes	4±2	4±3	0.846 ^a 0.809 ^b	4±2	4±2	0.768 ^a 0.745 ^b
Fertilization rate	72.74 %±19.60 %	68.37 %±14.90 %	0.177 ^a 0.049 ^b	70.60 %±19.10 %	72.86 %±15.60 %	0.523 ^a 0.728 ^b
Pregnancy rate (No of pregnant women)	17.52 % (<i>n</i> =17)	16.66 % (<i>n</i> =9)	0.999 ^a	15.53 % (<i>n</i> =16)	22.22 % (<i>n</i> =10)	0.484 ^a

^a Two samples *Z*-test (parametric)

^b Mann–Whitney *U* (non parametric)

*Fisher's exact test

Table 3 Significant associations for AMH and AMHR II polymorphisms concerning the subgroup analysis based on previous IVF trials and peak E2 levels

AMH polymorphism					
	Non-Carriers (Ile/Ile)		Carriers (Ser/Ser & Ile/Ser)		
	N	Mean ± SD	N	Mean ± SD	p-value
Basal FSH levels					
IVF trials ≤2	66	9.73 ± 10.3	42	8.01 ± 3.8	0.302 ^a , 0.371 ^b
IVF trials >2	20	8.31 ± 2.5	11	11.31 ± 3.75	0.012 ^a , 0.026 ^b
Fertilization rate	N	Mean ± SD	N	Mean ± SD	p-value
E2 hCG ≤1500	39	0.76 ± 0.2	20	0.65 ± 0.16	0.037 ^a , 0.010 ^b
E2 hCG >1500	41	0.69 ± 0.19	31	0.70 ± 0.14	0.787 ^a , 0.767 ^b
AMHR II polymorphism					
	Non-Carriers (A/A)		Carriers (G/G & A/G)		
	N	Mean ± SD	N	Mean ± SD	p-value
No of follicles					
IVF trials ≤2	70	6.56 ± 4.25	28	6.04 ± 4.03	0.579 ^a 0.619 ^b
IVF trials >2	18	7.39 ± 5.86	11	3.64 ± 1.80	0.019 ^a 0.050 ^b
Total gonadotropin dose	N	Mean ± SD	N	Mean ± SD	p-value
Peak E2 ≤1500	34	3832 ± 1403	14	3078 ± 983	0.074 ^a 0.093 ^b
Peak E2 >1500	35	2772 ± 935	12	3587 ± 801	0.010 ^a 0.012 ^b

a Significant associations for AMH polymorphism concerning the subgroup analysis based on previous IVF trials and peak E2 levels. In the subgroup of women who had more than two IVF attempts in the past, carriers of the polymorphism had statistically significant higher basal FSH levels compared to non-carriers. Furthermore, in the subgroup of women who reached peak E2 levels below 1500 pg/ml, fertilization rate was statistically significant lower in carriers of the polymorphism compared to non-carriers. b Significant associations for AMHR II polymorphism concerning the subgroup analysis based on previous IVF trials and peak E2 levels. In the subgroup of women who had more than two IVF attempts in the past, carriers of the polymorphism had statistically significant lower number of follicles compared to non-carriers. Furthermore, in the subgroup of women who reached peak E2 levels above 1500 pg/ml, total gonadotropin dose was statistically significant more in carriers of the polymorphism compared to non-carriers

^a Two samples independent t-tests

^b Mann–Whitney test

predominance to another in any of the other parameters examined. It should be mentioned that these differences remained after adjustment for age and BMI through multivariate analysis of variance.

Subgroup analysis based on age Associations of Gene Polymorphisms with patient and COS parameters and Pregnancy Outcome among the different genotypes of IVF/ICSI group.

We evaluated the association between the two polymorphisms and clinical, biochemical and COS parameters considering age. Thus, the case group of 151 women was divided into a subgroup aged ≤35 and a subgroup aged >35 years. The rationale was to remove the confounding factor of age, which is known to determine the level of AMH in the serum, while AMH and AMHR II SNPs as genetic traits remain stable throughout life of a woman. The purpose was to see if in a particular age group, with a relative damping of the factor of age, the expression of SNPs is an additional criterion for the efficiency of the IVF cycle. The following parameters within the above patient subgroups with different AMH and AMHR II gene polymorphisms were examined: BMI, infertility duration, previous IVF attempts, AMH, FSH, LH, E2 on the day of hCG administration, PRL, total gonadotropin dose used, stimulation days, number of follicles, number of oocytes retrieved, pregnancy rate. No significant differences were tracked in any parameter among the different genotypes of both polymorphisms.

Subgroup analysis based on AMH serum levels Associations of Gene Polymorphisms with patient and COS parameters and Pregnancy Outcome among the different genotypes of IVF/ICSI group.

The total of 151 women was divided, based on serum AMH levels, into two subgroups: women with AMH levels < 7.8 pmol/L and women with AMH levels ≥ 7.8 pmol/L, according to the Bologna criteria [24]. According to the ESHRE consensus of 2011, the cut-off values reported for AMH to discern infertile women in normal and poor responders are in the range from 0.5 to 1.1 ng/ml, otherwise, from 3.57 pmol/L to 7.85 pmol/L. Above or below this cut-off value, a normal or poor prognosis of an IVF cycle is expected, respectively. No significant differences were tracked in any parameter among the different genotypes of both polymorphisms.

Subgroup analysis based on IVF trials Associations of Gene Polymorphisms with patient and COS parameters and Pregnancy Outcome among the different genotypes of IVF/ICSI group.

The total of 151 women was divided into two subgroups based on previous IVF attempts: women with IVF trials ≤ 2 and women with IVF trials > 2. Statistical differences were observed only in women with IVF trials > 2, in which AMH polymorphism carriers had significantly higher serum FSH

levels compared to non-carriers ($P=0.012$), while concerning the AMHR2 polymorphism, carriers had significant lower number of follicles compared to non-carriers ($P=0.019$).

Subgroup analysis based on E2 levels at the day of hCG administration Associations of Gene Polymorphisms with patient and COS parameters and Pregnancy Outcome among the different genotypes of IVF/ICSI group.

The total of 151 women was divided into two subgroups based on peak E2 levels: women with peak $E2 \leq 1500$ pg/ml and women with peak $E2 > 1500$ pg/ml. Significant differences were observed in both subgroups:

Regarding the AMH polymorphism, women who had peak $E2 \leq 1500$ pg/ml and were carriers of the polymorphism demonstrated statistically significant lower fertilization rate in comparison with non-carriers ($P=0.037$). As for AMHR2 polymorphism, carriers of the polymorphism who had peak $E2 > 1500$ pg/ml required significant higher total gonadotropin dose compared to non-carriers ($P=0.010$).

Discussion

The present study aimed to investigate possible associations of the presence of AMH (Ile⁴⁹Ser) and AMHR2 (−482 A>G) SNPs with clinical, biochemical and COS parameters. Despite the vast literature regarding the implication of AMH and AMHR2 SNPs in various gynecological disorders, no consensus has been reached regarding the role of both SNPs in the context of assisted reproduction. Although no significant associations were found between AMH and AMHR2 SNPs and patient characteristics, ovulation induction parameters and controlled ovarian stimulation outcome apart from one parameter, fertilization rates, subgroup analysis revealed interesting associations among the different genotypes of IVF/ICSI group.

As far as is known, this is the second study which investigates the possible role of both polymorphisms in the field of assisted reproduction and the first one conducted in a Greek population. Serum concentration of AMH is widely used because it is considered an ideal marker of ovarian reserve. Low concentration of serum AMH is predictive of a reduced ovarian reserve and vice versa [25, 27–30]. Some studies have demonstrated an association between the serum AMH concentration and the number of oocytes retrieved after controlled ovarian stimulation [26, 31, 32]. Others suggested that AMH inhibits FSH action in the ovary [33]. This suggestion was based on studies showing that AMH decreased aromatase activity in the fetal ovary [34] and that follicles from AMH knock-out mice were more sensitive to FSH [35]. Although the exact physiological role of AMH is poorly understood, in vivo and in vitro studies in mice and in vitro studies in

human demonstrated that AMH inhibits the primordial follicle recruitment and cyclic selection [11, 12, 35, 36]. The presence of the Ile49Ser polymorphism in the gene encoding AMH may alter its bioactivity and function in the ovary, as Kevenaar et al. have already mentioned [18].

AMH exerts its biological activity via two receptors, AMHR1 and AMHR2. Interestingly, both receptors must be co-expressed with an appropriate type I receptor for signal transduction. Contrary to the AMH type II receptor, which is highly specific, the identity of the AMH type I receptor is not clear. Interestingly, three type I receptors of Bone Morphogenetic Proteins (BMPs), Alk2, Alk3 and Alk6 may transduce AMH signals, but none of them presents all the characteristics of an AMH type I receptor [36]. AMH binds to AMHR2. Upon ligand binding, the AMHR2 recruits and phosphorylates the AMHR1, which, in turn, induces subsequent downstream activation of a set of cytoplasmic effector proteins such as Smads 1, 5 and 8. These proteins act as messengers of hormonal signal to the cell nucleus, after forming heterodimers [37, 38]. However, in the presence of certain AMHR2 SNP, this signaling pathway may be disrupted. The polymorphism of the AMHR2 is located in the non-coding region of its promoter, a region which interacts with transcription factors in order to initiate the transcription process. Therefore, we hypothesized that the existence of AMHR2 (−482A>G) SNP in the promoter may inhibit binding of transcription factors and this may result in the inhibition of the transcription process. The existence of SNP in AMHR2 gene may render the AMH/AMHR2 complex less bioactive and this might lead to an increased recruitment of primordial follicles. This hypothesis is also supported by Kevenaar et al. [18].

Distribution of polymorphisms found in the present study, seem to be similar to that found by other researchers in the past, who studied the same AMH and AMHR2 polymorphisms in other European populations, such as the Dutch and the German ones. In fact, Kevenaar et al. found that AMH Ile49Ser was present in 25 % in the Dutch and 26.4 % in the Dutch & German altogether [18], whereas we found that AMH Ile⁴⁹Ser was present in 28 % in the Control and 35.7 % in IVF/ICSI group. This was also the fact regarding the distribution of AMHR2 polymorphisms: Kevenaar et al. found AMHR2 −482 A>G in 37.5 % in the Dutch and 34 % in Dutch & German altogether [18], whereas we found that AMHR2 −482 A>G was present in 32 % in the Control and 30.5 % in IVF/ICSI group. Furthermore, genotype distribution of the AMH and AMHR2 polymorphisms in the IVF/ICSI group and the control group were similar ($P=0.276$ and $P=0.907$ respectively). No polymorphism, alone or in combination, was highly detected in the IVF/ICSI group. However, it was also observed that carriers of AMH SNP present lower fertilization rate ($P_a=0.177/P_b=0.049$, Table 2).

Based on the above results, a possible association between AMH/AMHR2 SNPs with clinical, biochemical and COS

parameters was further evaluated by dividing women into subgroups. While comparing two age-based groups, two FSH-based groups and two AMH-based groups, no statistical differences in the distribution or in any clinical, biochemical and COS parameter between the different genotypes of both polymorphisms were detected. The fact that early follicular phase FSH levels did not differ among the AMH and AMHR II genotypes, may suggest a direct effect of AMH on the ovary, as shown by Kevenaar [18]. However, when the different genotypes of both polymorphisms in IVF/ICSI group were examined separately into subgroups based on IVF attempts and peak E2 levels, we found noteworthy differences regarding basal FSH levels, fertilization rate, number of follicles and total gonadotropin dose.

The question that rises is how credible serum AMH levels are in the prediction of fertility potential, since no differences were detected regarding the subgroup analysis based on AMH serum levels. In ART, women with extremely low AMH concentration (less than 0.1 to 0.4 ng/ml or less than 0.714 to 2.857 pmol/L respectively) demonstrate pregnancy potential according to a recent study. Weghofer et al. showed that the key factor affecting the chance of pregnancy is age. Patients aged >42 years with serum AMH within the range of 0.1–0.4 ng/ml had significantly lower clinical pregnancy rates per cycle compared to those aged <42 years with the same AMH range (3.7 % versus 11 % respectively, $P=0.031$). They concluded that women with AMH concentration even less than 0.1 ng/ml may still have a reasonable chance of achieving a pregnancy in order to undergo fertility treatment [39]. Thus, serum AMH may not be an ideal marker for the prediction of ovarian response. However, SNPs of AMH or AMHR II may reflect fertility potential in specific subgroups of women.

Another important finding of this study concerned the subgroup of women with more than two previous IVF attempts. Carriers of the AMH polymorphism exhibited significantly higher basal FSH levels ($P=0.012$), whereas carriers of the AMHR II polymorphism had significant lower number of follicles ($P=0.019$). A possible explanation regarding higher basal FSH levels found in carriers of the AMH polymorphism may be the accelerated recruitment of primordial follicles leading to an exhaustion of follicles from ovarian reserve. This finding is in accordance with several studies which confirm that basal FSH levels constitute a marker of ovarian reserve, as high basal FSH levels represent the decline of ovarian potential and are associated with a decreased fecundity and vice versa [40, 41]. Concerning the lower number of growing follicles that carriers of AMHR II polymorphism with more than two previous IVF attempts had, we suggest that the absence or the reduced number of normal functioning AMHR II hampers the physiological role of AMH, leading again to the exhaustion of follicles.

Basal FSH levels performed in early follicular phase (day 2 or 3) of the cycle had been long considered as the “golden” biomarker of ovarian reserve in the clinical practice, until AMH emerged as a new sufficient and even superior biomarker. A recent review published by Fleming et al. concluded that further research is needed in order to establish whether individualized treatment protocols based on serum AMH concentrations result in improved clinical outcome by reducing poor response rates, lowering the incidence of OHSS and increasing live birth rates [42]. On the other hand pharmacogenetic studies showed that gene polymorphisms of ESR1 and FSHR are associated with ovarian response and pregnancy outcome, representing a useful tool for the prediction of IVF treatment outcome. Our data shows that FSH concentration in peripheral blood is significantly lower in non-carriers of AMH polymorphism with more than two previous IVF attempts and this may suggest a better ovulation induction outcome, even in this subgroup of patients. Taking into account that AMH affects the modification of the FSH sensitivity of small antral follicles in the process of cyclic selection [11, 35], we can hypothesize that there is a biological path among FSHR-AMH/AMHR II complex that leads to a regulatory step of folliculogenesis.

A recent study suggested that FSH may induce down-regulation of AMH expression possibly via estradiol synthesis due to the negative correlation of AMH with concentrations of estradiol or this inhibition of AMH may be overcome by the suppression of AMH synthesis by factors other than FSH and possibly estradiol [43]. We have chosen the cut-off value of 1500 pg/ml for peak E2 levels to distinguish E2-based subgroups, as levels above it are generally considered a marker of a good ovarian stimulation. According to the above, carriers of AMH polymorphism with peak E2 levels on day of hCG administration ≤ 1500 pg/ml appeared to have significant lower fertilization rate ($P=0.037$) compared to non-carriers. This finding may support our theory of the accelerated recruitment of primordial follicles due to the presence of the polymorphism, which may result in suboptimal oocyte quality due to an adverse maturation process. We also found that carriers of AMHR II polymorphism with E2 levels on the day of hCG > 1500 pg/ml needed significant higher dose of gonadotropins compared to non-carriers ($P=0.010$). This correlation probably reveals a more “resistant” genotype to FSH action, which requires a stronger stimulus for a similar response.

One limitation of this study is that antral follicle count data are lacking. Furthermore, missing data regarding AMH levels (available in 109 out of 151 women) and AMHR II polymorphism (available in 148 out of 151 women) may confer potential bias. However, the remaining sample size was deemed sufficient for calculations, strengthening the results of the study. Finally, the implementation of three different COS protocols should be considered as a possible relevant bias factor, as there are concerns that they may influence fertility treatment outcome.

In conclusion, among non-carriers of AMH polymorphism, basal FSH levels were lower in those with more than two of previous IVF attempts and fertilization rate was statistically higher in those with peak serum E2 levels below 1500 pg/ml, whereas among non-carriers of AMHR II polymorphism, the number follicles was higher in those with more than two previous IVF attempts and total dose of gonadotropins was lower in those with peak serum E2 levels above 1500 pg/ml. Our observations support the notion that AMH and AMHR II polymorphisms are associated with certain parameters of controlled ovarian stimulation in specific subgroups of women, who undergo IVF/ICSI. Further assessment of AMH and AMHR II SNPs in combination with FSHR SNPs, such as *Ans*⁶⁸⁰*Ser* and *ESR1* SNPs, such as *PvuII* T/C, may provide a means for the prediction of ovarian response in specific subgroups of women entering an IVF/ICSI program. The effort could be even more tempting if the target is the identification of women of poor prognosis or on the contrary of high ovarian response, which are candidates for OHSS.

Summarizing, the present study reinforces the aspect that AMH and AMHR II SNPs may affect the signaling pathway of AMH, leading to an accelerated recruitment of follicles and may contribute to the individual variation in the FSH “threshold”. Although encouraging, the findings of the present study need to be confirmed by other researchers in well-designed larger-scale studies. Unfortunately, the exact molecular mechanisms that lie behind AMH and AMHR II SNPs are poorly understood. However, further investigations focusing on underlying complex mechanisms may enrich our understanding and help in applying this knowledge in the clinical setting in terms of a better prediction of ovarian response to gonadotropin stimulation.

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