

Original Paper

AMH and AMHR2 Polymorphisms and AMH Serum Level Can Predict Assisted Reproduction Outcomes: A Cross-Sectional Study

Carla Peluso^a Fernando L.A. Fonseca^b Guilherme G. Gastaldo^a
Denise M. Christofolini^a Emerson Barchi Cordts^a Caio P. Barbosa^a Bianca Bianco^a

^aHuman Reproduction and Genetics Center - Department of Collective Health, ^bLaboratory of Clinical Analysis - Department of Clinical Medicine, Faculdade de Medicina do ABC, Santo André/SP, Brazil

Key Words

AMH gene • AMHR2 gene • Ovarian reserve • Anti-Mullerian Hormone • Human Reproduction

Abstract

Background: In human assisted reproduction, the ovarian response to exogenous recombinant Follicle-stimulating Hormone (FSH) therapy is variable and difficult to predict. The standard protocol of ovarian hyperstimulation can result in satisfactory response; however, an unsatisfactory response necessitates FSH dose adjustment or results in ovarian hyperstimulation syndrome (OHSS). Polymorphisms in *AMH* and *AMHR2* genes appear to affect hormone biological activities, thus affecting follicle recruitment and development, leading to infertility. We aimed to evaluate *AMH* and *AMHR2* polymorphisms in infertile women, and correlate those findings with AMH, FSH and estradiol serum level response to controlled ovarian hyperstimulation (COH), as well as assisted reproduction outcomes.

Methods: A cross-sectional study comprising 186 infertile women that underwent one cycle of high complexity assisted reproductive treatment. Blood samples were collected and a TaqMan assay was used for *AMH* G146T/rs10407022 and *AMHR2* A-482G/rs2002555, A10G/rs11170555, C1749G/rs2071558 and G4952A/rs3741664 genotyping, and FSH, estradiol and AMH levels were measured. The findings were correlated to human reproduction outcomes.

Results: *AMH* rs10407022 and *AMHR2* rs2002555 polymorphisms were not associated with hormonal measurements, whereas *AMHR2* rs11170555 and rs3741664 were positively associated with AMH, estradiol and FSH levels. The genotype distribution of *AMH* and *AMHR2* genes according to Controlled Ovarian Hyperstimulation did not show a positive association. However, an association with AFC, degree of oocyte maturation (allele G of *AMHR2* rs2071558)

the number of embryos produced (alleles T and G of *AMH* rs10407022 and *AMHR2* rs2002555, respectively) and frozen embryo (allele G of *AMHR2* rs11170555) were found to be statistically associated. Considering COH, serum AMH and AFC were a positive predictor to OHSS. Regarding serum AMH and assisted reproduction outcomes, a positive correlation with all variables studied was found. Comparing AFC and AMH as predictors of human reproduction outcomes, the AFC was less effective than serum AMH. Considering pregnancy rates, no marker was positively associated. **Conclusion:** *AMHR2* polymorphisms were associated with estradiol, AMH and FSH measurements, as well as number and quality of embryos, while *AMH* polymorphisms was associated with number of embryos produced. Serum AMH was correlated with nearly all variables analyzed in assisted reproductive treatment, demonstrating that it represents a better biomarker of OHSS and human reproduction outcomes compared to *AMH* and *AMHR2* polymorphisms.

Copyright © 2015 S. Karger AG, Basel

Introduction

In human assisted reproduction, ovulation response to exogenous recombinant FSH therapy is variable and difficult to predict [1, 2]. The ability to identify patients with potential to develop hyper-response or inadequate response to standard treatment presents a valuable clinical aid.

Several parameters have been postulated as predictors of ovarian response. Ovarian function cannot be measured directly, and the use of serum markers [FSH [Follicle Stimulation Hormone], inhibin B, 17- β -estradiol and anti-Müllerian hormone [AMH]] and/or ultrasound variables [ovarian volume, measurement of antral follicles, ovarian stromal blood flow] have proven useful, although limited [3].

Anti-müllerian hormone [AMH], also called Mullerian-inhibiting substance MIS [4], is a dimeric glycoprotein and a member of the transforming growth factor β family that plays a role in the regulation of follicular development [5]. AMH is produced by granulosa cells of the early developing follicles in the ovary, and continues to be expressed in the growing follicles [6] until these follicles have reached a size of 4-6 mm and a differentiation state at which AMH becomes receptive for exogenous FSH [7], and may be selected for dominance [6-8].

Studies in knockout mice for the *AMH* gene have demonstrated that in the absence of this hormone, follicles are recruited at a faster rate and are more sensitive to FSH [9], suggesting that serum AMH could inhibit primordial follicle development and be induced by FSH. Moreover, studies in normo-ovulatory women demonstrated an association of the *AMH* gene polymorphisms, which are located in 19p13.3, [19p13.3, MIM 600957, Genebank ID 268], and its receptor *AMHR2* [12q13, MIM 600956, Genebank ID 269] with estradiol levels during the follicular phase of the menstrual cycle, suggesting a role in regulating FSH sensitivity [10]. Therefore, genetic variations in *AMH* and *AMHR2* genes may influence the hormonal function in folliculogenesis, resulting in infertility.

Rigon et al. [11] investigated *AMH* and *AMHR2* polymorphisms in women with idiopathic infertility and found that the genotype distribution was significantly different between cases and controls. Riggs et al. [12] demonstrated that AMH can be an ovarian response marker, providing utility in donor selection, and it can also predict the ovarian hyperstimulation syndrome. Previously, these authors showed that AMH was also correlated with ovarian reserve and the number of oocytes retrieved, and thus has a high positive predictive value in relation to age, hormonal levels of FSH, LH, estradiol and inhibin B [13]. However, AMH polymorphisms may affect hormone biological activities, which play an important role in controlling the recruitment and development of follicles [14]. Study of the gene polymorphism that regulate female reproductive function may help to clarify the mechanisms responsible for gonadal function and fertility in humans [11].

Based on these findings, we aimed to evaluate the polymorphisms G146T/Ile49Ser/rs10407022 of the *AMH* gene and A-482G/rs200255, A10G/rs11170555, C1749G/

rs2071558 and G4952A/rs3741664 of the *AMHR2* gene in Brazilian infertile women who had undergone assisted reproductive treatment, and correlate those findings with AMH, FSH and estradiol serum levels, controlled ovarian hyperstimulation (COH) response and assisted reproduction outcomes (e.g., antral follicle count (AFC), follicles visualized at USG, oocytes retrieved, MII (degree of oocyte maturation), embryos produced, transferred embryos, frozen embryos and pregnancy rate).

Materials and Methods

Subjects

We performed a prospective cross-sectional study that screened 430 infertile women from the Human Reproduction and Genetics Center of the Faculdade de Medicina do ABC, Santo André, Brazil, between September 2011 and September 2013, who had undergone the first cycle of high complexity assisted reproductive treatment. A total of 186 out of 430 patients (mean age 32.5 ± 3.5 years) met the inclusion criteria: only women with infertility caused by a male factor ($n=97$), tubal factor ($n=59$) or idiopathic infertility ($n=30$) were included in this study. All patients were younger than 38 years old, with normal serum levels of basal FSH (≤ 10.0 IU/ml), TSH (<4 mIU/L) and prolactin (<25 ng/ml), presence of both ovaries without any morphological abnormalities, normal ovulatory cycles (25-35 days), body mass index (BMI) ≤ 30 , no previous history of poor response and no evidence of endocrine disease, such as polycystic ovary syndrome. Patients with moderate/severe endometriosis (stage III and IV), previous ovarian history or underwent chemo/radiotherapy, low-complexity protocols and cases with severe male factors that underwent surgical procedures for sperm recovery were excluded from the study.

Investigation into the cause of infertility included a hormonal and biochemical profile, testing for sexually transmitted diseases, imaging tests, genetic investigation and/or immunological abnormalities, semen analysis of the partner, hysterosalpingography, hysteroscopy and laparoscopy (performed in all women up to 36 years old, as well as in patients over 36 years of age whenever there were symptoms or abnormalities in the imaging examinations). If no abnormalities were found in these exams, the infertility was classified as idiopathic.

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

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

A transvaginal ultrasound was used to scan ovaries, before the ovarian stimulation began, on the second day of the menstrual cycle. The antral follicle counting was accomplished in each ovary, and we used the total counting of follicles up to 10 mm. When the presence of polycystic ovaries was diagnosed the patient was excluded of the study.

As approved by the local Research Ethics Committee, the clinical data and peripheral blood samples were collected only after explaining the study aims and obtaining written informed consent.

Ovarian Stimulation

Ovulation was induced by recombinant rFSH an initial daily use of 100 IU or 200 IU that was administered for 10 days, starting on the second day of menstruation. As of the 6th day and until the 10th day, the antagonist was also administered. Between day 10 and 11, when the follicles reached a diameter of approximately 17 mm, as determined by transvaginal ultrasound, the patients were given human chorionic gonadotropin (hCG), and on day 13 oocyte retrieval was performed [16].

Regarding controlled ovarian hyperstimulation response, we considered: i) ovarian hyperstimulation syndrome (OHSS), characterized by multiple ovarian follicles (≥ 20 follicles) together with possible clinical symptoms such as ascites, hematological changes (hemoconcentration), pleural effusion, and liver and/or coagulation abnormalities according to the classification proposed by Golan et al. [17], as well as ≥ 4000 IU/mL of serum estradiol; ii) hyperresponse, when after 6 days of ovarian stimulation with gonadotropins, the

Table 1. AMH and AMHR2 gene polymorphisms and respective TaqMan assays

Gene	Localization	Polymorphism	rs	TaqMan Assay
AMH	19p13.3	T146G/ Ile49Ser	rs10407022	C_25599842_10
		A-482G	rs2002555	C_1673084_10
AMHR2	12q13	C1749G	rs2071558	C_1673083_1
		G4952A	rs3741664	C_11286518_1
		A intron 10G	rs11170555	C_25471905_10

development of $\geq 12 \leq 19$ follicles occurred, without OHSS clinical symptoms; iii) poor response, when after 6 days of ovarian stimulation with gonadotropins only up to 3 follicles smaller than 14 mm had developed; and iv) satisfactory response, when after 6 days of ovarian stimulation with gonadotropins, 4 to 12 follicles larger than 14 mm had developed.

Genotyping

Five milliliters of peripheral blood was collected in a tube containing EDTA and genomic DNA was extracted from lymphocytes according to a salting out method [18]. The detection of AMH and AMHR2 polymorphisms (G146T/rs10407022, A-482G/rs2002555; A10G/rs11170555; C1749G/rs2071558; G4952A/rs3741664, respectively) was performed using a TaqMan system and real time polymerase chain reaction (PCR), with primers and probes commercially available from Life Technologies (Foster City, CA, USA). Assays were performed using a TaqMan Genotyping Master Mix with 50 to 100 ng of DNA per reaction. The PCR conditions were 40 denaturation cycles of 15 seconds at 95°C and 1 minute of annealing/extension at 60°C (Table 1).

Hormonal Measurements

Fresh blood samples were collected on the 2nd or 3rd day of the menstrual cycle. Samples were centrifuged at 2000 g for 10 min, aliquoted in a cryopreservation tube and serum samples were stored at -80°C until AMH, FSH and estradiol measurements were taken.

Basal FSH and estradiol levels were measured by a competition method with a final fluorescent detection (ELFA, Enzyme Linked Fluorescent Assay, Mini-Vidas-BioMerieux, Hazelwood, and Missouri).

AMH measurement was performed in the same laboratory using the same assay by one operator. The AMH serum levels were measured using an enzyme-linked immunosorbent assay (ELISA) using the AMH Gen II kit by Beckman Coulter, Inc (Brea, CA, USA). The measurement was realized by automated Labotech (Alka Technology®). The reading was performed at a wavelength at 450 nm. To calculate the AMH value, we used the point to point method. Values are presented in concentrations of ng/ml (convert to SI units: 1 ng/ml = 7,14pM). The analytic sensibility was 0.008 ng/ml.

Statistical Analysis

Statistical analyses were accomplished using SPSS for windows version 18.0 (Chicago, IL). To compare the numeric variables and the polymorphism genotypic frequency; we used a Kruskal-Wallis/ANOVA test or a Mann Whitney/Student t-test. These tests were also employed to compare the measurements of serum AMH, AFC with ovarian stimulation outcomes and β -subunit of human chorionic gonadotropin β HCG results.

To verify the association between the genotypes with ovarian stimulation outcomes and the β HCG results, Chi-Square and Fisher tests were used. Spearman's correlation of was applied to verify the association between serum AMH, AFC and the studied quantitative variables.

A model of multinomial logistic regression was adjusted to identify risk factors for ovarian stimulation outcomes. The chi-square test was used to verify if the population was in Hardy Weinberg equilibrium.

The level of significance was considered as $p < 0.05$, or 5%.

Results

The mean estradiol, AMH and FSH serum levels of patients studied were: 46.1±13.1 pg/ml, 4.22±2.7 ng/ml, 6.4±2.1 mIU/ml, respectively.

Table 2. Association of the *AMH* polymorphism (G146T/rs10407022) with hormonal measurements and assisted reproduction outcomes. N: Number of subjects; SD: Standard Deviation; *p<0.05

Variables	G146T												p
	GG				GT				TT				
	N	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	
Total AFC	14	11.3	4.0	10	48	10.1	3.7	10	83	9.3	3.9	9	0.13
Estradiol	15	42.7	13.6	40.5	63.0	44.6	12.9	41.3	104.0	47.6	13.2	46.3	0.12
AMH	17	3.3	2.3	3.5	64.0	4.0	2.6	3.5	105.0	4.5	2.9	4.1	0.24
FSH	17	7.1	2.6	7.4	64.0	6.6	1.9	6.5	105.0	6.3	2.1	6.0	0.28
Follicles USG	13	5.9	5.6	4	54	6.5	4.0	6	95	8.3	6.0	6	0.13
oocytes retrieved	12	3.8	2.2	3.5	54	5.6	3.8	5	93	7.2	5.4	5	0.07
MII	11	3.5	1.6	3	51	5.2	3.5	4	90	6.0	4.6	5	0.13
N Embryos	9	1.7	1.1	2	46	2.8	1.6	2.5	76	3.6	2.5	3	0.013*
Embryo Transferred	8	1.6	0.5	2	45	1.8	0.5	2	76	1.8	0.6	2	0.56
Frozen Embryo	9	0.3	0.7	0	37	1.0	2.2	0	68	2.1	4.2	0	0.48

Table 3. Association of *AMH* and *AMHR2* gene polymorphisms and ovarian stimulation outcomes. N: number of individuals; p= Exact of Fisher test

Polymorphism	Genotype	Poor Response	Satisfactory Response	Hiper response	OHSS	p
G146T	GG	15.8%	6.4%	0.0%	12.5%	0.13
	GT	40.4%	33.9%	25%	12.5%	
	TT	43.9%	59.6%	75%	75%	
A-482G	AA	70.2%	60.6%	58.3%	50%	0.22
	AG	26.3%	24.8%	41.7%	37.5%	
A10G	GG	3.5%	14.7%	0.0%	12.5%	0.35
	AA	66.7%	61.5%	41.7%	50%	
C1749G	AG	26.3%	24.8%	41.7%	50%	0.55
	GG	7%	13.8%	16.7%	0%	
G4952A	CC	71.9%	72.5%	58.3%	50%	0.33
	CG	26.3%	23.9%	41.7%	50%	
	GG	1.8%	3.7%	0%	0%	
	GA	71.9%	74.3%	58.3%	50%	
		28.1%	25.7%	41.7%	50%	

Regarding the COH response, 30.6% (57/186) were poor responders, 58.6% (109/186) had a satisfactory response, 6.5% (12/186) were hyper responders; and 4.3% (8/186) developed ovarian hyperstimulation syndrome.

Considering the β HCG results, 32.8% (61/186) had a positive result and 67.2% (125/186) had a negative result.

G146T/rs10407022 polymorphism of the AMH gene

The genotype distribution of *AMH* G146T/rs10407022 polymorphism was in Hardy Weinberg equilibrium. Association of this polymorphism with AMH, estradiol and FSH serum levels did not show statistical significance (p=0.24; p=0.12; p=0.28, respectively) (Table 2).

The genotype distribution according to COH outcomes did not show a positive association, p=0.13, whereas 9.1% (17/186) presented the wild type genotype GG (variant Ile/Ile), 34.4% (64/186) were heterozygotes, and 56.5% (105/186) presented the polymorphic genotype TT (variant Ser/Ser) (Table 3). The frequency of the G allele was n=98 (26.3%), and n=274 (73.7%) for the allele T.

When we compared the genotype distribution according to human reproduction outcomes we did not find any statistically significant differences (AFC, p=0.13; follicles visualized at USG, p=0.13; oocytes retrieved, p=0.07; MII (degree of oocyte maturation), p=0.13; embryos transferred, p=0.56 and frozen embryos, p=0.48), except for the number of embryos produced, p=0.013. The polymorphic genotype TT presented higher mean values of the number of embryos produced (Table 2).

Table 4. Association of *AMHR2* polymorphism (A-482G; A10G; C1749G; G4952A) and serum estradiol, AMH, FSH and assisted reproduction outcomes. N: Number of subjects; SD: Standard Deviation; *p<0.05

Variables	A-482G/rs2002555												p
	AA				AG				GG				
	N	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	
Total AFC	92	9.6	3.8	9	37	10.1	4.3	10	16	9.8	3.4	9.5	0.81
Estradiol	115	45.3	11.9	43.0	48.0	47.9	14.9	45.6	19.0	46.8	16.3	46.5	0.65
AMH	117	4.2	3.0	3.6	50.0	4.3	2.3	4.1	19.0	4.0	2.6	3.8	0.67
FSH	117	6.7	2.2	6.6	50.0	6.0	1.9	5.6	19.0	6.6	1.9	6.0	0.12
Follicles USG	98	6.9	5.0	5.5	46	8.2	6.0	6.5	18	8.9	6.2	7.5	0.13
oocytes retrieved	95	6.2	4.9	5	46	6.4	5.1	6	18	7.3	3.5	7	0.24
MII	93	5.3	4.3	4	41	5.8	4.3	5	18	6.3	2.9	5.5	0.20
N Embryos	81	3.0	2.3	3	35	3.3	2.1	2	15	4.3	1.7	4	0.014*
Embryo Transferred	79	1.7	0.6	2	35	1.7	0.6	2	15	2.0	0.0	2	0.13
Frozen Embryo	73	1.4	3.6	0	29	2.0	3.7	0	12	1.7	3.2	0	0.69
Variables	A10G/rs11170555												p
	AA				AG				GG				
	N	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	
Total AFC	88	9.7	3.7	9	38	10.1	4.2	10	19	9.4	4.0	9.0	0.83
Estradiol	112	44.8	11.8	42.0	50	46.2	14.6	44.0	20	53.3	15.3	50.8	0.019*
AMH	114	4.4	3.0	3.9	51	4.4	2.3	4.1	21	2.8	2.3	1.7	0.011*
FSH	114	6.6	2.2	6.6	51	6.0	1.8	5.6	21	7.1	2.1	6.7	0.07
Follicles USG	94	6.8	4.9	5.5	48	8.7	6.7	7	20	7.6	4.3	6	0.27
oocytes retrieved	92	6.3	4.9	5	48	6.5	5.1	6	19	6.9	4.0	5	0.69
MII	90	5.4	4.2	4	43	6.0	4.3	5	19	5.8	3.4	5	0.53
N Embryos	79	3.0	2.2	3	37	3.2	2.1	2	15	4.1	2.2	4	0.09
Embryo Transferred	77	1.8	0.5	2	37	1.7	0.7	2	15	1.9	0.4	2	0.73
Frozen Embryo	72	1.4	3.6	0	30	1.9	3.7	0	12	2.2	3.0	1	0.046*
Variables	C1749G/rs2071558												P
	CC				CG				GG				
	N	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	
Total AFC	104	9.6	3.8	9	37	10.0	4.2	10	4	11.5	2.4	12.5	0.37
Estradiol	129	45.9	12.4	44.0	49	46.6	14.5	44.7	4	48.5	23.6	42.6	0.97
AMH	131	4.1	2.9	3.5	50	4.2	2.2	4.1	5	6.0	4.0	6.9	0.37
FSH	131	6.7	2.2	6.6	50	6.0	1.8	5.6	5	5.7	2.1	5.5	0.07
Follicles USG	111	6.8	4.8	6	47	8.7	6.8	7	4	10.8	2.2	10	0.049*
oocytes retrieved	108	6.2	4.7	5	47	6.4	5.2	6	4	10.5	2.5	10	0.07
MII	106	5.3	4.0	4	42	5.9	4.4	5	4	9.3	1.0	9.5	0.041*
N Embryos	92	3.1	2.3	3	36	3.1	2.0	2	3	6.3	2.1	7	0.06
Embryo Transferred	90	1.8	0.5	2	36	1.7	0.7	2	3	2.0	0.0	2	0.62
Frozen Embryo	82	1.4	3.4	0	29	1.9	3.7	0	3	4.3	5.1	3	0.27
Variables	G4952A/rs3741664												p
	GG				GA				AA				
	N	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	
Total AFC	105	9.6	3.8	9	40	10.2	4.2	10	-	-	-	-	0.41
Estradiol	131	46.3	12.7	44.0	51	45.8	14.5	43.3	-	-	-	-	0.72
AMH	133	4.1	2.9	3.4	53	4.5	2.4	4.2	-	-	-	-	0.15
FSH	113	6.7	2.2	6.6	53	6.0	1.8	5.6	-	-	-	-	0.033*
Follicles USG	113	6.9	4.8	6	49	8.9	6.6	7	-	-	-	-	0.10
oocytes retrieved	110	6.2	4.6	5	49	6.8	5.2	6	-	-	-	-	0.75
MII	108	5.3	4.0	4	44	6.2	4.4	6	-	-	-	-	0.22
N Embryos	93	3.1	2.2	3	38	3.3	2.2	2.5	-	-	-	-	0.63
Embryo Transferred	91	1.8	0.5	2	38	1.7	0.7	2	-	-	-	-	0.73
Frozen Embryo	83	1.4	3.4	0	31	2.1	3.9	0	-	-	-	-	0.53

When we considered the dominant model, it was noted that the presence of one polymorphic T allele continued to present higher values of embryos produced (p=0.011). In this case, we observed that the dominant allele is the polymorphic one. We also noted that the TT genotype presented higher mean levels of estradiol, p=0.048.

The correlation between this polymorphism and β HCG results also showed a negative association, p=0.472.

A-482G (rs2002555) polymorphism of the AMHR2 gene

The genotype distribution according to hormonal measurements of estradiol, AMH, and FSH, also did not present a positive association; p=0.65, p=0.67, p=0.12, respectively (Table 4).

COH response also did not demonstrate a positive relationship with genotypes (p=0.22), whereas 62.9% (117/186) were wild-type homozygous, 26.9% (50/186) were

Table 5. Comparison between serum AMH and AFC levels with embryo parameters and ovarian response. N: number of individuals; rho: coefficient correlation; SD: Standard Deviation; *Spearman correlation; **Kruskal-Wallis Test

	AMH			AFC		
	N	Rho	p	N	rho	P*
AFC	145	0.351	<0.001*	-	-	-
USG Follicles	162	0.263	0.001*	128	0.143	0.108
Retrieved Oocytes	159	0.183	0.021*	126	0.062	0.49
MII	152	0.215	0.008*	121	0.149	0.102
N Embryos	131	0.124	0.158	106	0.131	0.182
Transferred Embryo	129	0.033	0.712	105	0.012	0.904
Frozen Embryo	114	0.235	0.012*	93	0.292	0.004*

	N	Mean	SD	Median	p	N	Mean	SD	Median	P**
Poor response	57	3.9	2.6	3.69		42	10.5	3.6	10	
Satisfactory response	109	4	2.8	3.3	<0.001	90	9	3.6	8	0.005*
Hiper response	12	6.2	2.1	7		10	13.1	5	11	
OHSS	8	7	2	7.7		3	12.7	5	12	

heterozygous, and 10.2% (19/186) presented the polymorphic genotype GG, as shown in Table 3. The frequency of the wild type allele was n=284 (76.3%) and the frequency of the polymorphic allele was n=88 (23.7%).

Similar to the G146T/Ile49Ser polymorphism, we did not observe any positive association between the A-482G/rs2002555 polymorphism and human reproduction outcomes (AFC, p=0.81; follicles visualized at USG, p=0.13; oocytes retrieved, p=0.24; MII, p=0.20; embryos transferred, p=0.13 and frozen embryos, p=0.69), except for the number of embryos produced, p=0.014 (polymorphic G allele), as shown in Table 4.

In the analysis of the dominant model, we detected a higher mean value of embryos produced (p=0.005) and embryos transferred (p=0.046) in the presence of the polymorphic genotype GG compared to the A allele. Moreover, the A-482G polymorphism as not statistically significantly associated with the β HCG results, p=0.29.

A10G (rs11170555) polymorphism of the AMHR2 gene

Estradiol and AMH levels were positively associated with A10G genotypes: p=0.019 and p=0.011, respectively. Thereby, polymorphic genotype (GG) demonstrated higher mean values of estradiol and lower mean values of AMH, when compared to the AA genotype. Basal FSH did not display an association, p=0.07 (Table 4).

COH response was not associated with A10G polymorphism, p=0.35. Of all of the patients, 61.3% (114/186) were wild homozygous, 27.4% (51/186) were heterozygous, and 11.3% (21/186) presented the polymorphic genotype, as shown in Table 3. The allele A presented a frequency of n=279 (75%) and allele G presented a frequency of n=93 (25%).

The reproductive parameters were not associated with genotypes (AFC, p=0.83; follicles visualized at USG, p=0.27; oocytes retrieved, p=0.69; MII, p=0.53; embryos produced, p=0.09; embryos transferred, p=0.73). Frozen embryo number was correlated with polymorphic genotypes that produced higher rates compared to other genotypes, p=0.046 as shown in Table 4.

Moreover, A10G polymorphism resulted in statistically significant for β HCG, thereby patients with polymorphic genotype presented higher frequencies of positive β HCG, p=0.05.

C1749G (rs2071558) polymorphism of the AMHR2 gene

Basal FSH, AMH and estradiol did not show a positive association with C1749G polymorphism, p=0.97, p=0.37, p=0.07, respectively, as well as G146T and A-482G polymorphisms (Table 4).

COH response also did not demonstrate a positive relation, $p=0.55$, whereas 70.4% (131/186) were wild-type homozygous, 26.9% (50/186) were heterozygous, and 2.7% (5/186) presented the polymorphic genotype, as shown in Table 3. The allele frequency was $n=312$ (83.9%) for allele C and $n=60$ (16.1%) for allele G.

In contrast to other polymorphisms, we detected a positive association between follicles visualized at USG, $p=0.049$ and MII, $p=0.041$, while the other variables were not correlated (AFC, $p=0.37$; oocytes retrieved, $p=0.07$; embryos produced, $p=0.06$; embryos transferred, $p=0.62$ and frozen embryos, $p=0.27$). Polymorphic genotype carriers had higher mean values than wild type and heterozygous genotypes (Table 4).

For pregnancy rate, C1749G polymorphism could not predict β HCG results, $p=0.92$.

G4952A (rs3741664) polymorphism of the AMHR2 gene

Wild type genotypes had significantly higher basal FSH levels compared to that of heterozygous genotypes, $p=0.03$. However, estradiol and AMH serum levels were not positively correlated, with the G4952A polymorphism $p=0.72$ and $p=0.15$, respectively (Table 4).

As was the case for all other polymorphisms, G4952A could not predict COH response, $p=0.33$.

The frequency of genotypes were as follows: 71.5% (133/186) were wild type homozygous and 28.5% (53/186) were heterozygous. None of the patients presented the polymorphic genotype (Table 3). The allele frequency was 85.8% for the wild type allele ($n=319$) and 14.2% for the polymorphic allele ($n=53$).

G4952A polymorphism is also not a good predictor of assisted reproduction outcomes: we did not observe a statistically significant correlation with any of the variables studied (AFC, $p=0.41$; follicles visualized at USG, $p=0.10$; oocytes retrieved, $p=0.75$; MII, $p=0.22$; embryos produced, $p=0.63$; embryos transferred, $p=0.73$ and frozen embryos, $p=0.53$). As shown in Table 4.

Thus, the G4952A polymorphism did not demonstrate a positive association with β HCG results, $p=0.83$.

Serum AMH as a predictor of assisted reproduction outcomes

In a comparison of serum AMH levels with assisted reproductive parameters, we verified that all variables were statistically significant: AFC, $p<0.001$; follicles visualized at USG, $p=0.001$; oocytes retrieved, $p=0.021$; MII, $p=0.008$; number of frozen embryos, $p=0.012$; with the exception of number of embryos, $p=0.158$ and number of embryos transferred, $p=0.712$. Results are shown in Table 5.

Regarding the COH outcomes, we can attest that the AMH levels in patients who had suffered OHSS were almost double that of other groups; thus, the minimum value of AMH detected in these patients was almost four times higher compared to other patients, $p<0.001$. However, it was not possible to stipulate a cut-off value for OHSS, once good responder patients had a minimum of 0.5 ng/ml and a maximum of 17.5 ng/ml of serum AMH and the values of the two groups overlapped.

To identify if AMH is a predictor of COH response, a multinomial logistic regression model was performed. According to the results, for the growth of one unit of AMH (1.0 ng/ml), the chance of belonging to the hyper response group increased by 1.28 times, and the chance of belonging to the OHSS group increased by 1.36 times.

As for poor responders the chance of belonging to the hyper response group increased by 1.28, and the chance of belonging to the OHSS group increased by 1.37 times.

AFC as a predictor of assisted reproduction outcomes

When we use antral follicle counting as a predictor of COH response, AFC was positively correlated with hyperstimulation syndrome ($p=0.005$), as well as AMH. Considering assisted reproduction outcomes, we observed that AFC was positively correlated only with the number of frozen embryos, $p=0.004$. However, the other parameters were not correlated

(follicles visualized at USG, $p=0.108$; oocytes retrieved, $p=0.49$; MII, $p=0.102$; number of embryos produced, $p=0.182$; embryos transferred, $p=0.904$) (Table 5). β HCG results also had a negative correlation, $p=0.693$.

Discussion

To the best of our knowledge, this is the first study to associate *AMH* and *AMHR2* polymorphisms with AMH, estradiol, FSH serum levels, COH response and assisted reproduction outcomes in a Brazilian population.

Durlinger et al. [9] demonstrated that *AMH* knockout mice had follicles recruited at a faster rate than controls, and that they became more sensitive to FSH. *AMH* and *AMHR2* polymorphisms contribute to the individual variation in the FSH ovary threshold [19]. These polymorphisms appear to affect hormone biological activities, thus affecting follicle recruitment and development [14].

Kevenaer et al. [10] developed an association study with Dutch and German cohorts of normo-ovulatory women. The results revealed that, in Dutch women, carriers of the Ser/Ser variant (TT genotype) of the G146T/rs10407022 polymorphism had significantly higher estradiol levels on day 3 of their menstrual cycle when compared to women with the others genotypes. Subsequently, the Dutch and German cohorts were analyzed together. In the combined cohort, carriers of the Ser/Ser variant, again, had higher estradiol levels; however, this change was not associated with serum AMH and FSH. In the present study, we observed that only the A10G/rs11170555 polymorphism of *AMHR2* was associated with estradiol and AMH serum levels. Thus, carriers of polymorphic genotypes had higher mean estradiol levels, and lower serum AMH levels.

In contrast, a study with Chinese infertile women did not find a correlation between estradiol serum levels and G146T/Ile49Ser/rs10407022 polymorphism [20]. Similar to the results reported by Xu et al. [20], the present study did not find such correlations between A-482G, C1749G and G4952A polymorphisms of the *AMHR2* gene. In the same Dutch and German cohorts, it was shown that *AMHR2* genotypes were significantly associated with estradiol serum levels during the early follicular phase of the menstrual cycle [10].

None of these studies found an association between basal FSH and *AMH* and *AMHR2* genes. We demonstrated that only the polymorphism G4952A/rs3741664 was significantly associated with basal FSH, because non mutation carriers had higher FSH levels.

The Ser/Ser variant [TT genotype] is less effective in reducing individual FSH sensitivity of antral follicles. *In vitro* studies have demonstrated that Ser/Ser protein bioactivity is reduced compared with the Ile/Ile protein, and because of that, the T/Ser allele also had a lower total follicle number compared to the other groups [10]. However, we did not find any interconnection among G146T/Ile49Ser/rs10407022 or polymorphisms of the *AMHR2* gene and the antral follicle counting. Nevertheless, the C1749G polymorphism of the *AMHR2* gene showed a statistically significant difference, so that carriers of the polymorphic genotype had more follicles visualized by ultrasound and a better degree of oocyte maturation.

The G146T/rs10407022 polymorphism is located in the *AMH* gene promoter region, leading to an amino acid substitution in the protein: isoleucine to serine in position 49 of the *AMH* protein [Ile49Ser]. This region is responsible for protein stability and folding. Thus, mutations within the promoter region could affect *AMH* biosynthesis or bioactivity. This mutation in the *AMH* gene can inactivate the protein. The G146T/Ile49Ser/rs10407022 polymorphism did affect its bioactivity, but not the processing of *AMH* [21]. The reason why *AMH* and *AMHR2* polymorphisms can predict the number of embryos produced, while *AMH* serum levels could not predict this same variable are explained by our results. The presence of a serine at amino acid position 49 likely alters the protein folding, making the protein less bioactive in comparison to the protein that has isoleucine at this position [21].

Kevenaer et al. [10] suggested that *AMHR2* polymorphic genotype GG can result in diminished *AMH* signaling, and because of its location (promoter region), it may cause

disequilibrium with other SNPs. The diminished AMH signaling may result in increased primordial follicle recruitment. In a study conducted by Yassin et al. [22], it was found that the oocyte retrieval rate of the GG genotype of the *AMHR2A*-482G/rs2002555 was statistically lower than in the control group, suggesting that a homozygous mutation (-482GG genotype) may cause poor follicular development. However, they did not find a positive association with AMH G146T/Ile49Ser/rs10407022 polymorphism. We showed that the number of embryos produced were statistically associated with the polymorphic genotype (*AMH* 146TT and *AMHR2* -482GG) in cases of *AMH* G146T/Ile49Ser/rs10407022 and *AMHR2* A-482G/rs2002555 polymorphisms. We also showed that the *AMHR2* polymorphism A10G/rs11170555 was positively associated with the number of frozen embryos.

A mutation in the *AMH* gene and in its receptor, which prevents the AMH from properly exerting its function, would influence the inhibitory action on FSH. Therefore, it will be recruited by more follicles, and thus the number of embryos produced, and possibly frozen, would be relatively high.

There have been few studies reported that compared *AMH* and *AMHR2* polymorphisms and assisted reproduction outcomes. Moreover, most of those studies only analyzed the polymorphisms G146T/Ile49Ser/rs10407022 of the *AMH* gene and A-482G/rs2002555 of the *AMHR2* gene.

Furthermore, it has been shown that allele frequencies of the A-482G polymorphism were statistically significantly increased in Italian infertile patients compared with controls. The *AMH* G146T/Ile49Ser/rs10407022 polymorphism was associated with an increased frequency of T/Ser allele in infertile patients compared to controls, suggesting that variants of *AMH* and *AMHR2* genes appear to be associated with infertility [11].

The first study to indicate that AMH could predict COH response was reported by Seifer et al. [23]. They studied 107 infertile women that were divided into two groups: those who had ≤ 6 oocytes ($n=28$) and ≥ 11 oocytes retrieved ($n=79$). The results revealed a significant positive correlation between AMH and the number of oocytes retrieved, as well as mature oocytes. More recently, a study conducted on 81 infertile women from the Gaza Strip compared AMH with age, total number of oocytes, number of mature oocytes and number of embryos. The authors demonstrated that AMH was correlated with all of these variables, and they also showed that mean levels of AMH indicated a progressive increase in parallel to the total number of oocytes in poor, good and high responders [22].

In agreement with these studies, we demonstrated that serum AMH level is positively interconnected with AFC, follicles visualized at USG, oocytes retrieved, MII, and number of frozen embryos. However, AMH failed to predict pregnancy. A review by Broekmans et al [3] concluded that markers of ovarian reserves, including AMH, were not good predictors of pregnancy in IVF cycles. To confirm that AMH is a better marker of COH response and IVF outcomes, we compared it with AFC. Our results confirm previously reported findings [24-26] that attested that AFC is less effective than AMH in predicting IVF outcomes; AFC was only associated with number of frozen embryos. La Marca et al. [27] indicated that AMH has primarily been related to assisted reproduction because of its strong association with oocyte yield after COH. Therefore, this hormone should be capable of predicting ovarian response [24, 28-30].

Several studies have presented AMH as a predictor of ovarian response [24, 31, 32]. The ability to predict early ovarian response to COH would provide the opportunity to obtain essential information to improve further cycles [33]. A meta-analysis including 1,500 patients from nine studies established that AMH is a good predictor of excessive ovarian response [25]. Moreover, Lee et al. [28] reported that AMH is a better predictor than age, BMI, basal FSH or inhibin B. Our results corroborate with previous reports in the literature. We showed that AMH is strongly associated with OHSS, and for every time that normal responders increase 1.0 ng/ml of AMH, the chance of belonging to the OHSS group also increased by 1.28 times. This chance is 1.37 times when the reference is poor responder patients. Nevertheless, it is difficult to define a cutoff value for OHSS, especially for the lack of reference values for this hormone in the literature.

In summary, we showed that *AMH* polymorphisms is associated with the number of embryos produced, whereas *AMHR2* polymorphisms are associated with AMH, FSH and estradiol measurements, follicles visualized by ultrasound, degree of oocyte maturation, and number of embryos produced and frozen. Moreover, serum AMH level was associated with AFC, follicles visualized by USG, oocytes retrieved, degree of oocyte maturation and frozen embryos; additionally, AMH was strongly associated with OHSS. Additionally, AMH was shown to be a better biomarker than antral follicle counting and the polymorphisms studied in predicting IVF outcomes in Brazilian infertile women.

Disclosure Statement

CP, FLAF, GGG, DMC, EBC, CPB and BB disclosed no conflicts of interest.

Acknowledgments

The authors wish to thank FAPESP for granting Carla Peluso (#2011/15045-4) and Guilherme Gastaldo (#2013/06989-4) a student scholarship, and CNPq for granting Bianca Bianco (300825/2013-7), Caio Parente Barbosa (300816/2012-0) and Denise Maria Christofolini (301242/2013-5) a research productivity scholarship. This work was supported by FAPESP research grant #2011/08681-1 and 2014/06177-2.

References

- 1 Elchalal U, Schenker J: The pathophysiology of ovarian hyperstimulation syndrome — views and ideas. *Hum Reprod* 1997;12:1129–1137.
- 2 Fauser BCJM, Devroey P, Yen SSC, Gosden R, Crowley WF Jr, Baird DT, Bouchard P: Minimal ovarian stimulation for IVF. Appraisal of potential benefits and drawbacks. *Hum Reprod* 1999;14:2681–2686.
- 3 Broekmans F, Kwee J, Hendriks J, Mol BW, Lambalk CB: A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update* 2006;12:685–718.
- 4 Cate R, Mattaliano R, Hession C, Tizard R, Farber N, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, et al: Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. *Cell* 1986;45:685–698.
- 5 Di Clemente N, Josso N, Gouedard L, Belleville C: Components of the anti-Müllerian hormone signaling pathway in gonads. *Mol Cell Endocrinol* 2003;211:9–14.
- 6 Durlinger A, Gruijters M, Kramer P, Karels B, Ingraham J, Nachtigal M, Uilenbroek JT, Grootegoed JA, Themmen AP: Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 2002;143:1076–1084.
- 7 Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC, Themmen AP: Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod* 2004;10:77–83.
- 8 Visser JA, Themmen AP: Anti-mullerian hormone and folliculogenesis. *Mol Cell Endocrinol* 2005;234:81–86.
- 9 Durlinger A, Gruijters M, Kramer P, Karels B, Kumar T, Matzuk M, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP: Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology* 2001;142:4891–4899.
- 10 Kevenaar M, Themmen A, Laven J, Sonntag B, Fong S, de Jong F, Pols HA, Simoni M, Visser JA: Anti-mullerian hormone and anti-mullerian hormone type II receptor polymorphisms are associated with follicular phase oestradiol levels in normo-ovulatory women. *Hum Reprod* 2007;1547–1554.
- 11 Rigon C, Andrisani A, Forzan M, D'Antona D, Bruson A, Cosmi E, Ambrosini G, Tiboni GM, Clementi M: Association study of AMH and AMHRII polymorphisms with unexplained infertility. *Fertil Steril* 2010;94:1244–1248.
- 12 Riggs R, Kimble T, Oehninger S, Bocca S, Zhao Y, Leader B, Stadtmauer L: Anti-Müllerian hormone serum levels predict response to controlled ovarian hyperstimulation but not embryo quality or pregnancy outcome in oocyte donation. *Fertil Steril* 2011;95:410–412.

- 13 Riggs RM, Duran EH, Baker MW, Kimble TD, Hobeika E, Yin L, Matos-Bodden L, Leader B, Stadtmayer L: Assessment of ovarian reserve the anti-mullerian hormone: a comparison of the predictive value of anti-mullerian hormone, follicle stimulating hormone, inhibin B and age. *Am J Obstet Gynecol* 2008;199:202.
- 14 Yoshida Y, Yamashita Y, Saito N, Ono Y, Yamamoto H, Nakamura Y, Hayashi A, Terai Y, Ohmichi M: Analyzing the possible involvement of anti-Mullerian hormone and anti-Mullerian hormone receptor II single nucleotide polymorphism in infertility. *J Assist Reprod Genet* 2014;31:163-168.
- 15 WHO manual for the standardized investigation and diagnosis of the infertile male, 2004; Infertility: optimal evaluation of the infertile male: revised 2010; Infertility: report on Varicocele and infertility 2010.
- 16 Barbosa CP, Cordts E, Costa AC, Oliveira R, Mendonça M, Christofolini DM, Bianco B: Low dose of rFSH [100IU] in controlled ovarian hyperstimulation response: a pilot study. *J Ovarian Res* 2014;7:11-15.
- 17 Golan A, Ron-el R, Herman A, Soffer Y, Weinraub Z, Caspi E: Ovarian hyperstimulation syndrome: an update review. *Obstet Gynecol Surv* 1989;44:430-440.
- 18 Lahiri DK, Numberger JI: A rapid non-enzymatic method for preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res* 1991;19:5444.
- 19 De Castro F, Moron FJ, Montoro L, Galan JJ, Hernandez DP, Padilla ES, Ramírez-Lorca R, Real LM, Ruiz A: Human controlled ovarian hyperstimulation outcomes is a polygenic trait. *Pharmacogenetics* 2004;14:285-293.
- 20 Xu P, Shen S, Zhang X, Liang F, Xie G, Yi L, Gao Q, Wang Y: Haplotype analysis of single nucleotide polymorphisms in anti-Müllerian hormone gene in Chinese PCOS women. *Arch Gynecol Obstet* 2013;288:125-130.
- 21 Kevenaar M, Laven J, Fong S, Uitterlinden A, de Jong F, Themmen A, Visser JA: A functional anti-mullerian hormone gene polymorphism is associated with follicle number and androgen levels in polycystic ovary syndrome patients. *J Clin Endocrinol Metab* 2008;93:1310-1316.
- 22 Yassin MM, Sharif FA, Laqqan MM: Anti-Mullerian hormone as a predictor of ovarian reserve and ovarian response in IVF women from Gaza Strip. *Iran J Reprod Med* 2013;11:261-266.
- 23 Seifer DB, MacLaughlin DT, Christian BP, Feng B, Shelden RM: Early follicular serum mullerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles. *Fertil Steril* 2002;77:468-71.
- 24 La Marca A, Giulini S, Tirelli A, Bertucci E, Marsella T, Xella S, Volpe A: Anti-Müllerian hormone measurement on any day of the menstrual cycle strongly predict ovarian response in assisted reproductive technology. *Hum Reprod* 2007;22:766-771.
- 25 Broer SL, Mol B, Dolleman M, Fauser B, Broekmans FJ: The role of anti-Müllerian hormone assessment in assisted reproductive technology outcomes. *Curr Opin Obstet Gynecol* 2010;22:193-201.
- 26 Lehmann P, Velez MP, Saumet J, Lapensée L, Jamal W, Bissonette F, Phillips S, Kadoch IJ: Anti-Mullerian hormone (AMH): a reliable biomarker of oocyte quality in IVF. *J Assist Reprod Genet* 2014;31:493-498.
- 27 La Marca A, Sighinolfi G, Radi D, Argento C, Baraldi E, Artenisio AC, Stabile G, Volpe A: Antimullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). *Hum Reprod Update* 2010;16:113-130.
- 28 Lee T, Liu CH, Huang CC, Wu YL, Shih YT, Ho HN, Yang YS, Lee MS: Serum antimullerian hormone and estradiol levels as predictors of ovarian Hyperstimulation syndrome in assisted reproduction technology cycles. *Human Reprod* 2008;23:160-167.
- 29 Nardo L, Gelbaya TA, Wilkinson H, Roberts SA, Yates A, Pemberton P, Laing I: Circulating basal anti-mullerian hormone levels as a predictor of ovarian response in women undergoing ovarian stimulation for in vitro fertilization. *Fertil Steril* 2009;92:1586-1593.
- 30 Jayaprakasan K, Campbell B, Hopkisson J, Johnson I, Raine-Fenning N: A prospective, comparative analysis of anti-Mullerian hormone, inhibin-B, and three-dimensional ultrasound determinants of ovarian reserve in the prediction of poor response to controlled ovarian stimulation. *Fertil Steril* 2010;93:855-64.
- 31 Altmäe S, Hovatta O, Stavreus-Evers A, Salumets A: Genetic predictors of controlled ovarian hyperstimulation: Where do we stand today? *Human Reprod Update* 2011;17:813-828.
- 32 Arce JC, La Marca A, Klein BM, Andersen AN, Fleming R: Antimullerian hormone in gonadotropin releasing-hormone antagonist cycles: prediction of ovarian response and cumulative treatment outcome in good-prognose patients. *Fertil Steril* 2013;99:1644-1653.
- 33 Fréour T, Mirallie S, Bach-Ngohou K, Denis M, Barrière P, Masson D: Measurement of serum anti-Mullerian hormone by Beckman Coulter ELISA and DSL ELISA: comparison and relevance in assisted reproduction technology (ART). *Clin Chim Acta* 2007;375:162-164.