Clin. Lab. 2017;63:XXX-XXX

ORIGINAL ARTICLE

The Relationship Between the MTHFR C677T Genotypes to Serum Anti-Müllerian Hormone Concentrations and *In Vitro* Fertilization/Intracytoplasmic Sperm Injection Outcome

Seyedeh Z. Shahrokhi [1], Faranak Kazerouni [1], Firouzeh Ghaffari [2], Ali Rahimipour [1], Mir D. Omrani [3], Arezoo Arabipoor [2], Ramin Lak [4], Elaheh T. Ghane [5]

[1] *Department of Laboratory Medicine, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[2] *Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran*
[3] *Department of Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Velenjak Ave, Chamran Highway, Tehran, Iran*
[4] *Department of Microbiology, Faculty of Sciences, Shiraz branch, Islamic Azad University, Shiraz, Iran*
[5] *Department of Biostatistics, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

SUMMARY

Background: **The expression of the 5,10-methylentetrahydrofolate reductase (MTHFR) gene in human oocytes and preimplantation embryos suggests that the MTHFR gene is involved in folliculogenesis and female reproduction. Considering the importance of the MTHFR gene on female reproduction, the aim of this study was to evaluate the influence of MTHFR C677T polymorphism on ovarian marker reserve, particularly serum anti-Müllerian hormone (AMH) levels, and ovarian response as well as clinical pregnancy rates after *in vitro* fertilization (IVF)/ intracytoplasmic sperm injection (ICSI).**

Methods: **A total of 137 women who underwent ART treatment due to male factor infertility enrolled in this study. Genotyping of MTHFR C677T polymorphism and serum AMH concentrations were performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique and an ultrasensitive enzyme-linked immunosorbent assay (ELISA).**

Results: **Women with the TT genotype showed significantly higher AMH levels (4.5 ± 3.2 ng/mL) compared to carriers of other genotypes after ovarian stimulation. We observed a nonsignificant trend towards lower clinical pregnancy rates in patients with the TT (23.1%) versus CT (48.4%) genotypes (p = 0.2). No significant differences existed in terms of miscarriage and live birth rates among the groups. Multivariable logistic regression revealed that the duration of infertility and AFC were important predictive variables for the live birth rate.**

Conclusions: **Our results confirmed that the presence of the T mutant allele of the 677 polymorphism in the MTHFR gene led to an increased trend in AMH levels. Interestingly, we observed that the numbers of oocytes retrieved decreased in the mutated genotypes. We have not observed this trend in relation to oocyte maturity. The influence of the MTHFR C677T polymorphism on embryo quality and pregnancy rate after ART cycles remains unclear.**

(Clin. Lab. 2017;63:xx-xx. DOI: 10.7754/Clin.Lab.2016.161104)

Correspondence:
Faranak Kazerouni
Department of Laboratory Medicine
School of Allied Medical Sciences
Shahid Beheshti University of Medical Sciences
Tehran
Iran
Phone/Fax: +98 2122008155
Email: f_kazerouni@sbmu.ac.ir

Dr. Ghaffari
Department of Endocrinology and Female Infertility
Reproductive Biomedicine Research Center
Royan Institute for Reproductive Biomedicine ACECR
Tehran, Iran
Phone/Fax: +98 21 22306481
Email: ghafaryf@yahoo.com

Manuscript accepted December 23, 2016

KEY WORDS

serum anti-Müllerian hormone, ART outcome, MTHFR C677T polymorphism, male factor infertility, PCR-RFLP

INTRODUCTION

Prediction of the ovarian response to stimulation in assisted reproductive technology (ART) cycles is one of the ongoing challenges toward an optimal treatment strategy. Various hormone and clinical parameters such as age, baseline follicle stimulating hormone (FSH) levels, anti-Müllerian hormone (AMH) levels, and the antral follicle count (AFC) are used to predict patient response to ovarian stimulation [1]. Among these predictors, serum AMH levels are a novel clinical marker to evaluate the ovarian response [2,3]. Despite a correlation between these markers and ovarian response, the individual ovarian response to FSH cannot be absolutely predicted. Genetic variations are most likely the main cause of individual variability in the type of ovarian response to stimulation [4]. Recently, an association has been reported between the C677T polymorphism in the 5,10-methylentetrahydrofolate reductase (MTHFR) gene and abnormal response to ovarian stimulation during ART. Individuals that carry the MTHFR 677T alleles, particularly TT, have a decreased response to stimulation [5]. MTHFR is a key regulatory enzyme in folate metabolism, which is implicated in the availability of the methyl donors by catalyzing the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyl-tetrahydrofolate [6]. Within the MTHFR gene, a common C-to-T substitution at position 677 (C677T) in exon 4 results in a base change from alanine to valine at codon 222 [7]. Compared to wild-type (677CC) carriers, individuals with heterozygous (677CT) and homozygous (677TT) genotypes have been reported to display an activity loss of approximately 30% (heterozygous) and 70% (homozygous) [8,9]. Expression of the MTHFR gene in human oocytes and preimplantation embryos demonstrate its involvement in folliculogenesis [10] and maintenance of genomic stability in oocytes [11]. The effect of MTHTR C677T polymorphism in patients who undergo *in vitro* fertilization (IVF) treatment on ovarian response, embryo quality, and pregnancy rate is still a matter of debate [10,12-14]. Therefore, the use of biomarkers of functional ovarian reserve in combination with the MTHTR C677T polymorphism provide important information about the female reproductive system which cannot be accurately predicted by age and hormonal markers. In combination, they may help optimize treatment strategies and improve prediction of ovarian response to guide individualized treatment. Therefore, the present study aims to investigate the relationship between the MTHTR C677T polymorphism and ovarian response.

MATERIALS AND METHODS

Study population

This study was performed at Royan Institute, a referral infertility clinic, in Tehran, Iran from January 2015 to January 2016. We recruited 137 women who underwent ART treatment due to male factor infertility for participation in the study. According to the 2010 World Health Organization (WHO) criteria, male factor infertility was defined as a sperm concentration < 15 x 10^6/mL, progressive sperm motility < 32%, or normal sperm morphology < 4% [15]. Further criteria for inclusion were: regular menstrual cycle (cycle length, 25 - 35 days), 20 - 37 years of age, body mass index (BMI) 20 - 30 kg/m^2, presence of both ovaries, no evidence of endocrine disorders (thyroid dysfunction or hyperprolactinemia), no history of ovarian surgery, and no hormone therapy within the 3 months prior to the start of this study. We excluded subjects that had a history of endocrine abnormalities such as polycystic ovary syndrome (PCOS) and diagnosis of endometriosis. All participants provided written informed consent and the local Ethics Committee approved the study protocol and the research ethics board approval number was IR.ACECR.ROYAN.REC.1394.3.

Blood sampling

Patients provided blood samples for AMH measurement and genomic DNA extraction during days 2 to 3 of the menstrual cycle. In order to assay serum AMH levels, the blood samples were centrifuged and the sera were stored at -20°C until analysis. Serum AMH was measured by an UltraSensitive enzyme-linked immunosorbent assay (ELISA) kit (cat. AL-105-i, Ansh Laboratories, Webster, TX, USA), according to the manufacturer's instructions.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's protocol. Genotyping for the MTHFR gene C677T polymorphism was performed according to the polymerase chain reaction fragment length polymorphism (PCR-RFLP) technique. Briefly, a 198 bp region in exon 4 of the MTHFR gene was amplified with forward
5'-GGTCAGAAGCATATCAGTCATGAG-3'
and reverse
5'-CTGGGAAGAACTCAGCGAACTCAG-3' primers. Each reaction consisted of DNA template (3 µL), 0.5 mL of each of the primers, dNTP (0.25 µM), $MgCl_2$ (1 µL), 10 x PCR buffer (2.5 µL), ddH_2O (17 µL), and Taq polymerase (0.25 µL) in a total volume of 25 µL. The amplification was carried out in an Eppendorf thermal cycler with the following temperature profile: initial denaturation at 95°C for 5 minutes, followed by 30 cycles that consisted of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 35 seconds, and a final extension step at 72ºC

for 10 minutes. The amplified PCR products were digested with 2 IU of restriction enzymes Hinf I (Fermentas) at 37°C overnight, after which the fragments were separated by electrophoresis on a 3% agarose gel that contained Sybrsafe. The C to T transition at nucleotide 677 created digestion sites for Hinf I. The heterozygous (CT) was digested into three bands of 198, 175, and 23 bp and the mutant homozygous (TT) was digested into two bands of 175 and 23 bp. The wild-type (CC) DNA remained undigested.

Treatment protocol

Ovarian stimulation was performed by using a GnRH agonist and GnRH antagonist protocol. The details of ovarian stimulation protocols, follicle monitoring, oocyte retrieval, hCG triggering, and embryo transfers have been previously described [16]. Primary endpoints of the study were the total number of oocytes retrieved, the number of mature oocytes, and determination of the effect of variant genotypes of MTHFR C677T polymorphism on serum AMH levels. According to a widespread definition, poor ovarian response was < 4 oocytes produced in response to ovarian stimulation. We considered 4 - 15 produced oocytes to be a normal ovarian response whereas a high ovarian response had > 15 oocytes produced [17]. The secondary endpoints included clinical pregnancy, miscarriage and live bith rates. Clinical pregnancy was diagnosed by ultrasonographic observation of a gestational sac with or without heart pulsations at 6 - 7 weeks of gestation. Miscarriage rate was defined as a clinical pregnancy lost before 20 weeks of gestation. The live birth rate was considered to be the delivery of a fetus with signs of life after 20 completed weeks of gestation. The miscarriage and live birth rates were presented as total spontaneous miscarriage and total live births per total number of patients with embryo transfer (ET) in each group.

Statistical analysis

Results are reported as the mean ± standard deviation (SD) or number and percent. Statistical comparisons among groups were made with the one-way ANOVA test for parametric data and the Kruskal-Wallis test in case of non-parametric data. We used the chi-square test to compare categorical variables among the three groups. All analyses were performed using SPSS software Version 20 (IBM Corporation, USA) and p-values of < 0.05 were regarded as statistically significant.

RESULTS

We screened 190 patients for eligibility of which 18 did not meet the inclusion criteria and 15 declined to participate. The remaining 157 patients diagnosed with male factor infertility provided blood samples for C677T MTHER genotyping analysis. In total, we observed homozygosity for the C allele in 57.32% (n = 90), compound heterozygosity in 30.57% (n = 48), and homozygosity for the T allele in 12.1% (p = 19) of cases. Of these, 20 patients did not begin the IVF/Intra cytoplasmic Sperm Injection (ICSI) cycles and PCR-RFLP results showed that these patients had the following MTHER C677T genotype: CC (n = 9), CT (n = 6), and TT (n = 5). Statstical analysis of the 137 patients who received controlled ovarian stimulation in the IVF/IESI cycle and the cycle outcomes were compared according to the patients' MTHER C677T genotypes.

We compared basic characteristics, ovarian response, and cycle outcomes among the three genotype groups (Table 1). No significant differences existed among the three genotype groups in terms of age, BMI, basal FSH and LH levels, infertility duration, type of infertility, AFC, total amount of used gonadotropins for stimulation, day of stimulation until hCG injection, total number of retrieved oocytes, and the precentage of MII oocytes (Table 1). Subjects that carried the homozygous TT genotype showed higher AMH levels compared to carriers of other genotypes ($p < 0.02$). There were no significant differences in terms of numbers of good quality embryos, fertilization, clinical pregnancy, miscarriage, and live birth rates among the groups (Table 1).

The scatter plot and linear regression analysis were used to depict the correlation between serum AMH concentrations and numbers of oocytes retrieved (NOR) separately in the different MTHFR 677C>T genotypes (Figure 1). The results showed significant correlations between serum AMH concentrations and NOR in all three MTHFR genotype groups. Based on the AMH concentrations, there were significantly lower NOR in MTFHR 677 TT and MTFHR 677 CT individuals compared to those with the MTFHR 677 CC genotype. As seen in Figure 1, the mean oocyte numbers for different MTHFR 677C>T genotype individuals with AMH concentrations that ranged from 1 - 5 ng/mL were: 12.5 (677CC), 9.8 (677CT), and 8.1 (677TT; $p = 0.02$). Multiple linear regression analysis by the backward method was used for detection of significant factors that influenced the numbers of retrieved oocytes. All possible determinants - womens' age and BMI, MTFHR genotype (normal and mutated), basal serum FSH, LH levels, adjusted AMH level, AFC, type of stimulation protocol, and total doses of gonadotropins - were entered in the regression analysis. Results demonstrated that both the serum AMH level and AFC had significant positive effects, whereas the MTHFR mutated genotype had a significant negative effect on the total numbers of retrieved oocytes (Table 2). Other possible determinants included in the regression model did not have any significant impacts on the numbers of retrieved oocytes (Table 2).

The multivariable logistic regression test performed in a backward method was adjusted for age and BMI, then applied to determine the significant predictive variables for live births in the studied population. All possible important variables such as type and duration of infertility, serum AMH level, number of retrieved oocytes, number

Table 1. Demographic characteristics, ovarian response, and IVF/ICSI cycle outcomes as stratified by MTHFR genotypes.

Variables	CC (normal) (n = 81)	CT (n = 42)	TT (n = 14)	[a] p-value
Age (years)	29.3 ± 3.8	29.5 ± 4.5	30.5 ± 4.4	0.6
BMI (kg/m^2)	25.0 ± 3.9	25.8 ± 3.4	26.0 ± 3.5	0.4
Basal FSH level (mIU/mL)	5.8 ± 1.9	5.8 ± 2.6	6.5 ± 2.1	0.5
Basal LH level (mIU/mL)	4.6 ± 2.3	4.6 ± 2.9	5.6 ± 2.5	0.4
Basal AMH level (ng/mL)	3.3 ± 1.3	4.0 ± 2.5	4.5 ± 3.2	0.02 *
TSH level (µIU/mL)	1.67 ± 0.65	1.62 ± 0.55	1.90 ± 0.59	0.3
Prolactin level (ng/mL)	15.63 ± 5.98	16.19 ± 6.56	17.14 ± 6.76	0.6
Hemoglobin (ng/dL)	13.0 ± 1.0	13.3 ± 1.1	13.5 ± 1.2	0.1
MCV (fL/red cell)	84.7 ± 8.0	85.0 ± 6.0	85.6 ± 9.0	0.9
Infertility duration	5.0 ± 3.6	5.7 ± 3.6	4.6 ± 4.0	0.4
Type of infertility				0.1
Primary	77 (95.1)	39 (92.9)	12 (85.7)	
Secondary	4 (4.9)	3 (7.1)	2 (14.3)	
Azoospermia diagnosis	18 (22.2)	6 (14.3)	0 (0)	0.1
AFC	12.7 ± 3.2	12.9 ± 3.8	12.5 ±2.3	0.9
Type of stimulation protocol				0.4
Agonist	56 (70)	34 (81.0)	10 (71.4)	
Antagonist	24 (30)	8 (19.0)	4 (28.6)	
Total dose of used gonadotropins (75 IU/amp)	1720.4 ± 576.7	1832.1 ± 632.0	1864.2 ± 786.5	0.5
Duration of stimulation (days)	10.2 ± 2.1	10.5 ± 1.6	10.4 ± 1.2	0.7
No. of oocytes retrieved	13.1 ± 7.4	10.1 ± 6.2	12.2 ± 7.4	0.1
No. of MII oocytes	11.5 ± 6.5	8.8 ± 5.4	11.0 ± 6.5	0.08
No. of MI oocytes	0.3 ± 0.6	0.5 ± 0.9	0.5 ± 0.7	0.3
No. of GV oocytes	0.7 ± 1.4	0.4 ± 1.2	0.4 ± 0.9	0.5
MII (%) [b]	0.87 ± 0.13	0.86 ± 0.14	0.91 ± 0.11	0.5
No. of good quality embryos	4.4 ± 3.6	3.8 ± 2.9	5.0 ± 2.9	0.4
No. of embryos transferred	2.1 ± 0.6	2.1 ± 0.7	2.0 ± 0.5	0.9
No. of cycles not responsive to gonadotropins	3 (3.7)	1 (2.4)	0 (0)	0.6
No. of cycles with no embryos	4 (4.9)	2 (4.8)	0 (0)	0.7
No. of all frozen embryo cases	8 (9.9)	3 (7.1)	1 (7.1)	0.8
Fertilization rate without azoospermia cases	0.56 ± 0.24	0.61 ± 0.31	0.64 ± 0.24	0.4
Clinical pregnancy rate/ET	27/66 (40.9)	16/31 (44.4)	3/13 (23.1)	0.3
Clinical pregnancy rate/ET without including azoospermia cases	21/51 (41.9)	15/31 (48.4)	3/13 (23.1)	0.2
Miscarriage rate/ET	4 (6.1)	0 (0)	0 (0)	0.2
Live birth rate/ET	23 (34.8)	16 (44.4)	3 (23.1)	0.3

BMI - body mass index, MII - metaphase II, FSH - follicle stimulating hormone, LH - luteinizing hormone, AMH - anti-Müllerian hormone, MCV - mean corpuscular volume, AFC - antral follicle count, GV - germinal vesicle, ET - embryo transfer.
All quantitative variables are mean ± SD according to the Kruskal-Wallis and ANOVA tests when appropriate. All qualitative variables are shown as No. (%) by the chi-square test. [a] p-value (among the three genotype groups); [b] Number of MII oocytes in relation to total number of retrieved oocytes.

and quality of transferred embryos, total dose of gonadotropin, stimulation protocol, fertilization rate, and MTFHR genotype were entered in the model. Results showed that duration of infertility and AFC were important predictive variables for the live bitrh rate (Table 3). This meant that for each one unit increase in AFC, the live birth rate increased 1.15 times (p = 0.02).

Table 2. Multiple linear regression analysis by backward method for detection of significant factors influencing the number of retrieved oocytes.

Dependent variables	Non-standardized coefficient		Standardized coefficient		p-value
	ß	SE	ß	T	
Constant	8.2	2.3	-	3.4	0.001
AMH [a]	2.3	0.6	0.3	3.9	< 0.001
AFC	0.3	0.18	0.17	2.0	0.04
Mutated C677T MTHER genotyping	-2.8	1.1	-0.18	-2.3	0.02

[a] The AMH levels were adjusted for MTFHR genotype (adjusted r = 0.037, r^2 = 0.051, r = 0.022).

Table 3. Multivariable logistic regression analysis by the backward method for determination of the live birth predictive variables adjusted for age and BMI (n = 137).

Predictive factors	Beta	SE	Adjusted OR	CI	p-value
Duration of infertility	0.14	0.06	0.88	0.78 - 1.00	0.051
AFC	0.2	0.06	1.15	1.02 - 1.3	0.02

BMI - body mass index, OR - odds ratio, CI - confidence interval, AFC - antral follicle count.

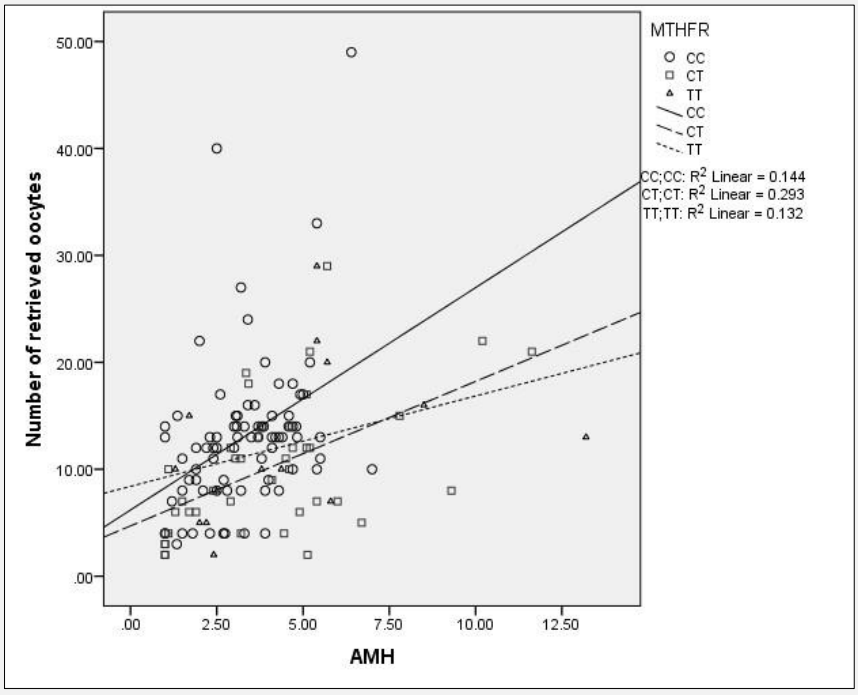

Figure 1. Scatter plot representing the correlation between anti-Müllerian hormone (AMH) serum concentrations and the number of oocytes retrieved (NOR) after controlled ovarian stimulation in 81 MTHFR 677CC (black circle, black regression line), 42 MTHFR 677CT (gray triangles, black dotted regression line), and 14 MTHFR 677TT (open squares, black dashed regression line) patients (r = Spearman's correlation coefficient).

Although AMH shows significant correlation with NOR in all MTHFR genotypes, homozygous MTHFR 677TT individuals have significantly fewer NOR for AMH (1 - 5 ng/mL) compared to individuals with the other MTHFR 677 genotypes.

DISCUSSION

The main finding of this study was the association of the C677T polymorphism in the MTHFR gene with a trend in serum AMH concentrations in women with normal ovarian function. The present study, which supported results reported by Pavlik et al. [18], found that despite increasing serum AMH levels in patients with mutations, the oocyte recovery and maturity rates in these patients did not increase. This finding could perhaps be due to an existing compensatory mechanism. Interestingly, we observed that the number of oocytes retrieved decreased when patients were stratified into two groups (normal and mutated genotypes). This trend did not apply in relation to oocyte maturity.

The MTHFR enzyme plays a key function in many biological processes considered important for cell division and embryo development. This enzyme regulates transfer of one-carbon units between DNA synthesis and methylation reactions [6]. Since the folic acid transport protein and MTHFR enzyme have been identified in human oocytes and preimplantation embryos [10], reduced MTHFR activity has been highlighted due to its important roles during oocyte growth [19]. Ovarian folliculogenesis is characterized by growth and differentiation of oocytes along with rapid proliferation of granulosa cells [20]. During this period, the requirement for folic acid and methionine is increased to support differentiation of granulosa cells which requires active DNA and protein synthesis [21]. Possibly at this time, reduced MTHFR activity and hyperhomocysteinemia, which are significant in individuals that have the TT genotype, may be delayed for follicular maturation. This leads to an increased rate of initial follicular recruitment thereby leading to elevated AMH levels [18]. However, hyperhomocysteinemia may also promote apoptosis [22] which can prevent these follicles from advancing toward cyclic recruitment. This possibility might have been the reason why we did not see a significant difference among the three genotypes regarding the numbers of MII oocytes.

D'Elia et al. conducted a retrospective study among 82 Brazilian women who underwent IVF treatment for male factor infertility. In their study, patients were divided into two groups according to the presence (n = 47) or absence (n = 35) of the mutant allele of the 677 polymorphism. Women with the CT and TT genotypes were combined together as the mutated group increasing the likelihood of obtaining a significant result. They demonstrated that the maturity of oocytes retrieved with the MTHFR C677T polymorphism and percentage of mature oocytes was lower in the mutant group compared to the normal group. The authors stated that high concentrations of intrafollicular homocysteine compromised oocyte maturation, as has been confirmed by previous studies [14]. Thaler et al. studied 105 women who underwent infertility treatment and correlated C677T MTHFR with ovarian response in those with advanced reproductive age. They observed that carriers of the MTHFR 677T allele who were over the age of 35 years required higher r-FSH doses for ovarian stimulation, produced significantly fewer retrieved oocytes, and had significantly lower concentrations of serum estradiol, which confirmed the reduced ovarian responsiveness to r-FSH. However, an increased percentage of patients, due to age-related decrease in ovarian reserve, required higher doses of FSH, regardless of MTHFR genotype [5]. Another study revealed no correlation between MTHFR 677T genotype and COH outcome, but observed an association between the MTHFR A1296C polymorphism wild type A allele with better COH outcome [4]. Since ethnic-specific variation in the distribution of polymorphisms has been previously reported [23,24], it seemed that the conflicting data was due to differences in the classification of patients and allelic frequencies between ethnic groups.

There are conflicting results regarding the impact of the MTHFR C677T polymorphism on IVF outcome. In this study, we have found higher clinical pregnancy and live birth rates in patients with the CT genotypes compared to the TT genotypes, but not the CC genotype. A trend existed towards lower clinical pregnancy rates in patients with TT genotypes but it did not reach statistical significance. Although women with the TT genotype produced good-quality embryos, it was likely that the embryos failed to implant for unexplained reasons, which resulted in implantation failure and early pregnancy loss. It was also reported that elevated plasma homocysteine levels due to impaired DNA methylation and gene expression might contribute to defective chorionic villus vascularization and subsequent early embryonic death [25].

In a recent study, Laanpere et al. reported that MTHFR C677T polymorphism was related to embryo quality and the chance of achieving clinical pregnancy in women after IVF/ICSI treatment. In their study of 39 female IVF patients and 225 fertile controls, the authors observed that women with the MTHFR 677 CT heterozygous genotype produced better quality embryos and had a greater chance for a positive hCG test and the achievement of clinical pregnancy compared with carriers of the CC and TT genotypes [12]. The better outcomes in women with the MTHFR 677 CT genotypes might be due to a favorable balance of folate distribution between both DNA synthesis and cellular methylation [26]. Similarly, Haggarty et al. observed that women with the heterozygous MTHFR 677 CT genotype had a small, yet significantly increased chance of a viable pregnancy after IVF treatment compared to those with the homozygous CC genotype [13]. In contrast to the previously mentioned studies, Dobson et al. studied 197 women undergoing IVF treatment and reported that neither the MTHFR C677T nor A1298C polymorphisms influenced the number, quality of embryos transferred, and short-term pregnancy outcomes [10]. The annotation with regard to the effect of different MTHFR 677C>T genotypes on embryo quality and pregnancy rate after ART cycles is complex since many factors can affect

the pregnancy rate after IVF/ICSI treatment. It seems that more, better designed studies are required in this regard.

As a limitation of our study, we used different protocols (agonist and antagonist) for ovarian stimulation which might affect the results. Although we could not use the same stimulation protocol for all study participants, multiple linear regression analysis was performed in order to evaluate the independent effect of each of the possible determinants on ovarian response. The results indicated that the effect of the MTHFR 677C>T genotypes on ovarian response was independent from the influence of the ovarian stimulation protocol.

CONCLUSION

We have found significant associations of the C677T polymorphisms in the MTHFR gene with serum AMH levels. Elevated AMH levels in 677TT patients is an important issue that clinicians should be aware of in order to reduce errors in determining the dose of the gonadotropin needed for ovarian stimulation.

Acknowledgement:
We would like to thank Dr. Reza Salman Yazdi for technical assistance during this work and all the participants in this study. This work was supported by Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Funding:
This work was supported by the Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Declaration of Interest:
The authors declare that they have no competing interests associated with the manuscript.

References:

1. La Marca A, Giulini S, Tirelli A, et al. Anti-Müllerian hormone measurement on any day of the menstrual cycle strongly predicts ovarian response in assisted reproductive technology. Hum Reprod 2007;22(3):766-71 (PMID: 17071823).

2. Van Rooij I, Broekmans FJ, te Velde ER, et al. Serum anti-Müllerian hormone levels: a novel measure of ovarian reserve. Hum Reprod 2002;17(12): 3065-71 (PMID: 12456604).

3. Seifer DB, MacLaughlin DT, Christian BP, Feng B, Shelden RM. Early follicular serum müllerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles. Fertil Steril. 2002;77(3):468-71 (PMID: 11872 196).

4. Rosen MP, Shen S, McCulloch CE, Rinaudo PF, Cedars MI, Dobson AT. Methylenetetrahydrofolate reductase (MTHFR) is associated with ovarian follicular activity. Fertil Steril 2007; 88(3):632-8 (PMID: 17572411).

5. Thaler CJ, Budiman H, Ruebsamen H, Nagel D, Lohse P. Effects of the Common 677C> T Mutation of the 5, 10-Methylenetetrahydrofolate Reductase (MTHFR) Gene on Ovarian Responsiveness to Recombinant Follicle-Stimulating Hormone. Am J Reprod Immunol 2006;55(4):251-8 (PMID: 16533336).

6. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci 2001;22(4):195-201 (PMID: 11282 420).

7. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995 May;10(1):111-3 (PMID: 7647 779).

8. Balaghi M, Wagner C. DNA methylation in folate deficiency: use of CpG methylase. Biochem Biophys Res Commun 1993;193(3): 1184-90 (PMID: 8323540).

9. De Cabo S, Hazen MJ, Molero ML, Fernández-Piqueras J. S-adenosyl-L-homocysteine: a non-cytotoxic hypomethylating agent. Experientia 1994;50(7):658-9 (PMID: 7518395).

10. Dobson A, Davis RM, Rosen MP, et al. Methylenetetrahydrofolate reductase C677T and A1298C variants do not affect ongoing pregnancy rates following IVF. Hum Reprod 2007;22(2):450-6 (PMID: 17053001).

11. Enciso M, Sarasa J, Xanthopoulou L, et al. Polymorphisms in the MTHFR gene influence embryo viability and the incidence of aneuploidy. Hum Genet 2016;135(5):555-68 (PMID: 27068821).

12. Laanpere M, Altmäe S, Kaart T, Stavreus-Evers A, Nilsson TK, Salumets A. Folate-metabolizing gene variants and pregnancy outcome of IVF. Reprod Biomed Online 2011;22(6):603-14 (PMID: 21507721).

13. Haggarty P, McCallum H, McBain H, et al. Effect of B vitamins and genetics on success of *in-vitro* fertilisation: prospective cohort study. Lancet 2006;367(9521):1513-9 (PMID: 16679164).

14. D'Elia PQ, dos Santos AA, Bianco B, Barbosa CP, Christofolini DM, Aoki T. MTHFR polymorphisms C677T and A1298C and associations with IVF outcomes in Brazilian women. Reprod Biomed Online 2014;28(6):733-8 (PMID: 24746944).

15. Lu, J., Y. Huang, and N. Lü, [WHO Laboratory Manual for the Examination and Processing of Human Semen: its applicability to andrology laboratories in China]. Zhonghua Nan Ke Xue 2010; 16(10) 867-71 (PMID: 21243747).

16. Madani T, Ghaffari F, Kiani K, Hosseini F. Hysteroscopic polypectomy without cycle cancellation in IVF cycles. Reprod Biomed Online 2009;18(3):412-5 (PMID: 19298742).

17. La Marca A, Grisendi V, Giulini S, et al. Individualization of the FSH starting dose in IVF/ICSI cycles using the antral follicle count. J Ovarian Res 2013;6(1):11 (PMID: 23388048).

18. Pavlik R, Hecht S, Ochsenkühn R, Noss U, Lohse P, Thaler CJ. Divergent effects of the 677C>T mutation of the 5, 10-methylenetetrahydrofolate reductase (MTHFR) gene on ovarian responsiveness and anti-Müllerian hormone concentrations. Fertil Steril 2011;95(7):2257-62 (PMID: 21481373).

19. Ebisch I, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13(2):163-74 (PMID: 17099205).

20. Maruo T, Laoag-Fernandez JB, Takekida S, et al. Regulation of granulosa cell proliferation and apoptosis during follicular development. Gynecol Endocrinol. 1999;13(6):410-9 (PMID: 10685335).

21. Megahed MA, Taher I. Folate and homocysteine levels in pregnancy. Br J Biomed Sci 2004;61(2):84-7 (PMID: 15250671).

22. Forges T, Monnier-Barbarino P, Alberto JM, Guéant-Rodriguez RM, Daval JL, Guéant JL. Impact of folate and homocysteine metabolism on human reproductive health. Hum Reprod Update 2007;13(3):225-38 (PMID: 17307774).

23. Wilcken B, Bamforth F, Li Z, et al., Geographical and ethnic variation of the 677C> T allele of 5, 10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. J Med Genet 2003;40(8):619-25 (PMID: 12920077).

24. Yang B, Liu Y, Li Y, et al. Geographical distribution of MTHFR C677T, A1298C and MTRR A66G gene polymorphisms in China: findings from 15357 adults of Han nationality. PLoS One 2013;8(3):e57917 (PMID: 23472119).

25. Heby O. DNA methylation and polyamines in embryonic development and cancer. Int J Dev Biol 1995;39(5):737-57 (PMID: 8645558).

26. Laanpere M, Altmäe S, Stavreus-Evers A, Nilsson TK, Yngve A, Salumets A. Folate-mediated one-carbon metabolism and its effect on female fertility and pregnancy viability. Nutr Rev 2010;68(2):99-113 (PMID: 20137055).