Divergent effects of the 677C>T mutation of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene on ovarian responsiveness and anti-Müllerian hormone concentrations

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Objective: To investigate the influence of the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T mutation on serum anti-Müllerian hormone (AMH) concentrations and on the numbers of oocytes retrieved (NOR) following controlled ovarian hyperstimulation (COH).

Design: Prospective cohort study.

Setting: University-based infertility clinic.

Patient(s): Two hundred and seventy women undergoing COH for IVF with or without intracytoplasmic sperm injection.

Intervention(s): None.

Main Outcome Measure(s): AMH levels were determined from blood samples collected after 10 days of GnRH superagonist treatment and before COH. The MTHFR 677C>T genotype was characterized by a TaqMan 5' nuclease assay.

Result(s): AMH serum concentrations correlated significantly with the NOR in all individuals studied. Average (±SD) AMH levels of TT carriers (2.85 ± 2.23 ng/mL) were significantly higher than those of homozygous CC (1.91 ± 1.59 ng/mL) or heterozygous CT individuals (2.23 ± 1.74 ng/mL). When evaluated by multiple regression analysis, AMH had a significant positive effect on NOR, whereas age and MTHFR 677TT genotype had significant negative effects.

Conclusion(s): The MTHFR 677TT genotype is associated with higher serum AMH concentrations and has a negative effect on NOR. This apparent paradox might be resolved in light of recent findings describing a negative feedback function of AMH in the coordination of follicle development. (Fertil Steril 2011;95:2257–62. ©2011 by American Society for Reproductive Medicine.)

Key Words: Anti-Müllerian hormone, MTHFR 677C>T mutation, ovarian hyperstimulation

The flavoenzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) has a central role in folate metabolism by catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The latter serves as a methyl group donor in the methylation of homocysteine to methionine (1). A common C→T exchange in the coding sequence of the MTHFR gene at nucleotide position 677 results in an alanine-to-valine substitution at amino acid position 222, thus increasing the thermolability and reducing the catalytic activity of the enzyme (2).

We have reported that mothers carrying the MTHFR 677C>T mutation exhibit a significantly lower risk of dichorionic twinning than those with the wild type CC genotype (3). Although this observation was shown to be in compliance with the inverse correlation of the ethnic distribution pattern of the T allele versus the incidence of dichorionic twins (3, 4), the underlying pathophysiologic mechanism remains unknown.

The vast majority of dichorionic twins result from the ovulation and fertilization of two separate oocytes (5, 6). This only occurs in a small percentage of human menstrual cycles. Growth, selection, and ovulation of oocytes are controlled by a complex regulatory network within the hypothalamic-pituitary-ovarian axis. Multiple follicle growth results when increased FSH concentrations are present at the time of follicle selection or when FSH concentrations exceed the threshold for too long (4, 7). Increased follicle recruitment has been observed in mothers of spontaneous twins (8), and some studies have shown elevated FSH in mothers of dizygous twins (8). Anti-Müllerian hormone (AMH) is expressed and secreted by preantral and small antral follicles (9), thus reflecting the number...
of follicles prior to cyclic recruitment. This appears to make AMH an excellent marker for the ovarian reserve (10–12), because AMH serum concentrations have been shown to be significantly correlated with the number of follicles (10) and oocytes (13) resulting from controlled ovarian hyperstimulation in IVF and intracytoplasmic sperm injection (ICSI) patients. Recently, AMH has been demonstrated to be not only a marker for ovarian reserve but also to influence follicular recruitment and growth. AMH was shown to have a role in several steps of feedback inhibition in regard to follicular development; it restrains initial recruitment of primordial follicles and also impairs folliculogenesis by inhibiting the responsiveness of growing follicles to FSH (14). Moreover, in human granulosa cells, AMH was shown to inhibit FSH-induced CYP19 mRNA and protein synthesis, resulting in a reduced E2 production (15).

In light of the emerging role of AMH as a regulator of follicular growth and selection, we asked whether the reduced rates of dichorionic twinning, which we had observed in mothers carrying the common MTHFR 677C>T mutation (3), might be due to an influence of this mutation on AMH serum concentrations. We report our findings in a well-defined cohort of women with normal ovarian function undergoing IVF-ICSI for male factor, tubal infertility, or both. AMH serum concentrations and results of controlled ovarian hyperstimulation were prospectively evaluated in these patients in regard to the individual MTHFR 677C>T genotype.

MATeRIALS AND METHODS

Patients

Two hundred and seventy women, who were treated with IVF or ICSI in our tertiary care infertility centre, were enrolled in this prospective study between July 2007 and March 2008. Maximum female age was 46 years. Only couples with tubal factor or male factor infertility, or both, were included. Tubal factor was defined as a bilateral blockage of tubes diagnosed by chromolaparoscopy.

Male factor infertility was classified according to the World Health Organization (1999)—that is, sperm concentration <20 million per milliliter, progressive sperm motility <50%, or normal sperm morphology <15% (16). Infertility workup included a standard gynecologic examination with a transvaginal sonographic examination and a pelvic examination on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). The fluorogenic, allele-specific oligonucleotide probes and PCR primers were provided by Applied Biosystems (TaqMan genotyping assay ID: C_1202883_20). PCR reactions were performed in 96-well plates in a total volume of 10 μL containing 5 μL TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μL of primer/TaqMan probe mix, and 10 ng genomic DNA. The PCR profile included an initial denaturation step at 95°C for 10 minutes, 40 cycles at 92°C for 15 seconds, and 60°C for 1 minute. For allelic discrimination, a postamplification plate reading was done with the ABI Sequence Detection Software supplied with the instrument.

Data and Statistical Analysis

Results are expressed as mean ± SD. Demographic data were first analyzed by using the Kruskall–Wallis test for the MTHFR 677C>T genotypes, followed by post hoc comparisons with the Mann–Whitney test. Student t test, Mann–Whitney U test, or chi-square test were performed when appropriate. For the determination of the Hardy–Weinberg equilibrium, the chi-square test was used. The relationship between AMH serum concentrations and the number of retrieved oocytes was assessed by univariate linear regression analysis. Assuming an additive genetic model, trend analysis was performed for the presence of zero, one, or two copies of the associated allele, incorporating the genotype variable as a continuous term in a linear regression model (19). Statistical significance was considered to be reached at P < 0.05. Statistical analysis of the data was performed with the SPSS version 16.0.1. (SPSS Inc., Chicago, IL).

RESULTS

Two hundred and seventy patients were prospectively enrolled in this study. IVF with or without ICSI was performed for tubal factor infertility (n = 93), male factor infertility (n = 201), or both.
Overall, homozygosity for the C allele was observed in 40.4% (n = 109), compound heterozygosity in 45.6% (n = 123), and homozygosity for the T allele in 14.1% (n = 38) of the subjects. The MTHFR 677C>T genotype distribution was in Hardy–Weinberg equilibrium ($\chi^2 = 0.121; P = 0.728$), and the allele frequencies of the study population were similar to those reported for Caucasians in the HapMap database (www.hapmap.org). Age, body mass index, smoking status, number of menstrual cycles per year, total amount of r-FSH required for stimulation, days of stimulation until hCG administration, and number of retrieved oocytes did not differ significantly among the three genotype groups (Table 1). The numbers (±SD) of oocytes recovered in women who were undergoing their first cycles (n = 88; 11.85 ± 6.56) versus subsequent cycles (n = 118; 11.14 ± 6.69) were found not to be different ($P = 0.385$).

When all individual factors potentially influencing the number of oocytes retrieved were evaluated by multiple regression analysis, AMH had a significant favorable effect, and age and the MTHFR 677TT genotype had a significant negative effect (Table 2). Body mass index, total amount of r-FSH used for stimulation, and days of stimulation until hCG administration, in contrast, did not significantly influence oocyte numbers (Table 2).

When total oocyte numbers of all patients were depicted as scatter plots and analyzed by linear regression, the numbers were found to be significantly correlated with individual AMH serum concentrations. For example, the numbers of oocytes retrieved were significantly lower than was to be expected, based on the AMH concentrations. AMH concentrations reportedly have a remarkable stability within menstrual cycles (26, 27). Nevertheless, some reports have shown a moderate decrease of AMH with follicular maturation (28), and this effect is apparently even more pronounced in patients undergoing controlled ovarian stimulation. By using a multiple linear regression model, AMH and age were identified as significant determinants of oocyte numbers. Whereas the MTHFR 677TT genotype was associated with increased AMH concentrations, multiple regression analysis revealed that this genotype had a negative influence on the number of oocytes.

Our study was done with patients undergoing IVF-ICSI treatment because of male factor or tubal factor infertility, or both. We carefully excluded women with an endocrine pathology, because this might influence AMH serum concentrations. For example, women with polycystic ovary syndrome have been shown to have significantly increased AMH concentrations (23–25).

When AMH concentrations of all patients were compared in relation to the MTHFR 677C>T genotype, values were significantly higher in 677TT carriers (2.85 ± 2.23 ng/mL) compared with either homozygous 677CC (1.91 ± 1.59 ng/mL; $P = 0.002$) or heterozygous 677CT individuals (2.23 ± 1.74 ng/mL; $P = 0.04$). AMH concentrations of 677CC and 677CT patients, in contrast, did not differ significantly (Fig. 2).

**DISCUSSION**

We report for the first time a significant effect of the MTHFR 677C>T mutation on AMH serum concentrations in a well-defined cohort of infertility patients with normal ovarian function. 677TT individuals were found to have significantly higher AMH concentrations. We confirmed previous reports of AMH being highly correlated with the numbers of oocytes retrieved after controlled ovarian stimulation. By using a multiple linear regression model, AMH and age were identified as significant determinants of oocyte numbers. Whereas the MTHFR 677TT genotype was associated with increased AMH concentrations, multiple regression analysis revealed that this genotype had a negative influence on the number of oocytes.

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**TABLE 1**

Demographic characteristics and ovarian response to recombinant FSH treatment as stratified by MTHFR 677 genotypes.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n = 270)</th>
<th>MTHFR 677CC (n = 109)</th>
<th>MTHFR 677CT (n = 123)</th>
<th>MTHFR 677TT (n = 38)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>35.9 ± 4.6</td>
<td>36.1 ± 4.4</td>
<td>36 ± 4.7</td>
<td>35.1 ± 4.8</td>
<td>0.589</td>
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<tr>
<td>Weight (kg)</td>
<td>63.8 ± 10.4</td>
<td>64.2 ± 11.8</td>
<td>63.5 ± 9.6</td>
<td>63.6 ± 8.4</td>
<td>0.839</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>167.7 ± 6.4</td>
<td>167.4 ± 6.4</td>
<td>168.1 ± 6.5</td>
<td>167.5 ± 6.3</td>
<td>0.362</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.6 ± 3.4</td>
<td>22.9 ± 3.9</td>
<td>22.4 ± 2.8</td>
<td>22.7 ± 3.4</td>
<td>0.964</td>
</tr>
<tr>
<td>AMH (ng/mL)</td>
<td>2.19 ± 1.78</td>
<td>1.91 ± 1.59</td>
<td>2.23 ± 1.74</td>
<td>2.85 ± 2.23</td>
<td>0.009</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>10.3 ± 2.1</td>
<td>10.2 ± 1.8</td>
<td>10.6 ± 2.5</td>
<td>9.9 ± 1.8</td>
<td>0.414</td>
</tr>
<tr>
<td>r-FSH (IU)</td>
<td>1839.7 ± 633.3</td>
<td>1827.5 ± 564.7</td>
<td>1866.6 ± 719.5</td>
<td>1787.8 ± 522.1</td>
<td>0.917</td>
</tr>
<tr>
<td>Starting dose r-FSH (IU)</td>
<td>190.5 ± 51.3</td>
<td>190.5 ± 51.8</td>
<td>190.7 ± 53.1</td>
<td>190.7 ± 45.1</td>
<td>0.989</td>
</tr>
<tr>
<td>MII (%)</td>
<td>0.75 ± 0.19</td>
<td>0.76 ± 0.16</td>
<td>0.74 ± 0.20</td>
<td>0.77 ± 0.22</td>
<td>0.985</td>
</tr>
<tr>
<td>(n = 153)</td>
<td>(n = 64)</td>
<td>(n = 64)</td>
<td>(n = 25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of oocytes</td>
<td>11.4 ± 6.6</td>
<td>11.4 ± 6.6</td>
<td>11.4 ± 6.4</td>
<td>11.3 ± 7.6</td>
<td>0.957</td>
</tr>
</tbody>
</table>

Note: Values are mean values ± SD unless otherwise indicated. Groups were compared by using the Kruskal–Wallis test for numerical data. BMI = body mass index; AMH = anti-Mullerian hormone; MII = metaphase II.

* All retrieved oocytes for subsequent ICSI procedure were assessed for their maturity. Numbers of metaphase II oocytes are given in relation to total number of received oocytes.

Our observation, that the MTHFR 677TT genotype influences AMH concentrations, is new, and the mechanism by which this particular mutation exerts this effect is currently unknown. Normal MTHFR activity is essential for the synthesis and balance of deoxynucleotide triphosphate (dNTP) precursor pools required for error-free DNA synthesis and repair (30). It is also essential for establishing and maintaining stable DNA methylation patterns, which regulate tissue-specific gene expression and chromatin conformation (31). The lower enzymatic activity of homozygous MTHFR 677T allele carriers may disturb biologic processes, including gene expression and protein function. Follicular maturation is characterized by a rapid proliferation and increased metabolic activity of granulosa cells (32). It therefore appears conceivable that such processes may be affected by a reduced MTHFR activity. AMH is secreted by granulosa cells of follicles that have undergone initial recruitment (14, 33). Only a small proportion of these follicles may enter cyclic recruitment. During controlled ovarian hyperstimulation, the follicles may develop into tertiary follicles and, after ovulation induction, release metaphase II oocytes. In our study, MTHFR 677TT patients showed higher AMH serum

### TABLE 2

Multiple linear regression analysis of possible determinants of the number of oocytes retrieved.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Nonstandardized coefficient</th>
<th>Standardized coefficient</th>
<th>T</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>–15.332</td>
<td></td>
<td>–0.429</td>
<td>0.668</td>
</tr>
<tr>
<td>Age (years)</td>
<td>–0.248</td>
<td>–0.172</td>
<td>–3.495</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.732</td>
<td>0.375</td>
<td>0.952</td>
<td>0.342</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>–0.313</td>
<td>–0.489</td>
<td>–1.121</td>
<td>0.263</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.247</td>
<td>0.238</td>
<td>1.133</td>
<td>0.258</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>–0.242</td>
<td>–0.078</td>
<td>–1.065</td>
<td>0.288</td>
</tr>
<tr>
<td>Total rec-FSH (IU)</td>
<td>0.000</td>
<td>–0.080</td>
<td>–1.008</td>
<td>0.314</td>
</tr>
<tr>
<td>MTHFR 677TT</td>
<td>–2.239</td>
<td>–0.117</td>
<td>–2.685</td>
<td>0.006</td>
</tr>
<tr>
<td>AMH</td>
<td>2.067</td>
<td>0.554</td>
<td>10.548</td>
<td>7.9E-22</td>
</tr>
</tbody>
</table>

* The dependent variable is the number of oocytes retrieved \( r = 0.718; r^2 = 0.496; P < 0.001 \).


**FIGURE 1**

Scatter plot depicting the correlation between AMH serum concentrations and the numbers of oocytes retrieved (NOR) after controlled ovarian hyperstimulation in 109 MTHFR 677CC (black circle, black regression line), 123 MTHFR 677CT (open squares, black dotted regression line), and 38 MTHFR 677TT (gray triangles, black dashed regression line) patients \( r = \) Spearman’s correlation coefficient. Whereas AMH is significantly correlated with NOR in all MTHFR genotypes, homozygous MTHFR 677TT individuals show significantly fewer NOR for given AMH concentrations than do individuals with the other MTHFR 677 genotypes.

**FIGURE 2**

AMH serum concentrations with regard to the MTHFR 677C>T genotypes. Box plots show median, interquartile range, outliers, and extreme cases of individual variables; AMH concentrations of MTHFR 677 TT individuals are significantly higher than in MTHFR 677CC \( (*P < 0.002) \) and in MTHFR 677CT individuals \( (*P < 0.04) \).
concentrations; however, this did not translate into higher numbers of oocytes retrieved. This discrepancy might be explained by higher amounts of AMH being released by initially recruited follicles of MTHFR 677TT individuals. Alternatively, follicle maturation could be retarded in these patients, leading to an increased rate of initial recruitment might be the result of a compensatory mechanism, ensuring adequate output of tertiary follicles despite of reduced FSH-action with the MTHFR 677 TT genotype. This possibility is supported by our finding that the rate of metaphase II oocytes did not differ among patients with the three genotypes (Table 1).

The concept of MTHFR activity affecting folliculogenesis is supported by previous reports by us (34, 35) and other groups (36), showing reduced ovarian responsiveness and lower numbers of oocytes retrieved in IVF-ICSI patients with reduced MTHFR activity caused by genetic variations of the respective gene. In our logistic regression model, the MTHFR 677TT genotype also was identified as a compensatory mechanism, ensuring adequate output of tertiary follicles that produce AMH, but do not advance toward cyclic recruitment. Indeed, an increased rate of initial recruitment might be the result of a compensatory mechanism, ensuring adequate output of tertiary follicles despite of reduced FSH-action with the MTHFR 677 TT genotype. This possibility is supported by our finding that the rate of metaphase II oocytes did not differ among patients with the three genotypes (Table 1).

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9. Weezen C, Laven JSE, Von Bergh ARM, Themmen AP. The role of AMH in the intrafollicular and interfollicular coordination of follicle development. In animal studies, female homozygous AMH knockout mice had more growing preantral and small antral follicles than did wild type mice. AMH inhibits initial recruitment of primordial follicles into the pool of growing follicles and also decreases the responsiveness of growing follicles to FSH (14). The increased AMH concentrations, which we observed in association with the MTHFR 677TT genotype, may result in a partial inhibition of FSH activity, and this could in part explain the negative effect of this genotype on oocyte numbers. An inhibition of FSH activity by increased AMH concentrations should reduce follicular recruitment and selection of multiple follicles. This effect would counteract the maturation and ovulation of multiple follicles and thus contribute to the reduced chances of dichorionic twinning that we have observed in mothers carrying the MTHFR 677TT allele (3).

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