

ORIGINAL ARTICLE

## ***MTHFR* C677T AND A1298C GENOTYPES AND HAPLOTYPES IN SLOVENIAN COUPLES WITH UNEXPLAINED INFERTILITY PROBLEMS AND IN EMBRYONIC TISSUES FROM SPONTANEOUS ABORTIONS**

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### **ABSTRACT**

The objective of this study was to analyze the methylenetetrahydrofolate reductases (*MTHFR*) C677T and A1298C genotype distributions in couples with unexplained fertility problems (UFP) and healthy controls, and to analyze the genotype and haplotype distribution in spontaneously aborted embryonic tissues (SAET) using allele specific polymerase chain reaction (PCR) in 200 probands with UFP, 353 samples of SAET and 222 healthy controls. The analysis revealed a significant overall representation of the 677T allele in male probands from couples with UFP ( $p = 0.036$ ). The combined genotype distribution for both *MTHFR* polymorphisms was also significantly altered ( $\chi^2$  21.73,  $p < 0.001$ ) although female probands made no contribution ( $\chi^2$  1.33,  $p = 0.72$ ). The overall representation of the 677T allele was more pronounced in SAET (0.5 vs. 0.351 in controls,  $p < 0.001$ ) regardless of the karyotype status (aneuploidy vs. normal karyotype). In addition, the

frequencies of the CA and CC haplotypes were significantly lower than in the control group ( $p = 0.021$  and  $p = 0.001$ , respectively), whereas the frequency of the TC haplotype was significantly higher than in controls ( $p < 0.0001$ ). The presented findings indicate that only male probands contribute to the association of *MTHFR* mutations with fertility problems in grown adults and demonstrate a high prevalence of mutated *MTHFR* genotypes in SAET.

**Keywords:** Methylenetetrahydrofolate reductase (*MTHFR*); Genotype; Haplotype; Infertility; Miscarriage.

### **INTRODUCTION**

Infertility is a worldwide reproductive health problem that affects approximately 15.0% of married couples. Half of these cases are due to factors that affect males [1], and about 60.0-75.0% of male infertility cases are idiopathic, since the molecular mechanisms underlying the defects remain unknown [2]. The female factors contribute the other half and they are also not well documented [3]. On the other hand, approximately 20.0% of recognized pregnancies are terminated as spontaneous abortions; their genetic etiology, apart from aneuploidy, is largely unknown [4].

Methylenetetrahydrofolate reductase (*MTHFR*) plays an important role in the process of DNA, RNA and protein metabolism, and is closely related with spermatogenesis [5-8]. The genetic polymorphisms

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of the *MTHFR* gene have been extensively studied, in particular C677T and A1298C, which have been identified as risk factors for several diseases such as arterial and/or venous thrombosis, adverse pregnancy outcome and congenital malformations [9-14]. These polymorphisms are significantly overrepresented in fetal samples from spontaneously aborted pregnancies [4,15-17]. However, when adult individuals affected with recurrent early pregnancy loss are tested for association with both *MTHFR* polymorphisms the study results are conflicting and the association is not always detected [18]. Especially ambiguous results from studies of female patients affected with recurrent early pregnancy loss indicate that hereditary thrombophilia may represent a less important factor capable of compromising fetal viability [19-22]. Consequently, routine testing for polymorphisms associated with hereditary thrombophilia is not part of the diagnostic protocol for individuals/couples seeking help for fertility problems. Unless targeted genetic testing is warranted due to a present typical clinical picture (*i.e.*, chromosome Y microdeletion analysis for males with azoospermia), only karyotyping can usually be offered to couples with no evident reason for infertility. Therefore, it may be of interest to investigate whether an association of *MTHFR* polymorphisms with infertility can be observed when couples seeking medical attention for infertility are compared to a control group of individuals. The aim of our study was to determine *MTHFR* C677T and A1298C genotype and haplotype distributions in couples with unexplained fertility problems (UFP) and healthy controls, and to analyze the genotype and haplotype distribution in spontaneously aborted embryonic tissues (SAET).

## MATERIALS AND METHODS

**Probands.** The total study sample included 775 probands representing three different groups: couples with UFP, SAET and healthy controls. Couples with UFP ( $n = 200$ , 100 females and 100 males), were referred to our laboratory from the Department of Reproductive Medicine and Gynecologic Endocrinology, University Clinical Centre Maribor, Maribor, Slovenia for routine karyotyping. At the time of the study enrolment, all probands were childless as a couple. Both female and male probands did not exhibit any other condition known to affect fertility

(*e.g.*, azoospermia in males or premature ovarian failure in females). Spontaneously aborted embryonic tissues (SAET) ( $n = 353$ ) were also sent for routine karyotyping from the Department of Obstetrics and Gynecology, University Clinical Centre Maribor, Maribor, Slovenia. The pregnancies ended between the 6th and 20th week of gestation, with the majority occurring earlier than week 15. Fetal tissues included chorionic villi, placenta, fetal skin and they all originated from at least a second pregnancy loss in each case. Because few fetal samples were from couples participating in the study, both groups were considered unrelated. The healthy controls ( $n = 222$ , 111 females, 111 males) were blood donors with a good reproductive history, who were recruited through the Department of Transfusiology, University Medical Centre Maribor, Maribor, Slovenia.

All samples were from individuals of Caucasian origin who were residents of different geographical areas of Slovenia. Informed consent was obtained from all participating individuals and ethical approval was granted prior to conducting the study.

**Cytogenetic Analysis.** A routine cytogenetic analysis was performed on metaphase chromosomes from embryonic tissues. Chromosomes were harvested according to standard cytogenetic methods and analyzed by G-bands [G-bands by trypsin using Giemsa (GTG)]. Karyotypes were defined according to ISCN (2009).

**DNA Extraction and Analysis.** Peripheral venous blood was collected in standard collection tubes containing EDTA as the anticoagulant. Genomic DNA was extracted from blood leukocytes with a simple salting-out method [23]. Genomic DNA from embryonic tissues was obtained using a QIAGEN Blood Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. All DNA samples were screened for the C677T/A1298C gene polymorphisms of *MTHFR* using an allelespecific polymerase chain reaction (PCR). Two reactions were performed per sample with the QIAGEN Multiplex PCR Kit (Cat. #206143; Qiagen) by amplifying one allele of each polymorphism (two duplex reactions). The mix included 1  $\times$  master mix, 1  $\mu$ M of each primer and 50 ng of genomic DNA. In reaction 1, alleles 677T and 1298A were amplified with *MTHFR*677T-F (5'-GAAGGTGTC TGC GGG CGT-3'), *MTHFR*C677T-R (5'-AGC AAC GCT GTG CAA GTT CTG-3'), *MTHFR*1298A-F (5'-AGG AGC TGA CCA GTG

AGGA-3') and *MTHFR*1298C-R (5'-TTC TCC CTT TGC CAT GTC C-3'). In reaction 2, alleles 677C and 1298C were amplified with *MTHFR* C677C-F (5'-GAA GGT GTC TGC GGG CGC-3'), *MTHFR*677T-R, *MTHFR*1298C-F (5'-AGG AGC TGA CCA GTG AGG C-3') and *MTHFR*1298C-R. The annealing temperature was 60°C in 30 amplification cycles. Successful PCR amplification was confirmed by electrophoresis on 3.0% agarose gel, stained with SYBR Green I, and photographed for documentation and allele scoring.

**Multiplex Ligation-Dependent Probe Amplification.** Cytogenetic analysis of all SAET was performed. For those samples where karyotyping was not possible the multiplex ligation-dependent probe amplification (MLPA) was performed using commercial MLPA kits containing subtelomeric probes (P036, P070; MRC Holland, Amsterdam, The Netherlands), according to the manufacturer's protocol. The MLPA data analysis was performed as previously reported [24].

**Statistical Analyses.** For various genotypes and haplotypes, an odds ratio (OR) and 95% confidence interval (95% CI) were calculated. Differences between the tested groups and control groups were assessed by the  $\chi^2$  test and the Fisher's exact test. Genotypes for the tested groups and the controls were assessed for departures from the Hardy-Weinberg equilibrium (HWE).

## RESULTS

### Couples with Unexplained Fertility Problems.

We analyzed 100 couples, a total of 200 individuals (average age 32.9 years, median age 33 years), 100 females (average and median age 32 years, range 21-44 years) and 100 males (average age 33.75 years, median age 34 years, range 23-44 years). The control group included 222 healthy blood donors (average and median age 43 years), 111 females (average and median age 42 years, range 27-66 years) and 111 males (average and median age 44 years, range 21-71 years). Genotypes and allele frequencies were compared separately for both *MTHFR* mutations between all groups of probands and controls. A statistically significant shift in allele frequency of the C677T mutation, an increase of the minor T allele frequency was observed. There were no significant differences in genotype frequencies for both muta-

tions, whereas HWE was observed in both groups and for both mutations.

When genotype frequencies were stratified according to gender we did not observe any differences in distribution of the data when female probands were compared to female controls and the HWE was maintained in all groups. However, in the group of male probands there was an increase of C677T mutation TT homozygotes and a corresponding decrease of CC homozygotes (Table 1). Consequently the T allele frequency was also significantly increased in male probands ( $p < 0.05$ , Table 1). The analysis of the A1298C mutation did not show any significant differences between male probands and male controls despite the fact that HWE was not maintained in the group of male probands (Table 1).

When combined genotype frequencies were compared between probands and controls, only males contributed to the significant difference in genotype distribution ( $p < 0.001$ ; see Table 2). Additionally, the haplotype distributions between the probands and controls were compared. The analysis was performed under the assumption that compound heterozygotes had minor alleles exclusively in the *trans* position. We did not observe any significant differences in haplotype frequencies between both groups of individuals.

**Spontaneously Aborted Embryonic Tissue Samples.** We also analyzed 353 samples of embryonic tissues from spontaneous abortions. All were karyotyped, and in samples without a viable cell culture, the genomic DNA was analyzed with the MLPA method to detect any aneuploidies. Major chromosomal aberrations, mostly trisomies and polyploidies, were detected in 131 samples (37.1%) whereas a normal karyotype was present in the remaining 222 samples (62.9%).

The genotype distribution of the *MTHFR* C677T mutation differed significantly in the SAET group compared to the control group (see Table 3). Hardy-Weinberg equilibrium was not present in the proband group. This deviation was caused by a substantial increase of the number of TT genotypes, which also significantly increased the 677T allele frequency (Table 3). Consequently, the CC genotype was under represented. Interestingly, HWE was also not present in the SAET samples for the A1298C mutation; however, genotype distribution did not differ significantly and neither did the allele frequencies (Table 3). When the SAET samples were divided according to the

**Table 1.** The distribution of *MTHFR* C677T and A1298C genotypes in male probands from couples with unexplained fertility problems and male controls.

| Genotype                   | Infertile Males <sup>a</sup><br>( <i>n</i> = 100) (%) | Male Controls <sup>a</sup><br>( <i>n</i> = 111) (%) | No Text             |                             |
|----------------------------|-------------------------------------------------------|-----------------------------------------------------|---------------------|-----------------------------|
|                            |                                                       |                                                     | OR (95% CI)         | <i>p</i> Value <sup>b</sup> |
| <i>MTHFR</i> C677T         |                                                       |                                                     |                     |                             |
| CC                         | 29 (29.0)                                             | 47 (32.3)                                           | 0.556 (0.314-0.987) | 0.046                       |
| CT                         | 51 (51.0)                                             | 50 (45.1)                                           | 1.270 (0.739-2.183) | 0.410                       |
| TT                         | 20 (20.0)                                             | 14 (12.6)                                           | 1.732 (0.831-3.608) | 0.189                       |
| CT + TT                    | 71 (71.0)                                             | 64 (57.8)                                           | 1.798 (1.014-3.189) | 0.046                       |
| C allele                   | 109 (0.545)                                           | 144 (0.649)                                         |                     | 0.036                       |
| T allele                   | 91 (0.455)                                            | 78 (0.351)                                          |                     |                             |
| $\chi^2$ : infertile males | 0.08                                                  | 0.02                                                |                     | 0.99 (df = 2)               |
| $\chi^2$ : male controls   |                                                       |                                                     |                     | 0.99 (df = 2)               |
| <i>MTHFR</i> A1298C        |                                                       |                                                     |                     |                             |
| AA                         | 44 (44.0)                                             | 48 (43.2)                                           | 1.031 (0.598-1.778) | 1.000                       |
| AC                         | 35 (35.0)                                             | 50 (45.0)                                           | 0.657 (0.377-1.145) | 0.160                       |
| CC                         | 21 (21.0)                                             | 13 (11.7)                                           | 2.004 (0.954-4.205) | 0.091                       |
| AC + CC                    | 56 (56.0)                                             | 63 (56.8)                                           | 0.970 (0.563-1.669) | 1.000                       |
| A allele                   | 123 (0.615)                                           | 146 (0.658)                                         |                     | 0.417                       |
| C allele                   | 77 (0.385)                                            | 76 (0.342)                                          |                     |                             |
| $\chi^2$ : infertile males | 6.81                                                  |                                                     |                     | 0.03 (df = 2)               |
| $\chi^2$ : male controls   |                                                       | 0.00                                                |                     | 1.00 (df = 2)               |

<sup>a</sup> Hardy-Weinberg equilibrium.

<sup>b</sup> Fisher's exact *T*-test.

presence of the aneuploidy, both subgroups showed comparable and significant increases in 677T allele frequency and genotype TT frequency. The allele and genotype frequencies of the A1298C mutation did not differ significantly between both subgroups of SAET samples. Hardy-Weinberg equilibrium was not present in the subgroup of SAET samples with normal karyotype for both *MTHFR* mutations.

The distribution of combined genotypes in SAET samples differed significantly from controls and the majority of the change was attributed to the increased presence of TTAC and TTCC genotypes (Table 4). Both subgroups of SAET samples showed little difference in their genotype distribution ( $p = 0.21$ ; Table 4). Finally, the haplotype frequencies were analyzed in compound heterozygotes. Three out of four possible haplotypes differed significantly between SAET samples and controls; haplotypes with 677C were under represented, whereas TC, the two minor allele

haplotypes were significantly over represented in SAET samples (see Table 5).

## DISCUSSION

The *MTHFR* C677T and A1289C polymorphisms are important thrombophilic factors that might be associated with infertility and recurrent spontaneous abortion (RSA) [25]. In the present study, we evaluated the *MTHFR* C677T and A1298C genotype and haplotype distributions in couples with UFP and healthy controls, and determined the *MTHFR* C677T and A1298C genotype and haplotype associations with compromised fetal viability with regard to the presence of aneuploidies.

The present study has certain limitations. In the case of the group with UFP, HWE was not present in all the tested groups (see results). However, in the control group, HWE was present, which means that

**Table 2.** The frequencies of the combined genotypes of *MTHFR* C677T and A1298C in couples with unexplained fertility problems and controls.

| Genotype <i>MTHFR</i> C677T/A1298C     | Infertile Couples (n=200) (%) | Controls (n=222) (%) | Infertile Females (n=100) (%) | Controls (n=111) (%) | Infertile Males (n=100) (%) | Controls (n=111) (%) |
|----------------------------------------|-------------------------------|----------------------|-------------------------------|----------------------|-----------------------------|----------------------|
| CC/AA                                  | 17 ( 8.5)                     | 21 ( 9.5)            | 8 ( 8.0)                      | 10 ( 9.0)            | 9 ( 9.0)                    | 11 ( 9.9)            |
| CC/AC                                  | 15 (12.5)                     | 49 (22.1)            | 20 (20)                       | 22 (19.8)            | 5 ( 5.0)                    | 27 (24.3)            |
| CC/CC                                  | 26 (13.0)                     | 24 (10.8)            | 11 (11.0)                     | 15 (13.5)            | 15 (15.0)                   | 9 ( 8.1)             |
| CT/AA                                  | 39 (19.5)                     | 45 (20.3)            | 17 (17.0)                     | 22 (19.8)            | 22 (22.0)                   | 23 (20.7)            |
| CT/AC                                  | 50 (25.0)                     | 47 (21.2)            | 25 (25.0)                     | 24 (21.6)            | 24 (24.0)                   | 23 (20.7)            |
| CT/CC                                  | 7 ( 3.5)                      | 8 ( 3.6)             | 3 ( 3.0)                      | 4 ( 3.6)             | 4 ( 4.0)                    | 4 ( 3.6)             |
| TT/AA                                  | 23 (12.0)                     | 25 (11.3)            | 11 (11.0)                     | 11 ( 9.9)            | 13 (13.0)                   | 14 (12.6)            |
| TT/AC                                  | 8 ( 4.0)                      | 1 ( 0.5)             | 3 ( 3.0)                      | 1 ( 0.9)             | 5 ( 5.0)                    | 0 ( 0.0)             |
| TT/CC                                  | 4 ( 2.0)                      | 2 ( 0.9)             | 2 ( 2.0)                      | 2 ( 1.8)             | 2 ( 2.0)                    | 0 ( 0.0)             |
| $\chi^2$ : all samples vs. controls    | 21.73, $p < 0.001$ (df=8)     |                      |                               |                      |                             |                      |
| $\chi^2$ : female samples vs. controls |                               |                      | 1.33, $p=0.720$ (df=8)        |                      |                             |                      |
| $\chi^2$ : male samples vs. controls   |                               |                      |                               |                      | 90.60, $p < 0.001$ (df=8)   |                      |

**Table 3.** The distribution of *MTHFR* C677T and A1299C genotypes in SAETs and controls.

| Genotype                                    | Abortions <sup>a</sup> (n = 353) (%) | Controls <sup>a</sup> (n = 222) (%) | No Text             |                                   |
|---------------------------------------------|--------------------------------------|-------------------------------------|---------------------|-----------------------------------|
| <i>MTHFR</i> C677T                          |                                      |                                     | OR (95% CI)         | $p$ Value <sup>b</sup>            |
| CC                                          | 108 (30.6)                           | 94 (42.3)                           | 0.600 (0.423-0.851) | 0.005                             |
| CT                                          | 138 (39.1)                           | 100 (45.1)                          | 0.783 (0.558-1.100) | 0.165                             |
| TT                                          | 107 (30.3)                           | 28 (45.1)                           | 3.014 (1.914-4.744) | <0.001                            |
| C allele<br>T allele                        | 354 (0.501)<br>352 (0.499)           | 288 (0.649)<br>156 (0.351)          |                     | <0.001                            |
| $\chi^2$ : abortions<br>$\chi^2$ : controls | 19.4                                 | 0.15                                |                     | <0.001 (df = 2)<br>0.980 (df = 2) |
| <i>MTHFR</i> A1298C                         |                                      |                                     |                     |                                   |
| AA                                          | 143 (40.5)                           | 91 (41.0)                           | 0.980 (0.697-1.380) | 0.931                             |
| AC                                          | 133 (37.7)                           | 97 (43.7)                           | 0.779 (0.554-1.096) | 0.162                             |
| CC                                          | 77 (21.8)                            | 34 (15.3)                           | 1.543 (0.992-2.399) | 0.065                             |
| A allele<br>C allele                        | 419 (0.593)<br>287 (0.407)           | 279 (0.628)<br>165 (0.372)          |                     | 0.264                             |
| $\chi^2$ : abortions<br>$\chi^2$ : controls | 19.57                                | 1.73                                |                     | <0.001 (df = 2)<br>0.630 (df = 2) |

<sup>a</sup> Hardy-Weinberg equilibrium.<sup>b</sup> Fisher's exact T-test.

**Table 4.** The frequency of the combined genotypes of *MTHFR* C677T and A1298C of spontaneously aborted embryonic tissues and control groups.

| Genotype <i>MTHFR</i> C677T/A1298C | Abortions (n=353) (%) | Normal Karyotype (n=222) (%) | Aneuploidy (n=131) (%) | Controls (n=222) (%) |
|------------------------------------|-----------------------|------------------------------|------------------------|----------------------|
| CC/AA                              | 50 (14.2)             | 31 (14.0)                    | 19 (14.5)              | 21 (9.5)             |
| CC/AC                              | 30 (8.5)              | 20 (9.0)                     | 10 (7.7)               | 49 (22.1)            |
| CC/CC                              | 28 (8.0)              | 18 (8.1)                     | 10 (7.7)               | 24 (10.8)            |
| CT/AA                              | 51 (14.5)             | 37 (16.7)                    | 14 (10.8)              | 45 (20.3)            |
| CT/AC                              | 62 (17.6)             | 33 (14.9)                    | 29 (22.3)              | 47 (21.2)            |
| CT/CC                              | 25 (7.1)              | 14 (6.3)                     | 11 (8.5)               | 8 (3.6)              |
| TT/AA                              | 42 (11.9)             | 29 (13.1)                    | 13 (10.0)              | 25 (11.3)            |
| TT/AC                              | 41 (11.6)             | 26 (11.7)                    | 15 (11.5)              | 1 (0.5)              |
| TT/CC                              | 24 (6.8)              | 14 (6.3)                     | 10 (7.7)               | 2 (0.9)              |

$\chi^2$ : all samples vs. controls (94.87,  $p < 0.001$ , df=8).

$\chi^2$ : normal karyotype vs. aneuploidy (10.83,  $p = 0.210$ , df=8).

**Table 5.** Comparison of haplotype frequencies in the *MTHFR* C677T and A1298C polymorphisms between the spontaneously aborted embryonic tissues and controls.

| Haplotype | Controls (n=444) (%) | Abortions (n=706) (%) | Odds Ratio (95% CI) | p Value <sup>a</sup> |
|-----------|----------------------|-----------------------|---------------------|----------------------|
| C/A       | 183 (41.2)           | 243 (34.4)            | 0.749 (0.586-0.956) | 0.021                |
| C/C       | 105 (23.6)           | 111 (15.7)            | 0.602 (0.447-0.811) | 0.001                |
| T/A       | 96 (21.6)            | 176 (24.9)            | 1.204 (0.907-1.597) | 0.226                |
| T/C       | 60 (13.5)            | 176 (24.9)            | 2.125 (1.543-2.927) | <0.000               |

<sup>a</sup> Fisher's exact T-test.

the difference in the tested groups is probably due to biological differences rather than recruitment bias. Such results have previously been reported in the studies with certain patient subgroups that were also without HWE for *MTHFR* polymorphisms [26,27]. The same observation was made in the group of SAET with absent HWE for both *MTHFR* polymorphisms before and after stratification for aneuploidy. In this case, similar results have not previously been reported. Larger samples may be needed in order to resolve these limitations. Despite limitations, our study was focused on the relationship between the *MTHFR* C677T/A1298C genotypes/haplotypes and fertility problems of grown adults and SAETs in a Slovenian population.

We found significant difference in the prevalence of *MTHFR* C677T/A1298C polymorphisms in probands with UFP compared to controls without an infertility history. Only male probands contributed to the

association, indicating that *MTHFR* mutation may be a gender-specific factor that affects fertility of grown adults. This single male contribution was expected, while it has been reported for male adults affected with infertility [6-8]. On the other hand, it may be consistent with the role of the *MTHFR* gene in spermatogenesis also mentioned in previously reported studies [5,7]. Altered DNA methylation patterns of the *MTHFR* promoter in sperm cells from infertile males indicate a possible mechanism that may help explain the observed association [28,29]. Also, we excluded from the study couples who were affected with known male infertility factors which possibly increased the likelihood of identifying the association with the *MTHFR* gene. Whether the *MTHFR* polymorphism C677T was directly involved in epigenetic phenomena or just identified an important genetic locus through linkage disequilibrium remains to be determined. Therefore,

the observed asymmetry between both genders may be a consequence of the differential role that the *MTHFR* gene may have in the gametogenesis. Consequently, no association was observed for female UFP probands despite the fact that the sample included individuals who were affected by RSA.

Interestingly, the observed association between *MTHFR* polymorphisms and couples with UFP was entirely the consequence of the increase in the frequency of the 677T allele, whereas the A1298C polymorphism contribution was not present. This difference in contribution of each *MTHFR* polymorphism influenced the combined genotype distributions (Table 2), as well as haplotype distributions, and it clearly shows the increased importance of the C677T polymorphism over the A298C polymorphism.

The present study also demonstrated that SAET exhibit both a significantly higher frequency of the *MTHFR* 677TT genotype and a lower frequency of the 677CC genotype, and a moderately higher frequency of the 1298CC genotype, compared to the controls. In addition, the *MTHFR* 677CT, 1298AA and 1298AC genotypes were similar between the SAETs and the controls. These results are of interest because they are different from the results from other studies. Moreover, Jeehyeon *et al.* [4] detected a higher proportion of aborted embryos with the 677CC genotype and a lower frequency of the 677CT genotype compared to their control group. On the other hand, Zetterberg *et al.* [15] detected a higher proportion of aborted embryos with the 677CT and 1298AC genotypes compared to their control group. This inconsistency among studies may partly be due to a different *MTHFR* genotype distribution between groups with different ethnic origins, or it may be affected by different criteria for SAET sample collection and from the selection criteria for the control sample.

Furthermore, we observed all nine possible combined genotypes of *MTHFR* C677T/A1298C from both the SAET and controls. This observation is contrary to the conclusions of Jeehyeon *et al.* [4] who stated that some combinations of C677T and A1298C alleles may have a severe effect on the fetus. Thus, the embryo is unable to survive, suggesting the pivotal role of the *MTHFR* gene in human development. However, we agree with Jeehyeon *et al.* [4] that more thorough analysis of the *MTHFR* polymorphism, based on embryos at a very early stage, should be conducted before a final conclusion is drawn.

The combined karyotype/MLPA analysis showed that approximately 37.0% of the SAET samples had a chromosomal abnormality, but the frequency of the *MTHFR* C677T and A1298C genotypes were not related to chromosomal status in the SAET samples. The observed association is again caused by the over representation of the 677T allele, which in turn, significantly changed the combined genotype distribution as well as the haplotype distribution. The minor allele combination (CT) was the main contributor to the association. The data also identified the C677T polymorphism as significantly more important in comparison to the A1298C with regard to the *MTHFR* influenced fetal viability.

In conclusion, our data highlight an interesting possibility that *MTHFR* polymorphisms may be implicated as male fertility factors in Slovenian couples with UFP. No such association was observed in female probands. With the analysis of SAET samples we confirmed the association of the *MTHFR* polymorphisms, especially the C677T, with the recurrent early spontaneous abortions.

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## REFERENCES

1. de Krester DM. Male infertility. *Lancet* 1997; 349 (9054): 787-790.
2. Filippini D, Feil R. Perturbation of genomic imprinting in oligozoospermia. *Epigenetics*. 2009; 4(1): 27-30.
3. Boivin J, Bunting L, Collins JA, Nygren K. An international estimate of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod*. 2007; 22(6): 1506-1512.
4. Jeehyeon B, Seung Joo S, Sun Hee C, Dong Hee C, Suman L, Nam Keun K. Prevalent genotypes of methylenetetrahydrofolate reductase (*MTHFR* C677T and A1298C) in spontaneously aborted embryos. *Fertil Steril*. 2007; 87(2): 351-355.

5. Singh K, Singh SK, Raman R. *MTHFR* A1298C polymorphism and idiopathic male infertility. *J Postgrad Med*. 2010; 56(4): 267-269.
6. Eloualid A, Abidi O, Charif M, El Houate B, Benrahma H, Louanjli N, *et al*. Association of the *MTHFR* A1298C variant with unexplained severe male infertility. *PLoS One* 2012; 7: e34111.
7. Safarinejad MR, Shafiei N, Safarinejad S. Relationship between genetic polymorphisms of methylenetetrahydrofolate reductase (C677T, A1298C, and G1793A) as risk factors for idiopathic male infertility. *Reprod Sci*. 2011; 18(3): 304-315.
8. Ouxi S, Renping L, Wei W, Lugang Y, Xinru W. Association of the methylenetetrahydrofolate reductase gene A1298C polymorphism with male infertility: a meta-analysis. *Ann Hum Genet*. 2012; 76(1): 25-32.
9. Martinelli I. Risk factors in venous thromboembolism. *Thromb Haemost*. 2001; 86(1): 395-403.
10. Fatini C, Gensini F, Battaglini B, Prisco D, Cellai AP, Fedi S. Angiotensin convertinase enzyme DD genotype, angiotensin type I receptor CC genotype, and hyperhomocysteinemia increase first-trimester fetal-loss susceptibility. *Blood Coagul Fibrinol*. 2000; 11(7): 657-662.
11. Wouters MG, Boers GH, Blom HJ, Trijbels FJ, Thomas CM, Borm GF. Hyperhomocysteinemia: a risk factor in woman with unexplained recurrent early pregnancy loss. *Fertil Steril*. 1993; 60(5): 820-825.
12. Steegers-Theunissen RP, Boers GH, Trijbels FJ, Finkelstein JD, Blom HJ, Thomas CM, *et al*. Maternal hyperhomocysteinemia: a risk factor for neural-tube defects? *Metabolism*. 1994; 43(12): 1475-1480.
13. Coulam CB, Jeyendran RS. Thrombophilic gene polymorphisms are risk factors for unexplained infertility. *Fertil Steril*. 2009; 91(4): 1516-1517.
14. Stegmann K, Ziegler A, Ngo ET, Kohlschmidt N, Schröter B, Ermert A, *et al*. Linkage disequilibrium of *MTHFR* genotypes 677C/T-1298A/C in the German population and association studies in probands with neural tube defects (NTD). *Am J Med Genet*. 1999; 87(1): 23-29.
15. Zetterberg H, Regland B, Palmer M, Ricksten A, Palmqvist L, Rymo L, *et al*. Increased frequency of combined methylenetetrahydrofolate reductase C677T and A1298C mutated alleles in spontaneously aborted embryos. *Eur J Hum Genet*. 2002; 10(2): 113-118.
16. Isotalo PA, Wells GA, Donnelly JG. Neonatal and fetal methylenetetrahydrofolate reductase genetic polymorphisms: an examination of C677T and A1298C mutations. *Am J Hum Genet*. 2000; 67(4): 986-990.
17. Volcik KA, Blanton SH, Northrup H. Examinations of methylenetetrahydrofolate reductase C677T and A1298C mutations – and in utero viability. *Am J Hum Genet*. 2001; 69(5): 1150-1153.
18. Christiansen OB, Nielsen HS, Kolte A, Pedersen AT. Research methodology and epidemiology of relevance in recurrent pregnancy loss. *Semin Reprod Med*. 2006; 24(1): 5-16.
19. Ren A, Wang J. Methylenetetrahydrofolate reductase C677T polymorphism and the risk of unexplained recurrent pregnancy loss: a meta-analysis. *Fertil Steril*. 2006; 86(6): 1716-1722.
20. Azem F, Many A, Ben Ami I, Yovel I, Amit A, Lessing JB, *et al*. Increased rates of thrombophilia in woman with repeated IVF failures. *Hum Reprod*. 2004; 19(2): 368-370.
21. Qublan HS, Eid SS, Ababneh HA, Amarin ZO, Smadi AZ, Al-Khafaji FF, *et al*. Acquired and inherited thrombophilia: implication in recurrent IVF and embryo transfer failure. *Hum Reprod*. 2006; 21(10): 2694-2698.
22. Ogino S, Wilson RB. Genotype and haplotype distributions of *MTHFR* 677C>T and 298A>C single nucleotide polymorphisms: a meta-analysis. *J Hum Genet*. 2003; 48(1): 1-7.
23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988; 16(3): 1215.
24. Stangler Herodež Š, Zagradišnik B, Kokalj-Vokač N. MLPA method for PMP22 gene analysis. *Acta Chim Slov*. 2005; 52(2): 105-110.
25. Kovalevsky G, Gracia CR, Berlin JA, Sammel MD, Barnhart KT. Evaluation of the association

- between hereditary thrombophilias and recurrent pregnancy loss: a meta analysis. *Acta Intern Med.* 2004; 164(5): 558-563.
26. Freitas AI, Mendonça I, Guerra G, Brión M, Reis RP, Carracedo A, *et al.* Methylenetetrahydrofolate reductase gene, homocysteine and coronary artery disease: the A1298C polymorphism does matter: Inferences from a case study (Maderira, Portugal). *Thromb Res.* 2008; 122 (5): 648-656.
27. Settin A, Elshazli R, Salama A, El Baz R. Methylenetetrahydrofolate reductase gene polymorphisms in Egyptian women with unexplained recurrent pregnancy loss. *Genet Test Mol Biomarkers.* 2011; 15(12): 887-892.
28. Rotondo JC, Bosi S, Bazzan E, Di Domenico M, De Mattei M, Selvatici R, *et al.* Methylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples of infertile couples correlates with recurrent spontaneous abortion. *Hum Reprod.* 2012; 27(12): 3632-3638.
29. Wu W, Shen O, Qin Y, Niu X, Lu C, Xia Y, *et al.* Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (*MTHFR*). *PloS One* 2010; 5: e13884.