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Methylenetetrahydrofolate reductase A1298C polymorphism and male infertility in a Romanian population group

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ABSTRACT

Introduction: *Folate metabolism is essential for proper cellular function, including DNA methylation. Within the folate pathway MTHFR (methylenetetrahydrofolate reductase) provides methyl groups for numerous reactions including DNA methylation. The MTHFR A1298C variant is associated with reduced enzyme activity, increased homocysteine and reduced folate plasma levels.*

Material and methods: *We analyzed the distribution of this single nucleotide polymorphism in the MTHFR gene in a case group of 66 infertile Romanian patients with idiopathic azoospermia or severe oligozoospermia and a control group of 67 Romanian men, to explore the possible association of the A1298C polymorphism and male infertility. Using the polymerase chain reaction – restriction fragment length polymorphism technique (PCR-RFLP), the allele and genotype distribution of SNP A1298C in the MTHFR gene were investigated in both patients and controls.*

Outcomes: *The frequencies of the polymorphism in infertile patients were not significantly higher than those in controls.*

Conclusions: *Our findings suggest that there is no significant association of SNP A1298C in the MTHFR gene with azoospermia or oligozoospermia, indicating that this polymorphism would not be a genetic risk factor for male infertility in our study group. It may be necessary to enlarge the study groups in order to obtain more significant conclusions and to evaluate other polymorphisms in genes that code for key enzymes in the folate and homocysteine metabolism, for being able to interpret the eventual complex gene-gene interactions with possible implications in the studied pathology.*

Key words: homocysteine, folate, azoospermia, DNA methylation

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INTRODUCTION

Couple infertility is a global health problem and according to the World Health Organization approximately one couple in seven is affected by fertility or subfertility problems (1). Male infertility in humans has been acknowledged as the cause of couple's inability to have children in 20-50% of total cases (2).

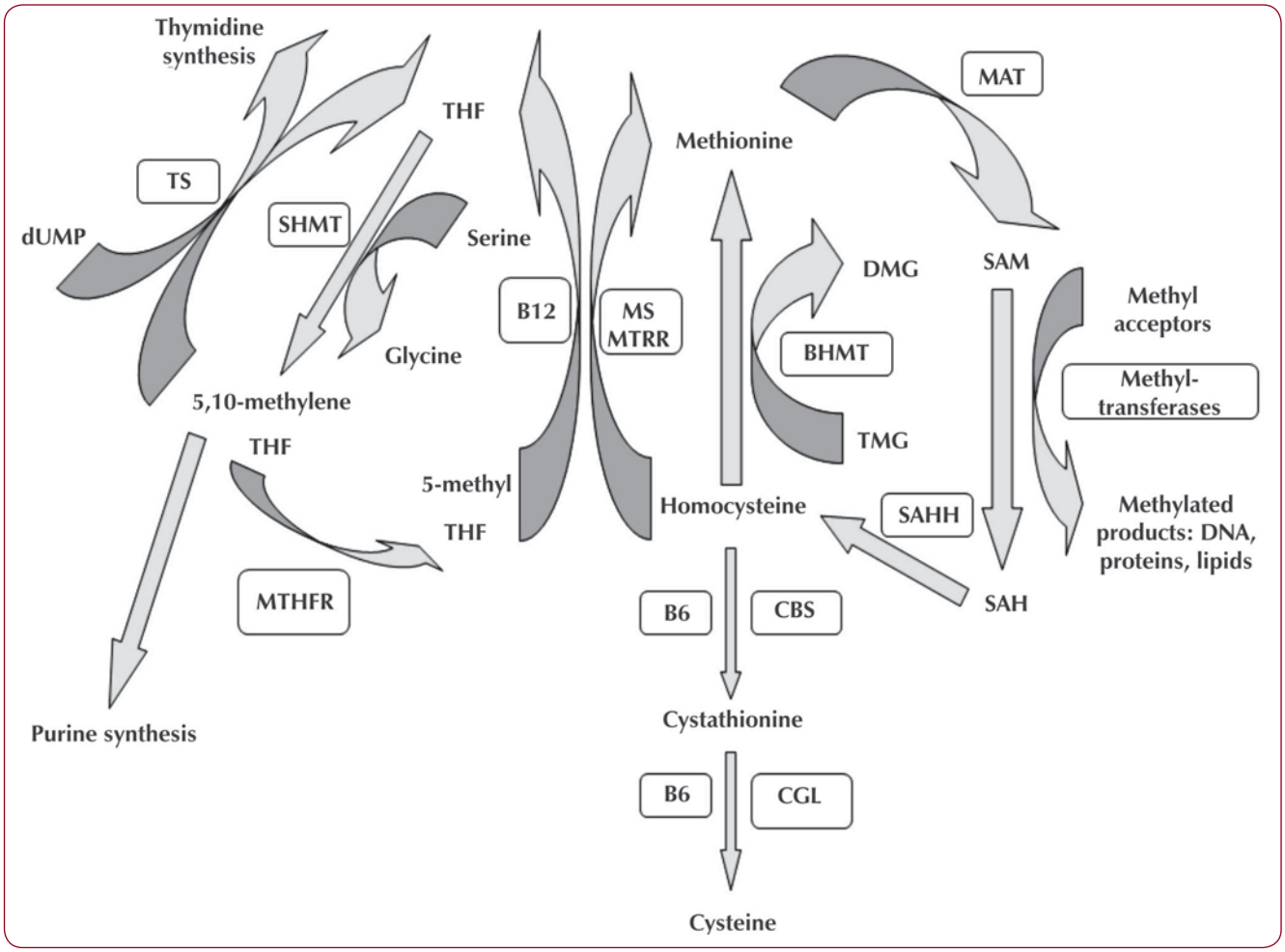
The most common non-genetic causes of male infertility are: hypogonadism, testicular maldescence, structural abnormalities of the male genital tract, genital infections, previous scrotal or inguinal surgery, varicoceles, chronic illness, medication and exposure to chemicals. However in about 40% of cases no cause was found related to infertility, hence launching the idea that a high number of idiopathic male infertility cases could be attributed to genetic factors. Genetic abnormalities were identified in men with unexplained oligozoospermia and azoospemia, including numerical and structural chromosomic abnormalities (3,4), deletions of the azoospermia factor region (AZF) of the Y chromosome or translocations between the Y chromosome and other chromosomes (5-7), mutations in the cystic fibrosis conductance regulator (CFTR) gene, commonly associated with congenital vas deferens abnormalities (8,9) and also other genetic factors (10). It was observed that some abnormalities associated with infertility are inherited, like reciprocal and Robertsonian translocations and CFTR mutations (11), while the majority of numerical chromosome abnormalities and AZF deletions are de novo events in the parental germ cells.

Folates are a group of inter-convertible coenzymes, differing by their oxidation state, number of glutamic acid moieties and one carbon substitutions. They are involved in amino acid metabolism, purine and pyrimidine synthesis and methylation of a large number of proteins, lipids, and nucleic acids as well. The relation between folate metabolism and the methionine/homocysteine pathway is particularly important. Homocysteine, a sulfhydryl-containing amino acid that is not used in protein synthesis, originates exclusively from the one-carbon metabolism of methionine, and it is remethylated into methionine with folates acting as methyl donors (12). In the last decade increased plasmatic levels of homocysteine have been found to be associated with an increased risk for several diseases, such as atherosclerotic, thromboembolic and neurodegenerative disorders, and also with early pathological events of life (13,14). The latter category of disorders includes the following: neural tube defects, late pregnancy complications such as preeclampsia, abruptio placentae, intrauterine growth retardation, preterm birth and intrauterine fetal death (15-19). However, although the recent progress in understanding the physiopathology of hyperhomocysteinemia – induced health events, there is only little information on the role of folates/homocysteine on male reproduction.

Within the folate metabolic cycle the MTHFR (methylenetetrahydrofolate reductase) gene encodes a key regulatory enzyme responsible for the reduction of 5, 10-methyltetrahyfrofolate, thus catalyzing the only reaction in the cell that ultimately generates 5-methyltetrahydrofolate, the biologically active folate derivative. The importance of folate metabolism is related to its function in providing one-carbon units for nucleic acids bases synthesis as well as for the synthesis of S-adenosylmethionine, the universal methyl donor for several biological methylation reactions.

Within the MTHFR gene several SNPs (single nucleotide polymorphisms) have been described. Martin et al. (20) resequenced the MTHFR gene product, and found a total number of 65 polymorphisms, 11 of which were non-synonymous cSNPs. A transition from cytosine to thymine at the 677 position of the MTHFR gene causes enzyme thermolability and reduced activity (21), therefore, impairments of MTHFR function, such as those associated with the presence of the C677T polymorphic site, are critical for altering nucleic acid metabolic pathways. Van der Put et al. (22) identified another polymorphism of the MTHFR gene, a 1298A-C mutation resulting in a gluto-ala substitution in position 429. It must be taken into account that whereas the C677T transition occurs within the predicted catalytic domain of the MTHFR enzyme, the A1298C transition is located in the presumed regulatory domain. The A1298C mutation resulted in decreased enzyme activity, which was more pronounced in the homozygous than heterozygous state.

The biochemical properties of the products of the C677T and A1298C polymorphisms



FOLATE METABOLISM: TS – rhymydylate synthase, SHMT – serine hydroxymethyltransferase, THF – tetrahydrofolate, MTHFR – methylenettrahydrofolate reductase, MS – methionine sunthase, MTRR – methionine sunthase reductase, BHMT – betaine-homocysteine methyltransferase, TMG – trimethylglycine, DMG – dimethylglycine, CBS – cystathionine beta-synthase, CGL – cystathionine gamma-lyase, MAT – methionine adenosyltransferase, SAM – S-adenosylmethionine, SAH – S-adenosylhomocusteine, SAHH – S-adenisylhomocysteine hydrolase

were studied by Yamada et al. (23), who observed that the A222V MTHFR enzyme corresponding to the C677T transition had an enhanced propensity to dissociate into monomers and lose its FAD (flavin adenin dinucleotide) cofactor on dilution, while the E429A protein had biochemical properties indistinguishable from the wildtype enzyme. ❑

MATERIALS AND METHODS

Our study was performed on a group of 66 infertile Romanian patients from which 54 were diagnosed with idiopathic azoospermia and 12 with severe oligozoospermia, and a control group of 67 Romanian men with at least 1 child.

Patients with a history of varicocele, congenital abnormalities, urogenital infections and undescended testicles were excluded from the test after examination by a specialist. Also after performing chromosomal and molecular analysis patients with chromosomal abnormalities, microdeletions in the AZF region of the Y chromosome were excluded from the study group. Informed consent regarding genetic testing was obtained from all study subjects. For genetic testing, 3ml of peripheral blood was extracted on EDTA to prevent blood clotting. Genomic DNA was extracted from blood leucocytes contained in a volume of 300μl using a commercially available extraction kit (Wizzard Genomic DNA Purification Kit, Promega®). The presence of the MTHFR A1298C polymorphism

was detected by means of molecular genetic techniques, respectively polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) by modifying a previously described protocol, van der Put et al. (22). The PCR amplification reaction was performed in a total volume of 25μl containing approximately 100ng of genomic DNA, 12.5μl PCR Master Mix (Fermentas MBI, Lituania®), 1μl BSA (Bovine Serum Albumine, Fermentas MBI, Lituania®) solution 2 mg/ml, 8 pM of each primer, forward and reverse (Eurogentec, Belgium®) and water free of nucleases to complete the 25μl volume. The PCR reactions were performed in a gradient thermocycler (MastercyclerGradient, Eppendorf®) by using the following primer pairs: 5'-CTTTGGGGAGCTGA AGGACTACTAC-3' and 5'-CACTTTGTGAC-CATTCCGGTTTG-3' under the following conditions were: an initial step consisting in denaturation for 5 min at 95°C, annealing for 2 min at 55°C, extension for 2 min at 72°C, followed by 35 cycles of denaturation at 95°C for 75 s, annealing at 55°C for 75 s, extension at 72°C for 90 s, and a final extension time of 6 min at 72°C. The amplified fragment of 163 bp was digested with MboII endonuclease (Fermentas MBI, Lituania®). The A1298C mutation abolishes an MboII restriction site. Digestion of the 163-bp fragment of the 1298 AA genotype gives five fragments, of 56, 31, 30, 28 and 18 bp, whereas the 1298CC genotype results in four fragments, of 84, 31, 30 and 18 bp. The digested fragments were resolved in a 3% MetaPhor gel (Lonza®, Basel, Switzerland), stained with ethidium bromide and then visualized on a UV transilluminator VilberLourmat Imaging System®, Marne-la-Vallée, France). The observed alleles and genotypes frequencies were calculated for both groups and the Chi-square test for deviation was performed in order to establish if the genotype distribution in the studied population were in Hardy-Weinberg equilibrium. A comparison of the results between the study group and control group was made and the differences were tested for significance using the Chi-Square test of the statistical software SPSS Statistics 17.0. ❑

OUTCOMES

The genotype and allelic frequencies obtained for the A1298C SNP are presented in table I. We performed association tests between the normal homozygote status (AA genotype) with the heterozygous status (AC genotype), mutant homozygous status (CC genotype) and carrier of at least one mutant allele (heterozygous AC and homozygous CC genotypes), respectively. The observed genotypes frequencies among the study groups were in agreement with Hardy-Weinberg equilibrium ($\chi 2=2.756$, $p=0.0969$) while the genotypes frequencies of the MTHFR A1298C polymorphism were 3% and 43.9% for the CC and AC genotype respectively, among the idiopathic azoospermia and severe oligozoospermia (AZF) group and 3% and 38.8%, respectively among the control group.

After applying the statistical Chi-square test to the observed genotypes all the p values obtained were >0.05, considered not statistically significant. Also, the odds-ratios for all the association tests have been around the value of 1.2, revealing that the A1298C SNP in the MTHFR gene is distributed similarly in the two study groups. In our study we obtained a similar allelic frequency (0.24) of the A1298C SNP found in previous published articles.

Genotypes	AZF group n (%)	Control group n (%)	OR (95%CI)	p value
Total no. of subjects	66 (100)	67 (100)		
AA	35 (53)	39 (58.2)		
AC	29 (43.9)	26 (38.8)	1.243 (0.6178-2.50)	0.542
CC	2 (3)	2 (3)	1.114 (0.1489-8.34)	0.916
AC+CC	31 (46.9)	28 (41.8)	1.234 (0.6217-2.448	0.548
Allele	**Alleles frequencies**			
Total no. of alleles	132	134		
A allele	0.75	0.77		
C allele	0.25	0.22	1.156 (0.6561-2.035)	0.616

TABLE 1. MTHFR A1298C genotype and allele frequencies

In the present study we evaluated for the first time the possibility of an association between the A1298C SNP in the MTHFR gene and male infertility in a Romanian population group. A few previous studies have evaluated the association of MTHFR C677T polymorphism in infertile patients from Germany, Netherlands, Italy, India, South Korea and China (24-30). Five of them (24,27-29) have reported an association between these polymorphism in the MTHFR gene and male infertility. However, the MTHFR A1298C SNP has been studied less. Varinderpal et al. (2) reported no association between the A1298C SNP and male infertility in an Indian study group; while another study done on Chinese infertile men also (29) concluded that there is no association between this SNP and the idiopathic cases of male infertility.

Considering the fact that folate deficiency has been shown to reduce the proliferation of various cell types (31) and also that it is already established that folate intake is very important for male infertility, future studies need to focus on the relation between idiopathic cases of infertility, genetic risk factors and the nutritional status of subjects; dietary habits which are particular in the country were the study is conducted influence plasmatic levels of homocysteine and folates.

It has already been shown that sperm concentration is increased by folic acid and zinc sulphate treatment (25). Also in the cause of altered folate status due to reduced MTHFR enzyme activity, epigenetic alterations in DNA must be taken into account as important etiological factors. DNA methylation typically occurs in CpG dinucleotide rich regions, CpG islands, highly conserved sequences in promoter regions or first exons of genes (32). Because of the strong correlation between DNA methylation in promoter regions and transcriptional repression (32), DNA methylation appears to be a fundamental as well as potentially reversible mechanism for epigenetic control of gene expression. There is accumulating evidence that hypermethylation is involved in carcinogenesis since this phenomenon contribute to suppression of gene transcription (33).

Compared to other types of pathologies, vascular, neurodegenerative were wide genome association studies are being used to determine possible risk factors, until this date there is only one study of this type published by Aston et al. (34) on male infertility, which investigated 370.000 SNPs and found 20 SNPs significantly associated with idiopathic forms of male infertility. However this pilot study emphasizes the fact that without proper financial support genome wide association studies are not feasible and that the candidate gene approach is still required if we are to uncover the molecular mechanisms of male infertility. ❑

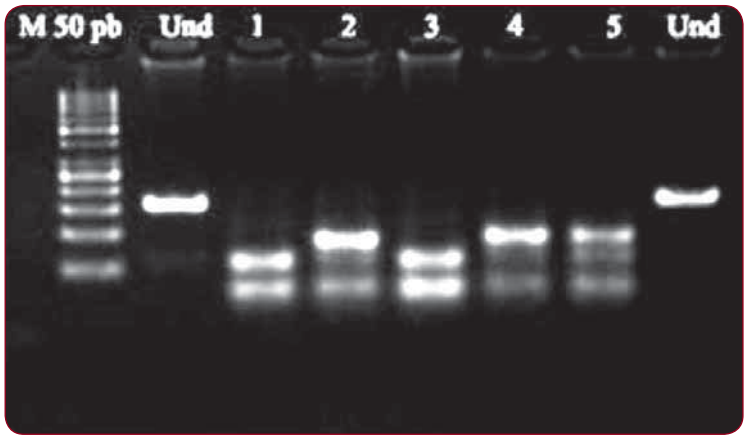


FIGURE 1. DNA electrophoresis of MTHFR A1298C on 3% MetaPhor gel: M 50 pb – DNA ladder; Und – undigested; 1,3 – homozygous normal allele; 2,4,5 – heterozygous allele

CONCLUSION

Despite our study had some limitations, like the impossibility to measure plasma levels of homocysteine and folates, our work provides data for the first time in a Romanian population group regarding risk factors for male infertility possibly attributed to abnormal folate status.

Considering the fact that the genotype and allelic distribution of the MTHFR A1298C polymorphism observed in our study was similar in the two groups we conclude that this polymorphism is not a genetic risk factor for male infertility in our Romanian population group. To better understand the etiology of male infertility future studies will need to be conducted on more subjects to obtain a higher statistical significance and to focus on indentifying and studying new candidate genes in order to obtain a deeper understanding of the complex gene-to-gene and gene-nutrient interactions which have a profound effect on the studied pathology.

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