Association study of folate-related enzymes (MTHFR, MTR, MTRR) genetic variants with non-obstructive male infertility in a Polish population

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Abstract

Spermatogenesis is a process where an important contribution of genes involved in folate-mediated one-carbon metabolism is observed. The aim of the present study was to investigate the association between male infertility and the *MTHFR* (677C > T; 1298A > C), *MTR* (2756A > G) and *MTRR* (66A > G) polymorphisms in a Polish population. No significant differences in genotype or allele frequencies were detected between the groups of 284 infertile men and of 352 fertile controls. These results demonstrate that common polymorphisms in folate pathway genes are not major risk factors for non-obstructive male infertility in the Polish population.

Keywords: MTHFR, MTR, MTRR, polymorphism, infertility

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Spermatogenesis is a multistep developmental process coordinated by sequential expression of various genes, with an important contribution of genes involved in folate-mediated one-carbon metabolism. This pathway is mandatory for thymidylate and purine biosynthesis, thus providing substrates for DNA synthesis in rapidly dividing male germ cells. Via involvement in homocysteine metabolism, folates participate in DNA, RNA and histone methylation reactions, taking part in regulation of transcription. The key enzymes implicated in the above mentioned metabolic pathways are: 5,10-methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR) and methionine synthase reductase (MTRR). It was found that polymorphisms defined within the coding sequences of these genes may affect metabolic pathways controlled by the enzymes.

Within the *MTHFR* gene, two functional single nucleotide polymorphisms (SNPs) were characterized. The *MTHFR* 677C > T variant (rs1801133) encodes a thermolabile protein variant with enzymatic activity decreased by 35% in heterozygotes and by 70% in the homozygous state. The *MTHFR* 1298A > C polymorphism (rs1801131) is associated with a 30% decrease in enzymatic activity. The *MTHFR* 677C > T and *MTHFR* 1298A > C SNPs were also shown to be associated with DNA hypomethylation

(Weiner *et al.*, 2014). In the *MTR* gene, an adenine to guanine transition at position 2756 (A > G, rs1805087) results in substitution of aspartic acid with glycine in codon 919 of the protein and is related to alterations in the folate metabolic pathway. The Asp919Gly substitution in the MTR enzyme results in its higher activity, leading to more effective homocysteine remethylation and methionine production (Ravel *et al.*, 2009). The *MTRR* gene includes a polymorphic locus *MTRR* 66A > G (rs1801394), which was shown to slightly reduce enzymatic activity, but was associated with decreased plasma homocysteine concentrations (Park *et al.*, 2005).

The available information on associations of the above mentioned SNPs in *MTHFR*, *MTR* and *MTRR* genes with male infertility reported from various populations is not consistent, and mostly evaluate *MTHFR* gene. Most of the studies available are from Asian populations (Lee *et al.*, 2006; A *et al.*, 2007; Park *et al.*, 2009), with some data from Caucasians: Italian (Stuppia *et al.*, 2003), Dutch (Ebisch *et al.*, 2003), Swedish (Murphy *et al.*, 2011), French (Ravel *et al.*, 2009; Montjean *et al.*, 2011), German (Bezold *et al.*, 2001), Spanish (Camprubi *et al.*, 2013) and only one from an East European (*i.e.* Slavic) population, from Russia (Weiner *et al.*, 2014). The results are still debatable, and the observed differences may not only depend on ethnic differences but also on environmental factors, *i.e.* folate intake, which in turn can influence DNA methylation and semen quality. The present study aimed at definition of associations of the common *MTHFR*, *MTR* and *MTRR* polymorphisms with male infertility in a Polish (*i.e.* Slavic) population.

The study was carried out in 284 consecutive, otherwise healthy male patients (aged 22–49 years, mean 32.7 ± 4.7) without any chromosomal abnormalities, undergoing semen analysis due to infertility workup. The inclusion criteria were as follow: no children from current or previous relations with at least a year history of at least a year of regular (2–3 weekly), unprotected sexual activity without conception; female partners aged up to 35 years with regular menstrual bleedings and/or progesterone levels in the luteal phase of the cycle > 10 ng/mL, normal transvaginal ultrasound examination, negative testing for *Chlamydia trachomatis* infection, without history of pelvic inflammatory disease or abdominal operations. Subjects were excluded from the study if semen analysis and clinical picture suggested obstructive azoospermia or testicular, epididymal, or accessory gland infection. Also, subjects with known systemic disease, BMI ≥ 30 kg/m^2, varicocele, history of mumps, testicular torsio or maldescence, trauma, as well as occupational hazards (exposure to solvents, pesticides, painting materials, heavy metals or radiation) were not taken into consideration.

The control group consisted of 352 healthy males (aged 21–56 years, mean 34.7 ± 8.7) recruited among consecutive men accompanying their female partners at term labor in the University Department of Feto-Maternal Medicine. Paternity was confirmed by women; however the possible paternal discrepancy was additionally checked based on blood group verification. Both the men undergoing infertility examination, as well as the fertile controls were Caucasians of Polish origin, recruited within the same geographical region. The study was approved by the local ethics committee and written informed consent was obtained from all subjects.

Genomic DNA was extracted from blood samples using GeneMATRIX Blood DNA Purification Kit (EURx, Poland). Pre-validated allelic discrimination TaqMan real-time PCR assays (Life Technologies, USA) were used for detection of the respective SNPs in *MTHFR* (rs1801131, rs1801133), *MTR* (rs1805087) and *MTRR* (rs1801394) genes. Amplification was performed in a 7500 Fast Real-Time PCR System with incorporated SDS software for SNP genotyping (Applied Biosystems, USA) using TaqMan GTXpress Master Mix (Life Technologies, USA). Fluorescence data was captured after 40 PCR cycles.

Allele and genotype frequencies were determined by direct counting of alleles. Concordance of genotype distribution with Hardy-Weinberg equilibrium was calculated using χ^2 test. Genotype and allele frequencies between the study groups were compared by means of Fisher's exact test. The effect of each polymorphism was tested in both a dominant and recessive model. All genotypes were distributed in concordance with Hardy-Weinberg equilibrium, both in infertile patients and control subjects. No significant differences between the study groups were noted, neither in genotype distribution, nor in allele frequencies. All genotyping results are given in Table 1.

Table 1: *Distribution of MTR, MTRR and Fluorescence data after 40 PCR cyclesMTHFR gene variants in infertile patients and control group.*

	Fertile n = 352		Infertile n = 284		p*	OR (95%CI)
	n	%	n	%		
MTR rs1805087 (2756A > G, Asp919Gly)						
Genotype						
AA	218	61.9%	178	62.7%	-	
AG	125	35.5%	93	32.7%	0.610	0.91 (0.78–1.46)
GG	9	2.6%	13	4.6%	0.271	1.77 (0.74–4.24)
(AG+GG) *vs.* AA	134	38.1%	106	37.3%	0.869	0.97 (0.70–1.34)
(AA+AG) *vs.* GG	343	97.4%	271	95.4%	0.193	0.55(0.23–1.29)
Allele						
A	561	79.7%	449	79.0%		
G	143	20.3%	119	21.0%	0.780	
MTRR rs1801394 (66A > G, Ile22Met)						
Genotype						
AA	70	19.9%	51	18.0%	-	
AG	171	48.6%	139	48.9%	0.666	1.12 (0.73–1.71)
GG	111	31.5%	94	33.1%	0.564	1.16 (0.74–1.83)
(AG+GG) *vs.* AA	282	80.1%	233	82.0%	0.608	1.13 (0.76–1.69)
(AA+AG) *vs.* GG	241	68.5%	190	66.9%	0.733	0.93 (0.77–1.50)
Allele						
A	311	44.2%	241	42.4%		
G	393	55.8%	327	57.6%	0.569	
MTHFR rs1801133 (677C > T, Ala222Val)						
Genotype						
CC	166	47.2%	143	50.4%	-	
CT	150	42.6%	113	39.8%	0.448	0.87 (0.63–1.21)
TT	36	10.2%	28	9.9%	0.783	0.90 (0.52–1.55)
(CT+TT) *vs.* CC	186	52.8%	141	49.6%	0.426	0.880 (0.64–1.20)
(CC+CT) *vs.* TT	316	89.8%	256	90.1%	0.895	1.04 (0.62–4.75)
Allele						
C	482	68.5%	399	70.2%		
T	222	31.5%	169	29.8%	0.502	
MTHFR rs1801131 (1298A > C, Glu429Ala)						
Genotype						
AA	156	44.3%	128	45.1%	-	
AC	156	44.3%	130	45.8%	0.933	1.02 (0.73–1.41)
CC	40	11.4%	26	9.2%	0.413	0.79 (0.46–1.37)
(AC+CC) *vs.* AA	196	55.7%	156	54.9%	0.873	0.97 (0.71–1.32)

No significant impact of the studied polymorphisms on male infertility was revealed in the present study. The original concept of the impact of *MTHFR* variants on male reproduction and initial positive association of the thermolabile 677T variant with infertility came from the German study of Bezold *et al.* (2001), who reported significant overrepresentation of TT homozygotes among male patients seeking fertility evaluation compared with control group (18.8 *vs.* 9.5%). This preliminary report, without detailed characterization of neither male infertility nor control subjects, has been subsequently followed by several studies in Caucasian populations, *i.e.* Dutch (Ebisch *et al.*, 2003), Italian (Stuppia *et al.*, 2003), Swedish (Murphy *et al.*, 2011), and Spanish (Camprubi *et al.*, 2013). Contrary to the original report, the results of all aforementioned studies were negative. It should be noted that most of them simply lacked sufficient power to verify the existence of the investigated association, as numbers of participants were low (Table 2). Nonetheless negative association results were accompanied by findings on a potential relationship of *MTHFR* genotype and sperm counts, but only in some studies. Ravel *et al.* (2009) did not find any association between *MTHFR* (677C > T, 1298A > C and 215GA - rs2066472) genetic variants and sperm counts in French infertile men, which was later confirmed by Montjean *et al.* (2011) in a larger cohort of mixed ethnicity. Similarly, none of the genotypes was associated with neither standard seminogram parameters nor presence of sperm DNA hypomethylation (Camprubi *et al.*, 2013). Finally, in the recent report from an East European population in Russia, Weiner *et al.* (2014) have observed the association of *MTHFR* genotype with azooospermia, but found no general impact of *MTHFR* 677C > T and *MTHFR* 1298A > C polymorphisms on male infertility. Summarizing the observations from Caucasian studies, including the present Polish one, it seems that *MTHFR* 677C > T and *MTHFR* 1298A > C polymorphisms are not associated with male infertility.

Table 2: *Previous studies on genes of folate-mediated one-carbon metabolism pathway in relation to male infertility.*

Genetic polymorphism studied	Association reported	Study population	Reference
MTHFR 677C > T	*MTHFR* 677C > T (TT homozygotes) with infertility	255 infertile men and 200 controls, ethnicity not given, Germany	Bezold *et al.*, 2001
MTHFR 677C > T	no association with infertility	93 infertile and 105 controls, Italian Caucasians	Stuppia *et al.*, 2003
MTHFR 677C > T	no association with subfertility	113 fertile and 77 subfertile males, Dutch Caucasians	Ebisch *et al.*, 2003
20 SNPs in 12 genes related to folate, homocysteine and B12 metabolism	no association of folate-related gene polymorphisms with infertility; *PEMT* (phosphatidylethanolamine		
N-methyltransferase) rs7946 and CD320 (transcobalamin receptor) rs173665 with infertility	153 infertile men and 184 controls, ethnicity not given, Sweden	Murphy *et al.*, 2011	
MTHFR 677C > T	no association with infertility or sperm counts	107 infertile men and 25 controls, ethnicity not given, Spain	Camprubi *et al.*, 2013
MTHFR 677C > T, 1298A > C; *MTR* 2756A > G; *MTRR* 66A>G; *SHMT1* 1420C > T; *MTHFD1* 1958G > A; *CBS* 844ins68	*MTHFD1* 1958G>Aand *MTR* 2756A > G with infertility (without correction for multiple testing only); *MTHFR* 677C > T with azoospermia;	275 infertile men and 349 controls, Russian Caucasians	Weiner *et al.*, 2014
MTHFR 677C > T, 1298A > C, 215GA; *MTRR* 66A > G, 524C > T; *CBS 919G > A*	no association with reduced sperm counts	70 azoospermia and 182 oligozoospermia cases, 114 normospermic controls, "French ethnic origin" stated	Ravel *et al.*, 2009
MTHFR 677C > T, 1298A > C	*MTHFR* 677C > T with infertility	373 infertile men + 396 controls, Korean Asians	Park *et al.*, 2005
MTHFR 677C > T, 1298A > C; *MTR* 2756A > G; *MTRR* 66A>G	*MTHFR* 677C > T and *MTRR* 66A>G with infertility; *MTHFR* 677C > T and *MTR* 2756A > G with azoospermia	360 infertile men and 325 controls, Korean Asians	Lee *et al.*, 2006
MTHFR 677C > T	*MTHFR* 677C > T with infertility	355 infertile and 252 fertile Chinese Asians	A *et al.*, 2007
MTHFR 677C > T, 1298A > C, 215GA; *MTRR* 66A>G,524C>T	*MTHFR* 677C > T with hyperhomocysteinemia; no association with sperm counts	522 men, mixed ethnic origin, France	Montjean *et al.* 2011
MTHFR 677C > T, 1298A > C	*MTHFR* 677C > T with non-obstructive azoospermia and severe oligozoospermia	156 infertile men and 233 controls, mixed ethnic origin, Brazil	Gava *et al.*, 2011
MTHFR 677C > T, 1298A > C; *DNMT3B* 46359C > T	no association with infertility	179 infertile and 200 fertile men, India	Dhillon *et al., 2007*
MTHFR 677C > T	*MTHFR* 677C > T with infertility, also confirmed by meta-analysis of data from available studies	522 infertile and 315 controls, India	Gupta *et al.*, 2011
MTHFR 677C > T, 1298A >	*MTHFR* 677C > T with in-	150 infertile and 150 con-	Mfady *et al.*, 2014

In contrast to Caucasian studies, investigations on the association of *MTHFR* polymorphism with male infertility conducted in populations of non-European descent gave several positive results. Two large studies from Korea presented a significant association of the *MTHFR* 677C > T (but not 1298A > C) polymorphism with infertility (Park *et al.*, 2005, Lee *et al.*, 2006). Moreover, these observations were supported by a study in Chinese patients, where *MTHFR* 677T status was found to be a risk factor for male infertility (A *et al.*, 2007). Data from Asian studies were also confirmed by other studies, including several reports revealing an impact of *MTHFR* 677C > T polymorphism on infertility: from an Arabic population, *i.e.* Jordanians, by Mfady *et al.* (2014), a Brazilian report on males of mixed ethnicity by Gava *et al.* (2011), or by Gupta *et al.* (2011) from India. However, negative data for Indians, for both *MTHFR* 677C > T and *MTHFR* 1298A > C, were also reported (Dhillon *et al.*, 2007). Such impact of ethnic differences is also reflected in meta-analyses. A stratified analysis by Wu *et al.* (2012) showed that a significant association between *MTHFR* 677C > T polymorphism and male infertility was present only in Asians (OR = 1.79 for two copies of T allele and OR = 1.42 for T allele carriers), but not in Caucasians. A meta-analysis published by Shen *et al.* (2012) on the *MTHFR* 1298A > C variant gave similar results. However, the authors joined genetically distinct ethnic groups for the analysis (Korean and Indian) as "Asians", which does not seem to be fully justified. These meta-analyses also are in accordance with the negative observations from the present study in a Polish-Caucasian population.

There is scarce data on two other polymorphisms evaluated in the present study, *i.e. MTR* and *MTRR*. The *MTR* 2756A > G polymorphism was not associated with male infertility in the aforementioned Korean (Lee *et al.*, 2006), Russian (Weiner *et al.*, 2014), as well as the Swedish studies (Murphy *et al.*, 2011). Our study does support these observations, as no impact of the *MTR* 2756A > G polymorphism on infertility in Polish males was found. However, the Korean study by Lee *et al.* (2006) found an association between *MTR* 2756GG genotype and an increased risk of azoospermia.

Similarly to the *MTHFR* polymorphism, the *MTRR* 66A > G polymorphism was found to impact male infertility in the Asian population. Lee *et al.* (2006) documented that the *MTRR* 66GG genotype promoted development of male infertility. Contrary to this, the Russian (Weiner *et al.*, 2014) and French (Ravel *et al.*, 2009) studies did not support the findings from the Korean population. Likewise, data from a Middle Eastern Arabic population demonstrated that the *MTRR* 66A > G genotype distribution was not different in fertile and infertile groups (Mfady *et al.*, 2014). Our results from a non-Russian, Slavic population did not reveal an association between the *MTRR* 66A > G polymorphism and male infertility. In conclusion, the present study did not reveal a significant association of the *MTHFR*, *MTR*, *MTRR* gene polymorphisms with non-obstructive male infertility in a Polish population.

Nonetheless, the observed discrepancy between the results of studies conducted in different populations may result from both genetic determinants and environmental factors, including differences in folate consumption in different regions. Reduced folate levels can result from mutations in folate pathway genes, as well as insufficient dietary intake. Folate deficiency affects spermatogenesis by producing DNA hypomethylation and resultant gene expression changes, as well as inducing uracil misincorporation in the course of DNA synthesis, and thus errors in DNA repair, strand breakage and chromosomal abnormalities (Ravel *et al.*, 2009). Deficiency of folates is also related with hyperhomocysteinemia, a risk factor for male infertility (Lee *et al.*, 2006). Hyperhomocysteinemia may not only result from low folate consumption, but also from genetic variants in genes of the folate pathway (Bialecka *et al.*, 2012). It was also demonstrated that folate treatment improved semen parameters, such as an increase in spermatozoa number and motility, as well as total normal sperm count.

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