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MTHFR C677T polymorphism, *GSTM1* deletion and male infertility: a possible suggestion of a gene–gene interaction?

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Abstract
Methylenetetrahydrofolate reductase (*MTHFR*) is a gene involved in the process of DNA synthesis and methylation. The *MTHFR* C677T polymorphism has been associated with male infertility. A prospective study was conducted on men seeking care at the infertility clinic in Milano to determine if the *MTHFR* C677T polymorphism is associated with infertility, and if such an association is modified by a common deletion of one of the glutathione transferases, *GSTM1*. One year after enrolment, 46 subjects reported having had a child, while 59 were still childless. Subjects carrying the *MTHFR* C677T homozygous variant polymorphism were at increased risk of being infertile after 1-year follow-up (OR 3.7, 95% CI = 1.4–10.4); carriers of the homozygous variant *MTHFR* genotype and of a functional copy of *GSTM1* appear to have a significantly higher risk of infertility ($n = 11$; OR = 22.0 95% CI = 3.8–127.9) than subjects who carry the wild-type genotype for both genes. Such risk becomes non-significant when the *GSTM1* deletion is also present ($n = 5$; OR = 1.1 95% CI = 0.2–5.1). A possible explanation of this unexpected result could lie in the known involvement of glutathione transferases in the metabolic pathways of both methylation and transulfuration. The interaction found deserves confirmation and replication in a larger population, since it may be relevant to several chronic diseases such as cardiovascular diseases and cancer.

Keywords: *Epidemiology, metabolism, fertility, MTHFR, GSTM1, male*

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Introduction

Methylenetetrahydrofolate reductase (*MTHFR*) is a polymorphic gene that regulates the metabolism of folate and methionine, and it is therefore involved in the process of DNA synthesis and methylation. Two polymorphisms (C677T and A1298C) have been detected in humans, with frequencies that vary according to geographical area. The *MTHFR* C677T polymorphism in its homozygous form is associated with high levels of homocysteine and low levels of folates in the blood. The association between the *MTHFR* C677T polymorphism and infertility has been investigated by four studies (Bezold et al. 2001, Ebisch et al. 2003, Stuppia et al. 2003, Singh et al. 2005), the first of which, conducted in Germany (Bezold et al. 2001), showed the frequency of the C677T homozygous variant in infertile men to be twice that of control subjects.

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Further studies in Italy (Stuppia et al. 2003) and the Netherlands (Ebisch et al. 2003) showed no association between fertility status and *MTHFR* C677T polymorphism. A recent study on Indian infertile men reported a significant association between infertility and *MHTFR* homozygosity (Singh et al. 2005). It has been suggested that hyperhomocysteinemia leading to precocious atherosclerosis of the testicular arteries could be the mechanism behind *MTHFR* polymorphism and infertility. Alternatively, low levels of folates associated with *MTHFR* polymorphism could be responsible for infertility by altering RNA and DNA synthesis (Stern et al. 2000, Friso et al. 2002). Recent data show that sperm counts in subfertile men improved after treatment with folic acid and zinc (Wong et al. 2002), and that this effect was restricted to wild-type *MTHFR* subjects (Ebisch et al. 2003). However, the biological mechanism of this differential response according to genotype has not been investigated further. An earlier study found no correlation between sperm folic acid concentration and sperm count (Landau et al. 1978).

One of the main difficulties in comparing the above studies on *MTHFR* and infertility is the difference in frequency of the *MTHFR* C677T homozygous variant found in different populations, which ranges from 9.5% in the German study to 27.6% in the Italian study. Moreover, in these studies, other genes that could affect methylation and DNA synthesis were not examined. One of these candidate genes is *GSTM1*, a glutathione transferase expressed in the testis (Listowsky et al. 1998), which has antioxidant properties (Norppa 1997, Brockmoller et al. 1998, Sram 1998, Autrup 2000). In a cross-sectional study on infertile men, the present authors observed higher sperm DNA–adducts in subjects with *GSTM1* deletion in comparison with infertile men with functional *GSTM1* (Paracchini et al. 2005). Because of this preliminary finding and of the high prevalence of *GSTM1* deletion in the general population, GSTM1 was chosen as a candidate gene in combination with MTHFR.

A prospective study was conducted on men seeking care at the infertility clinic in Milano to assess their fertility status after 1 year from clinical evaluation. The aims were to determine if an association exists between the *MTHFR* C677T polymorphism and infertility and to see if any such association is modified by a common deletion of one of the glutathione transferases, *GSTM1*.

Materials and methods

Study design

This study represents the follow-up of a cross-sectional study previously conducted (Gaspari et al. 2003). The 179 men consecutively recruited from January to May 2001 through the Infertility Clinic of the University of Milan, Italy, were contacted by telephone 1 year later and asked to furnish a biological sample for genetic analysis. A description of the baseline cohort under study has been published (Gaspari et al. 2003). Female causes of infertility were excluded from the initial study based on the information reported on the medical chart.

Among the 179 contacted subjects, 150 were traced, and of them 113 (75%) agreed to participate in the study; 49 attended the Institute for the collection of both mouth wash and a blood spot on a Guthrie card; 63 subjects asked to receive the material by mail with instructions on how to collect the mouth wash, and then returned the sample using a pre-stamped envelope; one subject gave 4 ml blood. All subjects signed a written informed consent to participate in the second part of the study. The subjects

were defined as infertile if they had a stable partner, but had no children after at least 1 year of unprotected intercourse, or after at least two attempts at artificial insemination during the year. Subjects were defined as fertile if they had a child, either physiologically or with assisted procedures, in the year after the initial visit. The ability to conceive, according to the above-mentioned criteria, was checked through a brief questionnaire administered at the time of the collection of the biological specimen for the subjects who came to the Institute, or on the telephone for all others. The questionnaire asked about any pregnancy that occurred in the previous year. In addition, a crosscheck was performed through the Municipal lists of the newborns in the previous year, and the fertility centre in Milan where the subjects were initially recruited.

One subject was excluded from the present analysis because he was undergoing chemotherapy treatment, one because he had no partner at the time of follow-up, and five because they documented that the infertility problem belonged to their partner. One subject did not inform us about whether or not he conceived a child; therefore, the final number of subjects included in the present study was 105.

The expected genotype frequencies were calculated using the available data on a population of 299 healthy subjects recruited between 1999 and 2002 among the health professionals working in the hospital.

Sample analysis

The original sample consisted of 105 subjects. One subject gave 4 ml peripheral blood, from which DNA was extracted. In 104 subjects, DNA was extracted from mouthwash using the Gentra-system Tissue Kit according to the manufacturer's protocol (Gentra Systems, Inc., Minneapolis, MN, USA). For 12 of these samples, a whole genome amplification of 1 μl extracted DNA was necessary to have sufficient material for genotyping (GenomiPhi Kit, Amersham Biosciences, Psicataway, NJ, USA), followed by a purification of the amplified DNA solution. For 14 subjects, DNA extraction from mouthwash was not successful. However, a Guthrie card was available for these subjects, and the DNA was amplified directly from DNA extracted from the blood spot. Two internal controls for a successful polymerase chain reaction (PCR) were included: the β-globin-specific product (268 bp), and the DNA sample from a volunteer with known functional *GSTM1* and known *MTHFR* genotype. All laboratory analyses were performed blind, and the tubes were coded with consecutive numbers by the epidemiologist before reaching the laboratory; the technician was unaware of the fertility status of the subjects under analysis.

GSTM1. The *GSTM1* genotype was determined by multiplex PCR using *GSTM1* (5′-GAACTCCCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTCAAATATACG-GTGG-3′) specific primer pairs together with a β-globin-specific primer pair (5′-CAACTTCATCCACGTTCACC-3′ and 5′-GAAGAGCCAAGGACAGGTAC-3′) (Ford et al. 2000) in a 50-μl reaction containing 5 μl 10X PCR buffer, 5 μl DNTPs (2 mM), 4 μl each GSTM1 primer (10 μM) and 3 μl each β-globin primer (10 μM), and 0.5 μl Taq DNA polymerase (DyNAzyme™ I DNA Polymerase, Fynnzymes, New England, BioLabs, Ipswich, MA, USA). Reaction was carried out for 35 cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 64°C for 1 min, and a primer extension temperature of 72°C for 2 min. The product of PCR

analysis was then analysed on ethidium bromide-stained agarose gel (2%). The absence or presence of the *GSTM1*-specific PCR products (215 bp) determined the genotype.

MTHFR. The C677T polymorphism is caused by the substitution of thymidine for cytosine in exon 4 of the *MTHFR* gene. This mutation results in an alanine to valine substitution at position 222 (Frosst et al. 1995). The *MTHFR* genotype was determined by PCR. Specific primer pairs located in exon 4 (MTHFR-1: 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′; MTHFR-2: 5′-AGG ACG GTG CGG TGA GAG TG-3′) (Ulrich et al. 2000) were used to amplify a portion of the *MTHFR* sequence in a 50-μl reaction containing 5 μl 10X PCR buffer, 5 μl DNTPs (2 mM), 2 μl each primer (10 μM) and 0.5 μl Taq DNA polymerase (DyNAzyme™ I DNA Polymerase). The reaction was carried out for 35 cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 68°C for 1 min, and a primer extension temperature of 72°C for 1 min. The product of PCR analysis was then digested by *HinfI* enzyme, in a 25-μl reaction containing 15 μl PCR fragment, 2.5 μl 10X buffer 2, and 10 units *HinfI* at 37°C overnight. The digestion products result in a 198-bp band for non-carriers (Ala/Ala), 198-, 175- and 23-bp bands for heterozygotes (Ala/Val), and 175- and 23-bp bands for homozygotes (Val/Val). The genotype was determined on ethidium bromide-stained agarose gel (3%).

Statistical analysis

Data were analysed using the SAS statistical package, version 8.0. Crude odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The association between fertility status and the simultaneous presence of polymorphisms in the two genes was also analysed by calculating the OR of being infertile with any combination of *GSTM1* and *MTHFR* polymorphisms. The ORs were then adjusted for age, smoking status and body mass index using conditional logistic regression. The expected frequency of the complete genotype was calculated by using the genotype frequency observed in the control population; the Monte Carlo χ^2-test (Roff & Bentzen 1989) was used to see if the difference between the observed and the expected genotype frequency was significant.

Results

One year after enrolment, 46 subjects reported having had a child, while 59 were still childless. The association between gene polymorphisms and fertility status is reported in Table I. The OR for infertility with the *MTHFR* heterozygous variant is 2.5 (95% CI = 1.1–6.1), and for the homozygous variant is 3.7 (95% CI = 1.4–10.4), with a significant trend from the heterozygous to the homozygous variant. The *GSTM1* deletion is not associated with infertility (OR = 0.7; 95% CI = 0.3–1.6). The results do not change after adjustment of the ORs for possible confounding factors. However, when fertility status is then considered in relation to the composite *GSTM1–MTHFR* genotype (Table II), subjects who carry the hetero- or homozygous variant *MTHFR* genotype and a functional copy of *GSTM1* have a significantly higher risk of infertility than subjects who carry the wild-type genotype for both genes. Such risk decreases and becomes non-significant when the *GSTM1* deletion is also present. The complete

Table I. Association between *MTHFR* and *GSTM1* genotype and fertility status.

Genotype	Infertile, *n* (%)	Fertile, *n* (%)	OR (95% CI)	OR (95% CI)*
MTHFR:				
Ala/Ala	11 (18.7)	18 (39.1)	1.0 (reference)	1.0 (reference)
Ala/Val	32 (54.2)	21 (45.7)	2.5 (1.1–6.1)	2.6 (1.0–6.6)
Val/Val	16 (27.1)	7 (15.2)	3.7 (1.4–10.4)	4.0 (1.2–13.5)
GSTM1:				
Present	25 (43.9)	16 (35.6)		
Deleted	32 (56.1)	29 (64.4)	0.7 (0.3–1.6)	0.8 (0.6–1.3)

*OR are adjusted for age ($\leq$35 versus $>$35 years), smoking habits (never/ever) and body mass index ($\leq$25.6 versus $>$25.6 kg m^{-2}). Totals may vary due to missing values.

genotype distribution of the infertile men is significantly different from the expected distribution derived from the control population (Monte Carlo $\chi^2 = 0.05$). The analysis on sperm morphology, measured as described (Gaspari et al. 2003), shows no differences between fertile and infertile subjects (data not shown).

Discussion

This paper reports for the first time the effect of the interaction between *MTHFR* and *GSTM1* on fertility of a cohort of men. One of the study's strengths is the definition of fertility status, ascertained after 1-year follow-up using a personal interview. The results suggest that subjects with the homozygous variant genotype of the *MTHFR* gene have a significant increased risk of being infertile in comparison with subjects who carry the wild-type or heterozygous *MTHFR* genotypes, as suggested by some (Bezold et al. 2001, Singh et al. 2005) but not all previous studies (Ebisch et al. 2003, Stuppia et al. 2003).

The involvement of *MTHFR* in spermatogenesis is believed to be through the regulation of DNA methylation (Stern et al. 2000, Friso et al. 2002); subjects homozygous for the *MTHFR* C667T polymorphism have lower genomic DNA

Table II. Association between the combined *MTHFR GSTM1* genotype and fertility.

MTHFR	*GSTM1*	Infertile, *n* (%)	Fertile, *n* (%)	OR (95% CI)	OR (95% CI)*	Expected genotype, *n* (%)
Ala/Ala	present	2 (3.5)	8 (17.8)	1.0 (reference)	1.0 (reference)	45 (15.0)
Ala/Val	present	12 (21.0)	6 (13.3)	8.0 (1.4–45.8)	8.6 (1.3–55.9)	78 (26.1)
Val/Val	present	11 (19.3)	2 (4.4)	22.0 (3.8–127.9)	20.3 (2.0–202.8)	29 (9.7)
Ala/Ala	deleted	9 (15.8)	10 (22.2)	1.0 (reference)	1.0 (reference)	37 (12.4)
Ala/Val	deleted	18 (31.6)	14 (31.1)	1.4 (0.5–4.4)	1.5 (0.5–4.6)	75 (25.1)
Val/Val Monte Carlo	deleted	5 (8.8)	5 (11.1)	1.1 (0.2–5.1)	1.3 (0.3–6.4)	35 (11.7)
Total		57	45			299
χ^2 *p* value (versus expected)		0.05	0.20			

*OR are adjusted for age ($\leq$35 versus $>$35 years), smoking habits (never/ever) and body mass index ($\leq$25.6 versus $>$25.6 kg m^{-2}). Totals may vary due to missing values.

methylation compared with those with wild-type *MTHFR*, possibly because of the reduced availability of 5-methyltetrahydrofolate required for S-adenosylmethionine (SAM) biosynthesis (Stern et al. 2000). Low DNA methylation is associated with low fertility rates (Cisneros 2004). Another effect of the *MTHFR* C667T polymorphism is auto-oxidation, leading to the production of toxic reactive oxygen metabolites such as hydrogen peroxide (Starkebaum & Harlan 1986, Loscalzo 1996), which could result in homocysteine-mediated DNA damage (Huang et al. 2000), and could be overcome by antioxidant agents such as folates. Folate availability is low when *MTHFR* is polymorphic.

Despite the theoretical background in support of a major role of *MTHFR* in spermatogenesis, the results in the literature show a weak, inconsistent association between *MTHFR* polymorphism and fertility. In order to understand the inconsistent results, the presence of another gene involved in the fertility process, which could interact with *MTHFR*, was hypothesized. Because of the authors' previous work showing *GSTM1* deletion to be a risk factor for genotoxicity in sperm (Paracchini et al. 2005), and the high prevalence of *GSTM1* deletion in the healthy population, the *GSTM1* deletion was chosen as a candidate common polymorphism. Although the numbers are small, especially when stratifying the sample for both *GSTM1* and *MTHFR* polymorphisms, the present results indicate that the *MHTFR* homozygous variant is a strong risk factor for infertility in conjunction with a functional *GSTM1*, and that this risk disappears when *GSTM1* is deleted. The difference in the composite *GSTM1–MTHFR* genotype is also present when looking at a large population of 300 healthy subjects.

This result is opposite to the authors' original hypothesis, which was based on the known antioxidant properties of *GSTM1*, and on their previous results (Paracchini et al. 2005). The mechanism behind the unexpected effect of the *GSTM1* genotype on infertility associated with the *MTHFR* variant is unknown. A possible explanation of this apparently paradoxical result could lie in the known involvement of GSTs in the metabolic pathways of both methylation and transulfuration (Figure 1) (Pajares et al. 1992, Castagna et al. 1995). One could speculate that the deletion of *GSTM1* might result in higher circulating levels of gluthatione (Strange & Fryer 1999), which seems to have a regulatory effect on SAM synthetase (Castagna et al. 1995). An increase in glutathione levels has been associated with an increase in SAM synthetase activity (Pajares et al. 1992), which consequently could increase SAM, a methyl donor required in almost all cellular methylation reactions, and homocysteine levels. Since both SAM and homocysteine are part of the methionine cycle, as well as the activity of *MTHFR*, it is possible that one consequence of higher glutathione could be an increase in methylation in subjects carrying the *MTHFR* polymorphism, thus counteracting the deleterious effect of such polymorphism on fertility. This hypothesis needs to be tested further, since the small sample size produced risk-point estimates with wide confidence intervals.

In conclusion, the results suggest for the first time, but in a small sample of subjects, that metabolic genes may interact with *MTHFR* C677T on the pathway to methylation. This result may be relevant to pathologies other than fertility, such as cardiovascular diseases and cancer, and therefore deserves replication in a larger population.

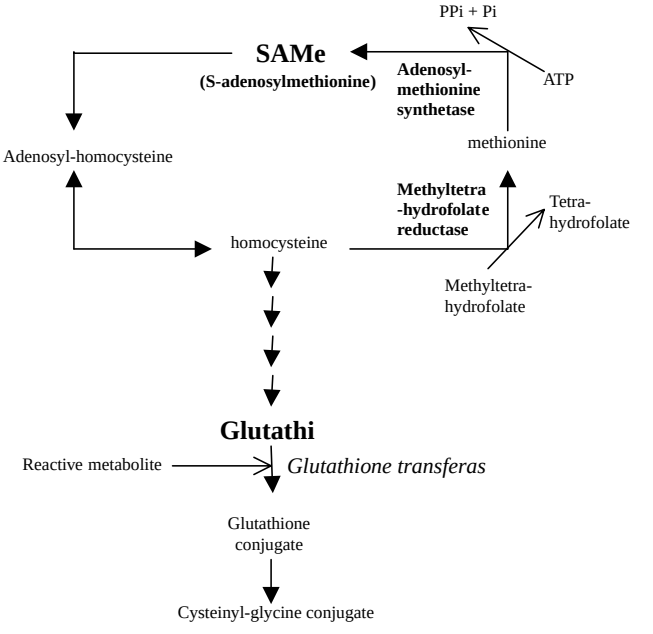

Figure 1. Schematic description of the relationship between the methylation and transulfuration metabolic pathways of homocysteine.

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