Polymorphisms in *MTHFR* and *MTRR* genes associated with blood plasma homocysteine concentration and sperm counts

Debbie Montjean, M.Sc.,[a,b,e,f,g] *Moncef Benkhalifa, Ph.D.,*[a,b] *Lionel Dessolle, M.D.,*[c] *Paul Cohen-Bacrie, D.Pharm.,*[b] *Stéphanie Belloc, D.Pharm.,*[b] *Jean-Pierre Siffroi, M.D., Ph.D.,*[d,e] *Célia Ravel, M.D., Ph.D.,*[c,f,g] *Anu Bashamboo, Ph.D.,*[f] *and Kenneth McElreavey, Ph.D.*[f]

[a] Advanced Technology Laboratory, La verrière; [b] Laboratoire d'Eylau, Unilabs, [c] AP-HP, Hôpital Tenon, [d] AP-HP, Hôpital Armand Trousseau, [e] ER9, Reproduction Humaine: Génétique et Thérapeutique, [f] Human Developmental Genetic Unit, Institut Pasteur, and [g] Université Pierre et Marie Curie, Paris, France

Objective: To investigate the relationship between *MTHFR* and *MTRR* genetic variants with respect to both blood plasma homocysteine concentration and sperm counts.
Design: Polymerase chain reaction followed by specific enzymatic digestion to determine the genotype of the individuals and blood plasma homocysteine quantification by high-performance liquid chromatography.
Setting: Research laboratory.
Patient(s): Two hundred sixty-eight men seeking infertility counseling and 254 partners of infertile women.
Intervention(s): We studied three *MTHFR* (c.1286A→C, c.665C→T and c.203G→A) and two *MTRR* (c.66A→G and c.524C→T) single-nucleotide polymorphisms and characterized sperm parameters in both oligozoospermic and normospermic men. A cohort of 522 men was examined for this study. A subgroup of 103 men was constituted for quantification of Hcy levels.
Main Outcome Measure(s): Semen samples were collected for determinations of sperm concentration, motility, and morphology according to World Health Organization guidelines as well as for DNA isolation. Blood samples of the corresponding individuals were obtained to quantify plasma homocysteine levels.
Result(s): We did not observe a relationship between homocysteinemia and sperm counts. The *MTHFR* c.665C→T variant is associated with mild hyperhomocysteinemia in blood plasma in the TT homozygous state.
Conclusion(s): No association was found between *MTHFR*/*MTRR* genetic variants and sperm counts. Although no association was observed with reduced sperm counts, the *MTHFR* 665TT genotype is associated with a significant increase in blood plasma homocysteine levels. (Fertil Steril® 2011;95:635–40. ©2011 by American Society for Reproductive Medicine.)

Key Words: *MTHFR*, *MTRR*, polymorphism, sperm counts, plasma homocysteine level

Folates are members of the B-class of vitamins, which are required for the synthesis of purines and pyrimidines. Folates in the form of 5,10-methylenetetrahydrofolate (5,10-CH2-THF) donate a methyl group to uracil, thereby converting it to thymidine, which is used for DNA synthesis and repair. The fidelity of DNA synthesis is dependent on the correct balance and availability of deoxynucleotides. The enzymes 5–10 methylenetetrahydrofolate reductase (*MTHFR*) and methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*) are two key enzymes of the homocysteine (Hcy) and folate metabolic pathway (1, 2). The *MTHFR* gene is located on chromosome 1 (1p36.3) (3), and its protein product converts 5,10-CH2-THF to 5-methyl-THF, which then donates a methyl group to homocysteine to produce methionine. Therefore, *MTHFR* maintains normal folates and circulating methionine and Hcy levels. It also plays a critical role in DNA synthesis and methylation reactions. Two polymorphisms in *MTHFR* have been shown to affect the functional activity of the enzyme. The *MTHFR* (c.665C→T; p.A222V) variant results in a thermolabile protein with enzymatic activity decreased by 35% in the heterozygote state and by 70% in the homozygote state (4, 5). This polymorphism has been reported to result in mild hyperhomocysteinemia particularly in patients with low folate intake (6). It has also been linked to an increased risk for certain cancers, thrombotic disease, and neural tube defects. However, individuals homozygous for the 665T polymorphism seem to be less at risk of developing other diseases, such as leukemia and colorectal cancer (7). The *MTHFR* (c.1286A→C; p.E429C) polymorphism is associated with a 30% decreased enzymatic activity but not with thermolability (5, 8). Both polymorphisms are thought to be associated with DNA hypomethylation (9). A further *MTHFR* polymorphism (c.203G→A; p.R68Q) has also been reported. The effect of this polymorphism on protein activity is unknown (10). The protein *MTRR* activates methionine synthase in a cobalamin-dependent manner. One polymorphism, *MTRR* (c.66A→G; p.I22M), replaces an isoleucine by a methionine residue and marginally reduces the protein's biological activity. The 66GG genotype is associated with a decrease in plasma Hcy levels. The association of this polymorphism with an increased risk of developing hyperhomocysteinemia is still a matter of debate (11). A second polymorphism in *MTRR* (c.524C→T; p.S175L) has

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Reprint requests: Debbie Montjean, M.Sc., Institut Pasteur, 25 rue du docteur Roux, 75015 Paris, France (E-mail: debbie.montjean@pasteur.fr).

been reported, and to date no disease association has been identified (10, 12).

Human spermatogenesis could be affected by changes in folate status by influencing DNA methylation patterns and thereby modifying gene expression or by inducing uracil misincorporation during DNA synthesis leading to errors in DNA repair, strand breakage, and possibly chromosomal anomalies (13). Although *MTHFR, MTRR*, and Hcy have been shown to play a role in spermatogenesis (14, 15), the relationship between the variants in these genes and spermatogenesis as well as with the plasma Hcy level remains unclear (10, 14, 16). We investigated whether there was an association between sperm parameters and the plasma Hcy level as well as with the known variants of *MTHFR* and *MTRR*.

We studied three *MTHFR* (c.1286A>C, c.665C→T and c.203G→A) and two *MTRR* (c.66A→G and c.524C→T) variants, quantified Hcy levels, and characterized semen parameters in a cohort of 522 men. A subgroup of 103 men was examined to determine whether there was a correlation between serum Hcy levels and genetic polymorphisms and/or sperm counts.

MATERIALS AND METHODS

Patients

The cohort consisted of 522 men with variable sperm counts. Individuals with known causes of infertility including genetic factors (chromosome anomalies, AZF microdeletions) (17), lifestyle factors (e.g., smoking, alcoholism, occupation), and clinical factors (varicoele, cryptorchidism) were excluded from the study. Written informed consent was obtained for genetic molecular investigations, and ethical approval was given by the local ethics committee. The mean age of the cohort was 37.4 (±6.7) years. Sperm counts and motility, vitality, and morphology analysis were performed according to the World Health Organization guidelines. The study population was divided into three groups: severe oligozoospermic (<5 million spermatozoa/mL; n = 89), mild oligozoospermic (5–20 million spermatozoa/mL; n = 179), and normospermic (n = 254) individuals. The cohort was constituted over a 3-year period in a single referral center. The group is of mixed ethnic origin as determined by self-assessment, and these origins reflect the ethnic diversity of the Paris region and includes individuals of North African, West African, Southeast Asian, and Caucasian descent.

Sperm DNA Isolation

Semen samples were collected by masturbation after 3–6 days of sexual abstinence. After liquefaction at 37°C, spermatozoa were selected and separated from somatic cells by centrifuging 1–2 mL of semen through a two-layer (45% and 90%) gradient of Percoll at 400 *g* for 20 minutes. The fraction in the 90% layer was washed in 2 mL of Tyrode (Eurobio, Courtaboeuf, France), and the tube was centrifuged at 400 *g* for 10 minutes. The pellet was collected with care to avoid any somatic cells contamination, and genomic DNA was isolated. The pellet was washed twice in 1 × phosphate-buffered saline (5,000 rpm for 5 minutes) and then resuspended in 20 μL (10 μL proteinase K, 20mg/mL +10 μL 1M DTT) for 1 hour at 56°C. Then 400 μL of digestion buffer (TrisCl, pH = 8, 10 mM; EDTA, 1 mM; SDS, 0.1%; and lN-Laururyl Sarcosine, 0.5%) was added for overnight incubation at 56°C. DNA samples were precipitated in 100% ethanol and washed 3 times in 70% ethanol at room temperature (8,000 rpm for 10 minutes). The pellets were air-dried and resuspended in 50 μL Tris-EDTA (10:1) buffer.

MTHFR and *MTRR* Polymorphisms Analysis

Three *MTHFR* (c.1286A→C, c.665C→T, and c.203G→A) and two *MTRR* (c.66A→G and c.524C→T) polymorphisms were analyzed by polymerase chain reaction (PCR) followed by specific enzymatic digestion. We used the following PCR mix: 200 mM of each dNTP, 1× Taq polymerase buffer, 2 μM of each primer sets, 1.5 mM $MgCl_2$, and 0.5 U of Biotaq DNA polymerase (Bioline, London, UK) in a 15-μL reaction volume. PCR and enzymatic digestion conditions were performed as described by Ravel et al. (10).

Plasma Hcy Level Quantification

Hcy concentrations were determined in blood plasma. Blood samples were collected in EDTA-containing vacutainer tubes. They were centrifuged (3,400 rpm for 15 minutes) to isolate the plasma, which was assayed by high-performance liquid chromatography in an ARCHITECT i2000 Immunoassay Analyzer (Abbott Laboratories, Wiesbaden, Germany). Normal homocysteinemia is between 4.44 and 13.5 μmol/L, and mild hyperhomocysteinemia is over 10 μmol/L.

The inter- and intra-assay variation for plasma Hcy quantification does not exceed 4%.

Statistical Analysis

Sperm parameters were summarized and compared by means according to Hcy levels and the genotype. Nonparametric tests (Mann-Whitney and

TABLE 1

A summary of the polymorphisms included in this study, together with their allelic frequencies in the three groups of individuals with differing sperm counts.

	MTHFR A222V			MTHFR E429A			MTHFR R68Q			MTRR I22M			MTRR S175L		
		Allele frequency			Allele frequency			Allele frequency			Allele frequency			Allele frequency	
	n	C	T	n	A	C	n	G	A	n	A	G	n	C	T
No. of severe oligozoospermic men screened	89	0.69	0.31	80	0.67	0.33	56	1	0	13	0.43	0.57	74	0.55	0.45
No. of mild oligozoospermic men screened	179	0.65	0.35	191	0.66	0.34	106	1	0	40	0.28	0.72	147	0.64	0.36
No. of normospermic men screened	254	0.65	0.35	176	0.7	0.3	99	1	0	77	0.36	0.64	168	0.59	0.41
Total no. of men screened for each polymorphism	522	—	—	447	—	—	261	—	—	130	—	—	389	—	—

Note: No significant differences in allelic frequencies were observed between the different groups.

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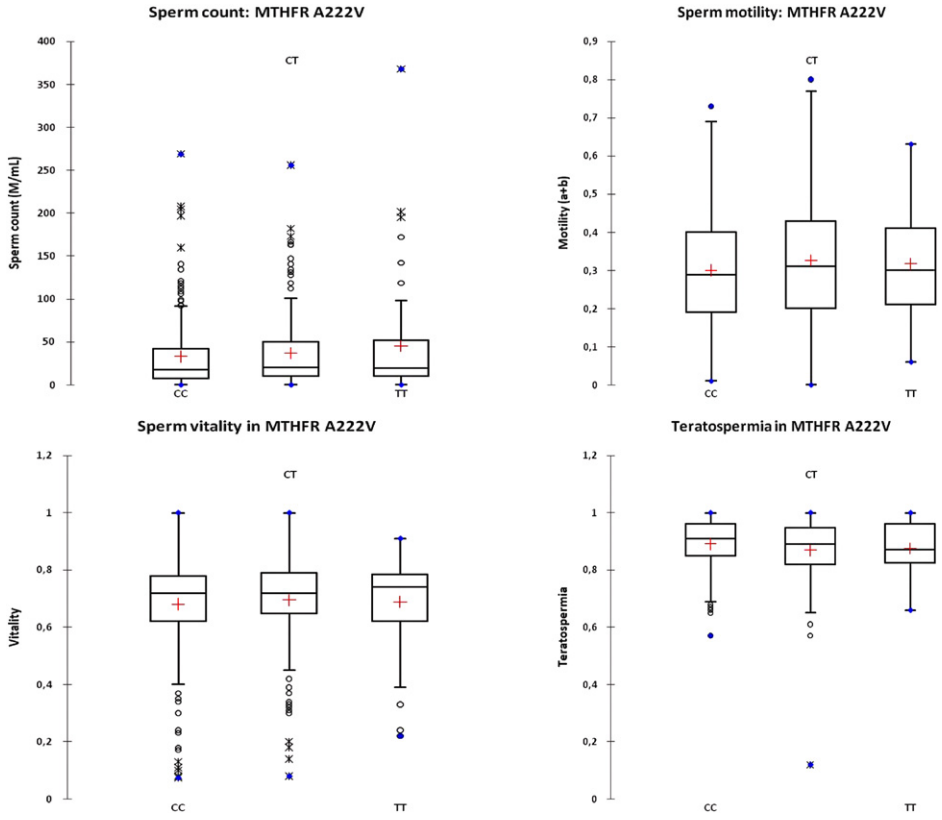
FIGURE 1

Box plot showing sperm parameters according to *MTHFR* 665 genotype. Similar results were found for other *MTHFR* and *MTRR* genotypes.



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Kruskall-Wallis) were used. Once the variables had been transformed and normalized, linear regression was used to model the distribution of sperm parameters as a function of Hcy concentration. All the analyses were performed using XLSTATstatistical software. $P \leq .05$ was considered statistically significant.

DNA Fragmentation Analysis

The detection of DNA strand breaks (DNA fragmentation) was performed by a flow-cytometry–based TUNEL labeling assay (Epics XL-MCL cytometer, Beckman Coulter).

RESULTS

MTHFR/MTRR Polymorphisms and Sperm Parameters

We investigated the association between three MTHFR (c.665C>T, c.1286A>C, c.203G>A), two MTRR (c.66A>G, c.524C>T) polymorphismsz and sperm parameters. Table 1 summarizes the sperm counts and allelic frequencies. Men carrying the *MTHFR* 665CC genotype show lower sperm counts than others (mean sperm counts: CC = 32.9 M/mL, CT = 36.9 M/mL, and TT = 45.194 M/mL), but this difference is not statistically significant: P=.959 (CT vs. TT) and P=.454 (CC vs. TT). Furthermore, we found no association between other sperm parameters (motility, vitality, fragmentation, and morphology) and genetic polymorphisms in *MTHFR* (Fig. 1) or *MTRR* (data not shown).

MTHFR/MTRR Polymorphisms, Hcy Levels, and Sperm Counts

The plasma Hcy levels were quantified to determine possible associations between variations in Hcy levels with either the *MTHFR/MTRR* genotype and/or semen parameters. A group of 103 men with a defined *MTHFR/MTRR* genotype and with blood serum available was studied. The clinicobiological parameters of these men are described in Table 2. Although five polymorphisms were studied, only the *MTHFR* 665TT genotype showed a correlation with serum Hcy levels. This variant was associated with increased Hcy levels as compared with the CC and CT genotypes (1.4-fold, P<.001, 665TT vs. 665CC, P<.001, odds ratio [OR] = 4.5, 95% confidence interval [CI], [1.53–13.27]; and 665TT vs. 665CT, OR = 4.38, P<.02, 95% CI, [1.32–14.52]; Fig. 2). However, the Hcy levels did not correlate with the sperm count and were indistinguishable in all the categories: severe oligozoospermia versus mild oligozoospermia, P=.3; mild oligozoospermia versus normospermia, P=.18; and severe oligozoospermia versus normospermia, P=.89. Although it is not statistically significant, mild hyperhomocysteinemia is more frequent in severe oligozoospermic men as compared with in normospermic men (OR = 2.55, χ^2 = 1.72, P<.2) and generally in all oligozoospermic men as compared with normospermic ones (OR = 1.78, χ^2 = 1.54, P<.2). The correlation analysis indicated no apparent relationship between sperm counts and blood plasma Hcy levels in our cohort (R^2 = 0.00, P=.829; Fig. 2).

TABLE 2

Clinicobiological parameters of men whose plasma Hcy level has been quantified.

	Severe oligozoospermic patients	Moderate oligozoospermic patients	Normospermic Men	*P* value: Severe oligo- vs. moderate oligospermic	*P* value: Moderate oligo- vs. normospermic	*P* value: Severe oligo- vs. normospermic
No. of men	16	34	53			
Mean sperm count ± SD	1.78 ± 1.54	12.74 ± 3.64	81.2 ± 68.8	**< 2.2e-16**	**2.1e-9**	**2.9e-11**
Mean Hcy level ± SD	8.3 ± 3.16	9.5 ± 4.8	8.2 ± 3.4	0.32	0.2	0.88
Mean motility level ± SD	13% ± 8.2%	26% ± 13.6%	44% ± 14.4%	**1.33e-4**	**1.82e-7**	**5.4e-14**
Mean vitality ± SD	60% ± 15.4%	61% ± 19%	75% ± 10.6%	0.86	**1.45e-3**	**6.02e-3**
Mean abnormal morphology ± SD	94% ± 5.5%	90% ± 6.6%	83% ± 9.4%	0.04	0.27	**7.5e-6**
Mean DNA fragmentation ± SD	19% ± 10.6%	25% ± 13.7%	25% ± 18.7%	0.5	0.9	0.4

Note: Sperm counts are expressed in million spermatozoa/mL and Hcy levels in μmol/L. Severe oligozoospermia is characterized by sperm counts under 5 million spermatozoa/mL; mild oligozoospermia, 5–20 million spermatozoa/mL; and normospermia, >20 million spermatozoa/mL. Bold text indicate a statistically significant difference.

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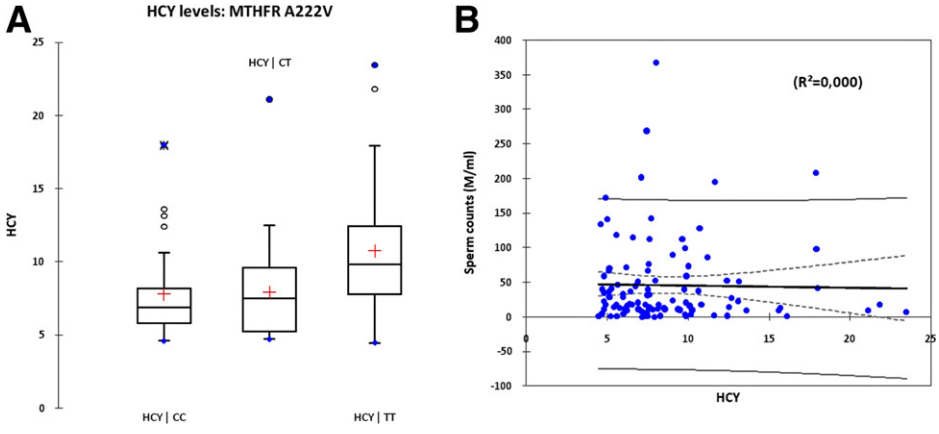
DISCUSSION

The relationship between folate metabolism and infertility is controversial. Mice that lack *MTHFR* exhibit hyperhomocysteinemia and show global DNA hypomethylation. Although these mice also show delayed maturation of the external genitalia, they do appear to be fertile (18). However, extensive backcrossing of these mice to a BALB/c background results in spermatogenic failure during early postnatal development, leading to male factor infertility (19). Fertility could be restored in some of these animals by supplementing the diet with betaine, which serves as an alternative methyl donor for the remethylation of homocysteine. These data indicate that at least in mice, folate metabolism plays a key role in the maintenance of normal spermatogenesis.

In the human, the relationship between folate metabolism and fertility is unclear. Several case-control studies suggest that *MTHFR* variants are associated with sperm quantity: Bezold et al. (16) noted that 20% of men with low sperm counts had a *MTHFR* 665TT genotype that was twice the frequency observed in the normospermic group. Other studies have found a similar correlation (20–23), whereas some failed to find any association between *MTHFR* variants and sperm numbers (10, 24). The distribution of the *MTHFR* c.665C→T variant shows considerable ethnic and geographic variation (25–27). Environment and nutrients are also thought to influence the impact of *MTHFR* polymorphisms (28). These factors and variation in the cohort size from one study to another may explain the conflicting findings.

FIGURE 2

Effect of *MTHFR* 665 polymorphism on plasma Hcy level and impact of Hcy level variation on sperm counts. (**A**) Box plot showing Hcy levels according to *MTHFR* 665 genotype. (**B**) Linear regression of sperm counts according to Hcy levels. *Gray lines* represent the 95% CIs of the sperm counts distribution and predictions. Hcy levels are expressed in μmol/L.



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In this study we set out to establish whether the *MTHFR/MTRR* variants were associated with serum Hcy levels and sperm counts in a given individual. The *MTHFR* variants 665CT and to a lesser extent 1298AC reduce the biological activity of the proteins by up to 80% for the *MTHFR* 665TT genotype and can lead to an increase in plasma Hcy levels (6, 8). The *MTRR* 66AA genotype has also been associated with increased homocysteinemia (11). Here we found that only the *MTHFR* 665TT variant is associated with mild hyperhomocysteinemia (10.742 μmol/L) compared with the 665CC and 665CT variants (665TT vs. 665CC, $P<.001$ OR = 4.5, 95% CI [1.53–13.27]; and 665TT vs. 665CT, OR = 4.38, $P<.02$, 95% CI [1.32–14.52]).

Hyperhomocysteinemia is associated with an increased risk of cardiovascular disease (29) and neural-tube defects (30), but its relationship with spermatogenesis remains unclear. A study of 65 men showed that administration of 15 mg of folate supplement per day for 3 months resulted in an increase in mean sperm count from 15.02 to 22.58 million spermatozoa/mL as well as improved sperm motility (17.7% to 27.8%) (15). A 74% increase in total sperm counts after 26 weeks of folate treatment (5 mg per day) has also been reported (31). These data suggest that folate/Hcy metabolism may play an important role in the maintenance of human male factor fertility. In our study we did not detect an association between the serum Hcy level and sperm concentration ($R^2 = 0.00$, $P=.829$). This is similar to published data where no correlation was found between blood plasma Hcy and sperm counts. However, a statistically significant positive correlation ($r = 0.21$, $P\leq.01$) has been observed between seminal plasma Hcy level and sperm numbers (32, 33). Therefore, further studies with larger cohorts are required to establish the correlation between Hcy levels in seminal fluid and serum, the *MTHR/MTRR* genotypes. The *MTHFR/MTRR* genotype may indicate individuals who would benefit from folate supplement to improve sperm quantity.

Recent studies have established a link between sperm DNA methylation defects and male factor infertility, where hypospermatogenesis is associated with aberrant methylation of imprinting genes (34, 35). A relationship between *MTRR* and *MTHFR* polymorphisms, serum Hcy levels, and DNA methylation status has also been suggested (9, 36). Indeed, in one study, *MTHFR* genotypes were associated with a DNA hypomethylation state either independently of folate availability (*MTHFR* c.1286A→C) or with low levels of folate dietary intake (9). Hyperhomocysteinemia could influence DNA methylation profiles during spermatogenesis, and it would be of interest to study DNA methylation profile in the germ line of patients with defined *MTHFR/MTRR* genotypes and homocysteine values.

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REFERENCES

1. Donnelly JG. Folic acid. Crit Rev Clin Lab Sci 2001;38:183–223.
2. Fowler B. Homocysteine: overview of biochemistry, molecular biology, and role in disease processes. Semin Vasc Med 2005;55:77–86.
3. Goyette P, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG, et al. Human methylenetetrahydrofolate reductase: isolation of cDNA mapping and mutation identification. Nat Genet 1994;7:45–51.
4. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3.
5. Van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neuraltube defects? Am J Hum Genet 1998; 62:1044–51.
6. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 1996;93:7–9.
7. Schwahn B, Rozen R. Polymorphisms in the methylenetetrahydrofolate reductase gene: clinical consequences. Am J Pharmacogen 2001;13:189–201.
8. Weisberg IS, Jacques PF, Selhub J, Bostom AG, Chen Z, Curtis Ellison R, et al. The 1298A→C polymorphism in methylenetetrahydrofolate reductase (*MTHFR*): in vitro expression and association with homocysteine. Atherosclerosis 2001;156:409–15.
9. Castro R, Rivera I, Ravasco P, Camilo ME, Jakobs C, Blom HJ, et al. 5,10-methylenetetrahydrofolate reductase (*MTHFR*) 677C→T and 1298A→C mutations are associated with DNA hypomethylation. J Med Genet 2004;41:454–8.
10. Ravel C, Chantot-Bastaraud S, Chalmey C, Barreiro L, Aknin-Seifer I, Pfeffer J, et al. Lack of association between genetic polymorphisms in enzymes associated with folate metabolism and unexplained reduced sperm counts. PLoS One 2009;4:e6540.
11. Gaughan DJ, Kluijtmans LA, Barbaux S, McMaster D, Young IS, Yarnell JW, et al. The methionine synthase reductase (*MTRR*) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. Atherosclerosis 2001;157:451–6.
12. O'Leary VB, Mills JL, Pangilinan F, Kirke PN, Cox C, Conley M, et al. Analysis of methionine synthase reductase polymorphisms for neural tube defects risk association. Mol Genet Metab 2005;85:220–7.
13. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13:163–74.
14. Ebisch IM, Van Heerde WL, Thomas CM, Van der Put N, Wong WY, Steegers-Theunissen RP. C677T methylenetetrahydrofolate reductase polymorphism interferes with the effects of folic acid and zinc sulfate on sperm concentration. Fertil Steril 2003;80:1190–4.
15. Bentivoglio G, Melica F, Cristoforoni P. Folinic acid in the treatment of human male infertility. Fertil Steril 1993;60:698–701.
16. Bezold G, Lange M, Peter RU. Homozygous methylenetetrahydrofolate reductase C677T mutation and male infertility. N Engl J Med 2001;344:1172–3.
17. McElreavey K, Krausz C, Bishop CE. The human Y chromosome and male infertility. Results Probl Cell Differ 2000;28:211–32.
18. Chen Z, Karaplis AC, Ackerman SL, Pogribny IP, Melnyk S, Lussier-Cacan S, et al. Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. Hum Mol Genet 2001;10:433–43.
19. Kelly TL, Neaga OR, Schwahn BC, Rozen R, Trasler JM. Infertility in 5,10-methylenetetrahydrofolate reductase (*MTHFR*)-deficient male mice is partially alleviated by lifetime dietary betaine supplementation. Biol Reprod 2005;72:667–77.
20. Park JH, Lee HC, Jeong YM, Chung TG, Kim HJ, Kim NK, et al. *MTHFR* C677T polymorphism associates with unexplained infertile male factors. J Assist Reprod Genet 2005;22:361–8.
21. Singh K, Singh SK, Sah R, Singh I, Raman R. Mutation C677T in the methylenetetrahydrofolate reductase gene is associated with male infertility in an Indian population. Int J Androl 2005;28:115–9.
22. Lee HC, Jeong YM, Lee SH, Cha KY, Song SH, Kim NK, et al. Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility. Hum Reprod 2006;21:3162–70.
23. Dhillon VS, Shahid M, Husain SA. Associations of *MTHFR* DNMT3b 4977 bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylation of GSTM1 in non-obstructive infertility in Indian men. Mol Hum Reprod 2007;13:213–22.
24. Stuppia L, Gatta V, Scarciolla O, Colosimo A, Guanciali-Franchi P, Calabrese G, et al. The methylenetethrahydrofolate reductase (*MTHFR*) C677T polymorphism and male infertility in Italy. J Endocrinol Invest 2003;26:620–2.
25. Stevenson RE, Schwartz CE, Du YZ, Adams MJ Jr. Differences in methylenetetrahydrofolate reductase genotype frequencies, between whites and blacks. Am J Hum Genet 1997;60:229–30.
26. Rozen R. Genetic predisposition to hyperhomocysteinemia: deficiency of methylenetetrahydrofolate reductase (*MTHFR*). Thromb Haemost 1997;78: 523–6.
27. Botto LD, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am J Epidemiol 2000;151:862–77.
28. Toffoli G, De Mattia E. Pharmacogenetic relevance of *MTHFR* polymorphisms. Pharmacogenomics 2008;9: 1195–206.

29. Refsum H, Ueland PM, Nygård O, Vollset SE. Homocysteine and cardiovascular disease. Annu Rev Med 1998;49:31–62.
30. Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, et al. Homocysteine metabolism in pregnancies complicated by neural-tube defects. Lancet 1995;345:149–51.
31. Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, Steegers-Theunissen RP. Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. Fertil Steril 2002;77:491–8.
32. Boxmeer JC, Smit M, Utomo E, Romijn JC, Eijkemans MJ, Lindemans J, et al. Low folate in seminal plasma is associated with increased sperm DNA damage. Fertil Steril 2009;92:548–56.
33. Wallock LM, Tamura T, Mayr CA, Johnston KE, Ames BN, Jacob RA. Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers. Fertil Steril 2001;75:252–9.
34. Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. Lancet 2004;363:1700–2.
35. Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, et al. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. Hum Mol Genet 2007;16:2542–51.
36. Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci U S A 2002;99:5606–11.