

# Methylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples of infertile couples correlates with recurrent spontaneous abortion

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**STUDY QUESTION:** Is the methylation status of the methylenetetrahydrofolate reductase (MTHFR) promoter region in semen samples associated with 'recurrent spontaneous abortion' (RSA)?

**SUMMARY ANSWER:** MTHFR promoter hypermethylation is more frequent in semen samples from RSA couples than in semen samples from infertile couples with no history of RSA (NRSA) and affects the whole sperm population significantly more often.

**WHAT IS KNOWN ALREADY:** Modifications to the MTHFR gene such as polymorphisms and promoter methylations are associated with male infertility.

**STUDY DESIGN, SIZE AND DURATION:** Retrospective cohort study of semen samples from 20 RSA couples, 147 NRSA couples and 20 fertile men between 2011 and 2012.

**MATERIALS, SETTING AND METHODS:** DNA from the semen samples of RSA, NRSA and fertile men were analyzed by methylation-specific PCR amplification using primers which anneal to the methylated or unmethylated cytosine-phosphodiester bond guanine (CpG) islands within the promoter region of MTHFR. The specificity of the PCR products was assessed by DNA sequencing.

**MAIN RESULTS AND THE ROLE OF CHANCE:** The methylated MTHFR epigenotype (including samples where it co-existed with unmethylated MTHFR epigenotypes) was detected in 75% of RSA men, 54% of NRSA men and 15% of fertile men. MTHFR methylation was observed in the whole sperm population in semen samples from 55% of RSA men compared with 8% in NRSA men ( $P < 0.05$ ) and 0% in fertile men ( $P < 0.05$ ). DNA sequencing analysis was fully concordant with the PCR results and revealed that when MTHFR methylation occurred, CpG islands within the promoter region were 100% methylated (hypermethylation of MTHFR promoter).

**LIMITATIONS, REASONS FOR CAUTION:** The relatively small sample size of RSA infertile couples.

**WIDER IMPLICATIONS OF THE FINDINGS:** The hypermethylation of the MTHFR gene promoter should be taken into consideration as a novel putative risk factor in RSA etiology.

**STUDY FUNDING/COMPETING INTEREST(S):** Our institution has received an FAR research grant from the University of Ferrara, Ferrara, Italy. No competing interests declared.

**Key words:** MTHFR / infertile couples / RSA infertility / methylation / epigenetics

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## Introduction

'Recurrent spontaneous abortion' (RSA) is defined as the miscarriage of two or more consecutive pregnancies before 20 weeks of gestation and affects ~1% of infertile couples (Rai and Regan, 2006). In the female, recurrent abortions are associated with genetic, anatomical and endocrine abnormalities, psychological, infectious and immunological causes, thrombophilic disorders and antiphospholipid syndrome (Rai and Regan, 2006). However, half of cases remain unexplained (Tang and Quenby, 2010). Currently, there is only limited data on a possible male cause. Some studies have indicated that abnormal organization or fragmentation of sperm DNA may negatively affect embryo development and possibly increase miscarriage (Evenson et al., 1999; Agarwal and Said, 2003; Brahem et al., 2011; Absalan et al., 2012). Additional evidence suggests that sperm chromosomal aneuploidies and apoptosis, and genetic and epigenetic anomalies could be correlated to spontaneous pregnancy loss (Carrell et al., 2003; Bernardini et al., 2004; Puscheck and Jeyendran, 2007; Ostojic et al., 2008).

DNA methylation is the main epigenetic phenomenon which regulates the correct development of sperm. Indeed, it is needed in order to enable the correct compaction of chromatin in the sperm head and to permanently silence the promoters of genes involved in genetic imprinting (Rajender et al., 2011).

Methylenetetrahydrofolate reductase (MTHFR) is one of the key regulatory enzymes of methylation. It maintains the bioavailability of endogenous methyl donor groups required for a variety of substrates, such as DNA, RNA, proteins and lipids. A balance between exogenous, by means of diet and endogenous methyl group donors enables molecule methylation to proceed efficiently in different cells, including sperm cells (Wong et al., 2000; de Vogel et al., 2011). MTHFR gene polymorphisms are known causes of reduced MTHFR enzyme activity resulting in low availability of methionine and global DNA hypomethylation (Chen et al., 2001; Terruzzi et al., 2011). In human sperm, MTHFR polymorphic variants correlate with reduced sperm counts, leading to male infertility in some populations (Gava et al., 2011; Gupta et al., 2011; Kumar et al., 2011), and recurrent spontaneous abortion (RSA) (Govindaiah et al., 2009). In addition, MTHFR promoter hypermethylation is associated with impaired spermatogenesis in infertile men and men with idiopathic infertility (Khazamipour et al., 2009; Wu et al., 2010).

Altogether, these data underline the physiological role of MTHFR in spermatogenesis and suggest that its dysregulation can lead to male infertility.

The objective of the present study was to assess the methylation status of the MTHFR gene promoter in sperm samples from male partners in RSA couples, that is, RSA men. These RSA men' results were compared with those obtained from semen samples from infertile couples with no history of RSA, that is, NRSA men and fertile controls.

## Materials and Methods

### Patients recruitment

Infertile couples were recruited at the Infertility Center of the Obstetric and Gynecological Clinic, University of Ferrara, Italy.

Twenty infertile couples (men aged  $35 \pm 5.4$  years) who presented with RSA (two or more embryo losses before the 12th week of gestation) were enrolled in this study.

A complete evaluation of the RSA females including sonography, physical examination, cytogenetic, immunological, infection and reproductive hormonal assays was carried out. Clinical examination of the RSA men included investigations to look for chromosomal, anatomic, hormonal, immunological and infective pathologies. Hematological and biochemical assays including a hemogram with erythrocyte sedimentation rate and urine analysis were also performed.

RSA couples whose results fell within the normal range of the aforementioned assays were considered as idiopathic RSA patients.

One hundred and forty-seven (men aged  $35.3 \pm 4.4$  years) consecutive infertile couples (according to the WHO infertility definition, i.e. lack of pregnancy after 12 months or more of regular, unprotected sexual intercourse in reproductive age) undergoing evaluation for infertility at the Infertility Center with NRSA, and 20 fertile couples with proven fertility (one child within the last 12 months) (WHO, 2010) were also enrolled in this study. NRSA men were free from known causes of infertility including genetic factors (chromosomal abnormalities and microdeletions in the azoospermia factors region of the Y chromosome), lifestyle factors (e.g. smoking, alcoholism, occupation) and clinical factors (varicocele, cryptorchidism). Written informed consent was obtained from all study subjects.

### Semen analysis

Semen samples from RSA men, NRSA men and fertile controls were evaluated by standard semen analysis using the (WHO, 2010) criteria. Samples were collected by masturbation and ejaculation into sterile glass cups after 3–5 days of abstinence. After sperm fluid liquefaction at 37°C for 30 min, sperm concentration and motility were evaluated in the Meckler chamber. Sperm morphology and leukocyte, white blood cell, round cell and epithelial cell counts were assessed with the use of pre-stained slides (TestSimples, Waldeck, Munster, Germany). Semen samples were classified normal when concentration, motility and morphology were  $\geq 15 \times 10^6$  sperm/ml,  $\geq 32\%$  (sperm progressive motility) and  $\geq 4\%$  normal, respectively.

### Spermatozoa purification

Following complete liquefaction semen samples were centrifuged on a density gradient consisting of two overlaid layers, 80 and 40% (down to up), of PureSperm100/PureSperm Buffer (Celbio, Milan, Italy) to separate spermatozoa from seminal plasma and cellular contaminants (leukocytes, round cells and miscellaneous debris) as previously reported (Houshdaran et al., 2007). Spermatozoa were washed once in PureSperm Wash and then resuspended in 0.3 ml of PureSperm Wash. A microscopic examination of the purified sperm was performed to control the quality of cell preparations. Following spermatozoa purification, samples were stored at  $-20^\circ\text{C}$  until processing for molecular analysis.

### DNA isolation

DNA was isolated from purified spermatozoa, as previously described (Martini et al., 1996). Briefly, spermatozoa digestion was in 50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl pH 8.0, (Sigma-Aldrich, Milan, Italy) containing  $1 \mu\text{g}/\mu\text{l}$  proteinase K and 0.5% SDS at 55°C for 3–4 h. DNA was purified with a phenol-chloroform-isoamyl alcohol mixture (25:24:1) and then precipitated with 1/10 volume of 3 M sodium acetate and two volumes of cold ethanol at  $-20^\circ\text{C}$  overnight.

## Sodium bisulfite treatment of spermatozoa DNA

DNA from purified spermatozoa was treated with sodium bisulfite, which converts unmethylated cytosines of DNA to uracil, while leaving the 5-methylcytosines unchanged, using the Epitect Bisulfite kit (Qiagen, Milan, Italy) as previously described (Wu *et al.*, 2010). Samples were then purified using DNA purification columns (Epitect Bisulfite kit, Qiagen) and subsequently PCR amplified.

## Methylation-specific PCR

One hundred nanograms of DNA were amplified by PCR using specific primers designed to distinguish methylated and unmethylated DNA within the promoter region of MTHFR after bisulphate treatment, as previously reported (Wu *et al.*, 2010). PCR was performed in a total volume of 25  $\mu$ l. PCR for both primer sets contained 1  $\times$  PCR buffer, 2 mM MgCl<sub>2</sub>, 200 mM dNTPs, 0.5  $\mu$ M of each primer and 1.25 U of Taq DNA Polymerase (Roche, Monza, Italy). The PCR conditions were as follows: an initial denaturing step at 95°C for 5 min followed by 40 cycles at 95°C for 40 s, 64°C for MTHFR methylated primers (62°C for MTHFR unmethylated primers) for 40 s and 72°C for 1 min and a final extension at 72°C for 5 min. PCR results were analyzed using 2% agarose gel electrophoresis, stained with ethidium bromide. One control sperm DNA sample was methylated using an Sssl methyltransferase (New England Biolabs, Milan, Italy) according to the manufacturer's protocol and used as a positive control in the PCRs.

## DNA sequencing of PCR products

Three PCR products from each MTHFR epigenotype pattern detected in the sperm samples, i.e. only methylated, only unmethylated and co-existing methylated and unmethylated epigenotypes in the same semen sample ( $n = 9$ ), were purified and cloned into the pCR2.1 vector using the TA Cloning kit (Invitrogen, Milan, Italy) as previously reported

(Wu *et al.*, 2010). Ten clones from each plate were selected (total clones,  $n = 90$ ) and sequenced with an automated ABI Prism 3730  $\times$  1 Genetic Analyser (Applied Biosystems, Monza, Italy).

## Statistical analysis

Analysis of variance (ANOVA) was used to explore the relationships between fertility status and potentially important covariates, such as age, BMI, parity, abstinence time, testicular volume and ejaculate volume. Differences in sperm concentration, motility and morphology between infertile groups and fertile controls, between RSA groups and NRSA groups, between normal recurrent spontaneous abortion (nRSA) and abnormal recurrent spontaneous abortion (abRSA) groups and between normal non-recurrent spontaneous abortion (nNRSA) and abnormal non-recurrent spontaneous abortion (abNRSA) groups were tested using Mann–Whitney test.

The observed MTHFR epigenotype frequencies (i.e. methylated, unmethylated or methylated/unmethylated) were compared between groups using the chi-square test. All statistical analyses were carried out using Graph Pad Prism version 5.0 for Windows (Graph Pad, La Jolla, CA, USA).  $P$ -values  $< 0.05$  were considered statistically significant.

## Results

### Semen classification

Standard semen analysis was conducted on samples collected from 20 RSA men, 147 NRSA men and 20 fertile men (Table 1). In RSA men, 14/20 (70%) of sperm fluids had normal semen parameters, hereafter referred to as nRSA men, and 6/20 (30%) had abnormal semen parameters, hereafter referred to as abRSA men, i.e. oligozoospermic ( $n = 2$ ), astenozoospermic ( $n = 1$ ), teratozoospermic ( $n = 2$ ) and astenoteratozoospermic ( $n = 1$ ) (Table 1).

**Table 1** Results of semen analysis from men from infertile couples with a history of recurrent spontaneous abortion (RSA males), men from infertile couples with NRSA males and fertile controls.

Parameters	Fertile controls ( $n = 20$ )	RSA males ( $n = 20$ )		NRSA males ( $n = 147$ )	
		Normal ( $n = 14$ )	Abnormal ( $n = 6$ )	Normal ( $n = 108$ )	Abnormal ( $n = 39$ )
Age (years) <sup>a</sup>	35 $\pm$ 4.9	35.8 $\pm$ 4.9	33 $\pm$ 6.5	35.3 $\pm$ 4.4	35 $\pm$ 4.4
BMI (kg/m <sup>2</sup> ) <sup>a</sup>	22.8 $\pm$ 1.7	22.1 $\pm$ 1.9	22.2 $\pm$ 2.2	21.9 $\pm$ 2.8	21.1 $\pm$ 2.2
Parity (number) <sup>a</sup>	1.7 $\pm$ 0.7	0*	0*	0.4 $\pm$ 0.6*	0.2 $\pm$ 0.4*
Abstinence time (days) <sup>a</sup>	4.1 $\pm$ 0.8	3.8 $\pm$ 0.8	4 $\pm$ 0.6	4.1 $\pm$ 0.7	4.1 $\pm$ 0.8
Testicular volume (ml) <sup>a</sup>	22.8 $\pm$ 4	21.4 $\pm$ 3.6	18.9 $\pm$ 1.7	22.5 $\pm$ 3.2	20 $\pm$ 3.1
Ejaculate volume (ml) <sup>a</sup>	4 $\pm$ 1	3.5 $\pm$ 1	2.8 $\pm$ 1.1	3.8 $\pm$ 1.1	2.6 $\pm$ 1.3*
Concentration (10 <sup>6</sup> /ml) <sup>b</sup>	110 (96.2–130)	93 (82.2–106.2)*,Δ,§	38.5 (6.5–72.7)*,†	99.5 (90–110)*,‡	42 (37–50)*
Progressive motility (%) <sup>b</sup>	52 (51–54.5)	50.5 (44.5–54.5)*,Δ,°	37.5 (33–45.5)*,†	63 (51–70.75)*,‡	36 (33–43)*
Morphology (%) <sup>b</sup>	23.5 (20–30.7)	24 (16.7–29.2) <sup>Δ</sup>	13 (7–22.5)*,†	25 (22–28) <sup>‡</sup>	19 (13–25)*

RSA and NRSA males are divided in those with normal and abnormal semen parameters.

<sup>a</sup>Mean  $\pm$  SD.

<sup>b</sup>Values are given as median and interquartile range (IQR).

\* $P < 0.05$  versus fertile controls.

<sup>Δ</sup> $P < 0.05$  versus abRSA.

<sup>†</sup> $P < 0.05$  versus nNRSA.

<sup>‡</sup> $P < 0.005$  versus abNRSA.

<sup>§</sup> $P < 0.0001$  versus abNRSA.

<sup>°</sup> $P < 0.05$  versus abRSA.

In NRSA men, 108/147 (73%) of ejaculates had normal semen parameters, hereafter referred to as nNRSA men and 39/147 (27%) of samples had one or more abnormal semen parameters, hereafter referred to as abNRSA men, i.e. oligozoospermic ( $n = 5$ ), astenozoospermic ( $n = 13$ ), teratozoospermic ( $n = 2$ ), oligoastenozoospermic ( $n = 6$ ), oligoteratozoospermic ( $n = 2$ ), astenoteratozoospermic ( $n = 4$ ) and oligoastenoteratozoospermic ( $n = 7$ ) (Table 1).

In fertile men, 20/20 (100%) of sperm fluids had normal semen parameters (Table 1).

## DNA methylation at the MTHFR promoter region

PCR analyses of sperm DNA samples showed three different MTHFR epigenotype patterns: methylated (M), unmethylated (U) and M and U co-existing in the same semen sample (M/U) (Supplementary data, Fig. S1). Overall, 75% (15/20) of semen samples from RSA men showed M and M/U epigenotypes compared with 54% (79/147) from NRSA men ( $P = 0.0166$ ) and 15% (3/20) from fertile controls ( $P < 0.0001$ ) (Fig. 1). Specifically, the M epigenotype was detected in 55% (11/20) of semen samples from RSA men compared with 8% (12/147) from NRSA men ( $P < 0.0001$ ) and 0% (0/20) from fertile controls ( $P < 0.0001$ ) (Fig. 1).

For a more detailed analysis, the MTHFR epigenotype frequencies were evaluated in nRSA and abRSA men and compared with those obtained from nNRSA, abNRSA men and fertile controls (Fig. 2). M and M/U epigenotypes were detected in 86% (12/14) of semen samples from nRSA men, compared with 58% (63/108) from nNRSA men ( $P = 0.0238$ ) and 15% (3/20) from fertile controls ( $P < 0.0001$ ) and in 50% (3/6) of semen samples from abRSA men compared with 41% (16/79) from abNRSA men ( $P = 0.0459$ ). The M epigenotype was detected in 71% (10/14) of semen samples from nRSA

men compared with 6% (6/108) from nNRSA men ( $P < 0.0001$ ) and 0/20 from fertile controls ( $P < 0.0001$ ) and in 1/3 (33%) from abRSA men compared with 15% (6/39) from abNRSA men (Fig. 2).

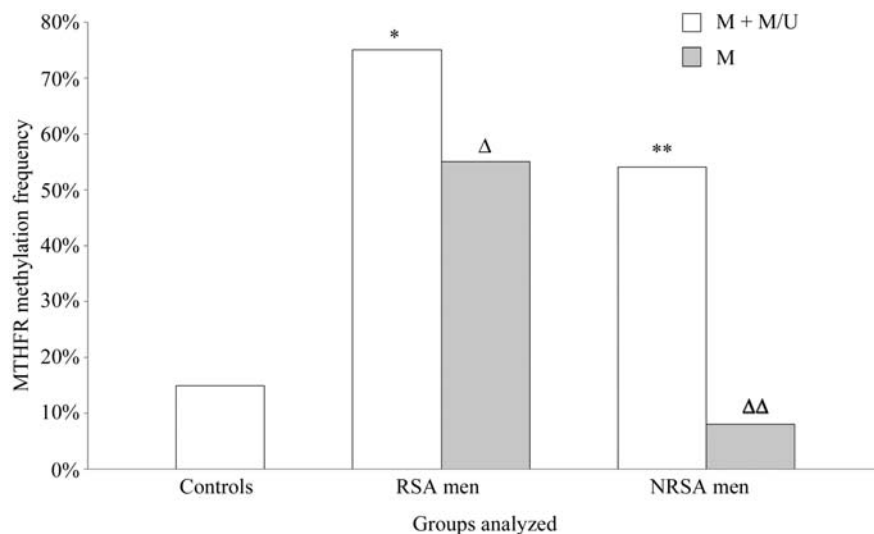
The statistical analysis of M and M/U MTHFR epigenotype frequencies and M MTHFR epigenotype frequencies between nRSA and abRSA and between nNRSA and abNRSA is reported in Figure 2.

## DNA sequencing analysis of MSP products

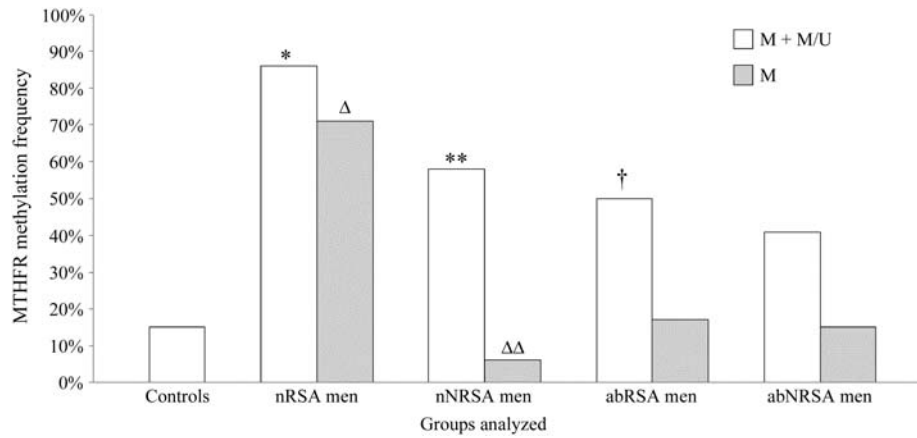
A total of 90 clones derived from 3 U, 3 U/M and 3 M PCR products (10 from each) from RSA and NRSA men and controls were randomly selected for DNA sequencing. Sequenced samples were 100% concordant with PCR results (Fig. 3). Moreover, DNA sequencing analysis showed that the 18 cytosine-phosphodiester bond guanine (CpG) islands within the MTHFR promoter region were fully methylated in almost all the methylated PCR products analyzed (hypermethylation of MTHFR gene promoter) (Fig. 3).

## Discussion

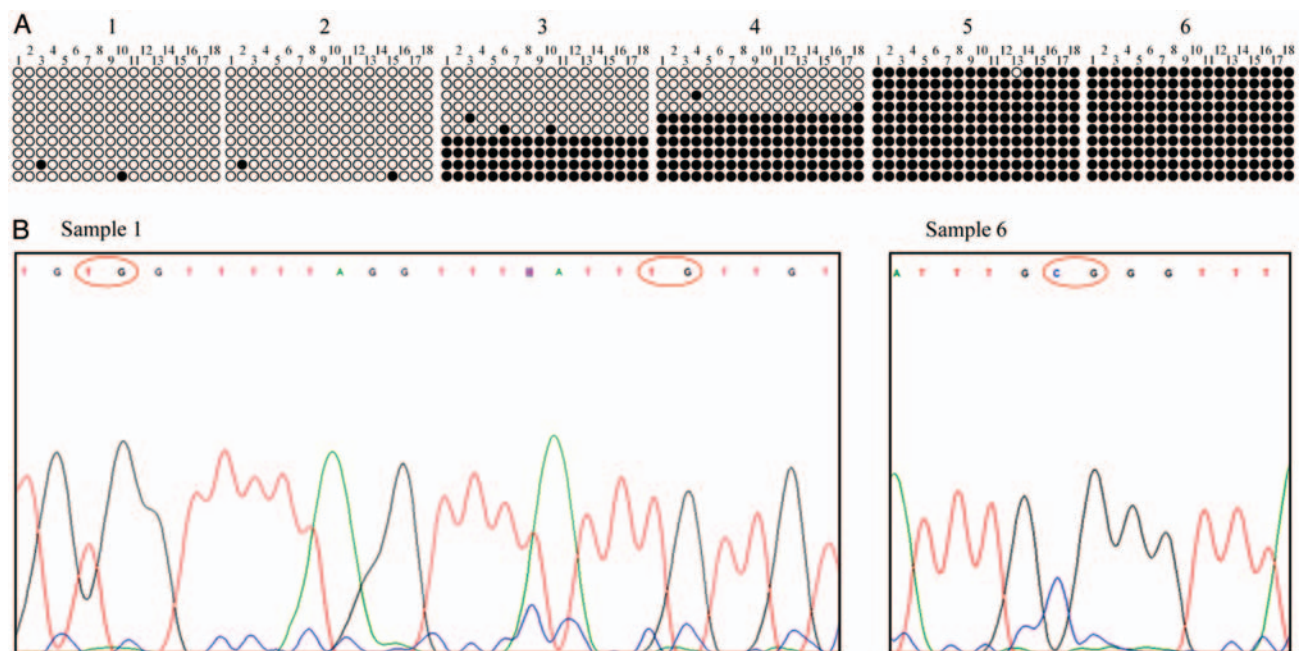
The above results indicate that MTHFR promoter hypermethylation is a common epigenetic event affecting sperm from infertile couples. These data confirm and extend a previous recent study which demonstrated that MTHFR promoter hypermethylation frequently occurred in sperm DNA from infertile couples, i.e. idiopathic infertile couples (Wu et al., 2010). Furthermore, since hypermethylation of the MTHFR gene promoter in sperm was significantly greater in a well-characterized group of RSA infertile couples than in NRSA men, these data show, for the first time, that MTHFR hypermethylation is associated with RSA and could be implicated in its cause. Moreover, a more detailed analysis of MTHFR epigenotype frequency detected in the semen samples revealed that the methylated MTHFR epigenotype



**Figure 1** Frequency of MTHFR methylation in semen samples from fertile controls, men from infertile couples with a history of RSA men and men from infertile couples with no history of recurrent spontaneous abortion (NRSA men). White bars indicate the frequency of methylated (M) plus methylated/unmethylated (M/U) MTHFR epigenotypes; gray bars indicate the frequency of the methylated (M) MTHFR epigenotype. \*M + M/U frequency significantly different from controls and NRSA men, \*\*M + M/U frequency significantly different from controls.  $P < 0.05$  chi-square test. ΔM frequency significantly different from controls and NRSA men, ΔΔ M frequency significantly different from controls.  $P < 0.05$  chi-square test.



**Figure 2** Frequency of MTHFR methylation in normal semen samples from controls, men from infertile couples with a history of recurrent spontaneous abortion (nRSA) and men from infertile couples with no history of recurrent spontaneous abortion (nNRSA) and abnormal semen samples from the two infertile groups (abRSA and abNRSA men). White bars indicate the frequency of methylated (M) plus methylated/unmethylated (M/U) MTHFR epigenotypes; gray bars indicate the frequency of the methylated (M) MTHFR epigenotype. \*M + M/U frequency significantly different from controls, nNRSA men and abNRSA men.  $P < 0.05$  chi-square test.  $\Delta$ M frequency significantly different from nNRSA men and abNRSA men,  $\Delta\Delta$ M frequency significantly different from abRSA men.  $P < 0.05$  chi-square test.  $\dagger$ M + M/U frequency significantly different from abNRSA men.  $P < 0.05$  chi-square test.



**Figure 3** Representative results of bisulfite-PCR analysis subjected to DNA sequencing of the MTHFR promoter region. **(A)** Filled-in and blank circles represent methylated and unmethylated CpG islands, respectively. The 18 CpG islands within the MTHFR promoter region are numbered on the upper side of the circles. Samples 1 (fertile men derived) and 2 (NRSA men derived) are from unmethylated MTHFR epigenotypes. Samples 3 (NRSA men derived) and 4 (RSA men derived) are from unmethylated/methylated MTHFR epigenotypes. Samples 5 and 6 (both RSA men derived) are from methylated MTHFR epigenotypes. **(B)** DNA sequencing of the MTHFR promoter region. Sample 1: two unmethylated CpG islands within the gene promoter are indicated (red circle). Sample 6: one methylated CpG island within the gene promoter is indicated (red circle).

was highly prevalent in 55% of RSA men compared with 15% of NRSA men. This result indicates that when MTHFR hypermethylation occurs, it frequently affects the whole sperm population of RSA men.

Whether hypermethylation of MTHFR arises from testicular adult germinal stem cell pools, or from defects occurring during progenitor diploid germ cell expansion or haploid germ cell differentiation remains to be determined. However, the recent detection of MTHFR gene promoter hypermethylation in the testis of azoospermic infertile men suggests that the epigenetic modification of this gene in adult germinal stem cells can occur (Khazamipour et al., 2009). In this context, MTHFR hypermethylation epigenotype patterns (M and M/U) detected in sperm samples may reflect the MTHFR hypermethylation status of adult germinal stem cells throughout the seminiferous tubules. Therefore, our study indicates that MTHFR gene promoter hypermethylation is a widespread epigenetic modification which probably occurs in adult germinal stem cells in RSA men.

It is known that the hypermethylation of gene promoters acts to repress the transcription process, thus silencing gene expression (Rajender et al., 2011). The lack of MTHFR enzyme hampers the bioavailability of methyl group donors required for the methylation of different substrates including DNA (Chen et al., 2001; Terruzzi et al., 2011). This, in turn, may affect the two essential roles of DNA methylation in spermatogenic cells, the global genome methylation process and the genomic imprinting of paternal genes. In normospermic men, decreased global sperm methylation has been related to poor pregnancy rates and outcomes during IVF (Benchaib et al., 2003). DNA methylation is also essential in genomic imprinting regulation, which ensures the silencing of paternal genes at the level of imprinted loci (Piedrahita, 2011). Correctly imprinted maternal and paternal genes are needed to regulate the major functions at the fetomaternal interface, such as nutrient transport, trophoblast proliferation, invasion and angiogenesis (Piedrahita, 2011). In rodents and humans, impaired methylation imprints generate small placentae with abnormalities in proliferation, apoptosis and differentiation of the trophoblast (Georgiades et al., 2001; Serman et al., 2007; Diplas et al., 2009; Lim and Ferguson-Smith, 2010).

Taken together, these data underscore the loss of MTHFR activity as a potential risk factor for the abnormal methylation of sperm DNA. Accordingly, the full methylation of CpG islands (hypermethylation), within the MTHFR promoter region as detected in this study, is a predictive mark of MTHFR gene silencing. Thus, if MTHFR hypermethylation occurs throughout the whole sperm population, as is frequently detected in RSA men, all sperm DNA could be globally hypomethylated and, in turn, account for recurrent pregnancy failures. Such conditions may be sufficient to explain recurrent pregnancy loss in some idiopathic infertile couples as well as in the four RSA idiopathic couples included in this study whose causes remain unexplained even following extensive evaluation. Nevertheless, the synergistic effect of the male risk factor, i.e. sperm DNA hypomethylation, which was detected in 11 RSA men in this study combined with female risk factors, i.e. anatomical, endocrine and immunological causes affecting corresponding RSA women, cannot be discarded.

Diversely from what was previously observed by Wu et al. (2010), no association between MTHFR promoter hypermethylation and abnormal semen characteristics was found in our study, either in RSA or NRSA men. On the contrary, a higher prevalence of MTHFR hypermethylation was detected in semen samples from nRSA (86%) when

compared with those from abRSA men (50%), as well as from nNRSA (58%) when compared with those from abNRSA men (41%). The reason for this difference could be the small number of abnormal semen samples as against the normal semen samples used in this study. Nevertheless, this result seems to suggest that MTHFR hypermethylation is an independent factor from conventional semen parameters. Therefore, methylation analysis of MTHFR should be taken into consideration as a useful parameter in assessing sperm ability to induce pregnancy, especially when normal semen characteristics (concentration, motility and morphology) are shown.

In conclusion, we have demonstrated not only that hypermethylation of the MTHFR gene promoter frequently occurs in sperm from infertile couples but that it is more prevalent in RSA men than in NRSA men and frequently affects the whole of the sperm population. These data indicate a new male factor associated with RSA infertility and may contribute to elucidating its cause. Therefore, hypermethylation of the MTHFR gene promoter should be taken into consideration as a novel putative risk factor in RSA etiology. Despite awareness of the limitations of this study due to the small sample size, the results encourage further larger studies in this promising research field. Finally, our findings may open new strategies for exploring ways of restoring MTHFR inactivity in RSA men by modulating DNA methylation by means of exogenous methyl donors, for instance by diet or drugs.

## Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

## Authors' roles

J.C.R. and S.B. equally contributed to the MTHFR experiments on DNA from sperm samples; E.B. and M.D.D. carried out the analysis and processing of semen samples; M.D.M. provided the statistical analysis; R.S. carried out the DNA sequencing analysis; A.P. and R.M. enrolled the patients and provided the clinical information on infertile couples; M.T. critically revised the manuscript.

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## Conflict of interest

None declared.

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