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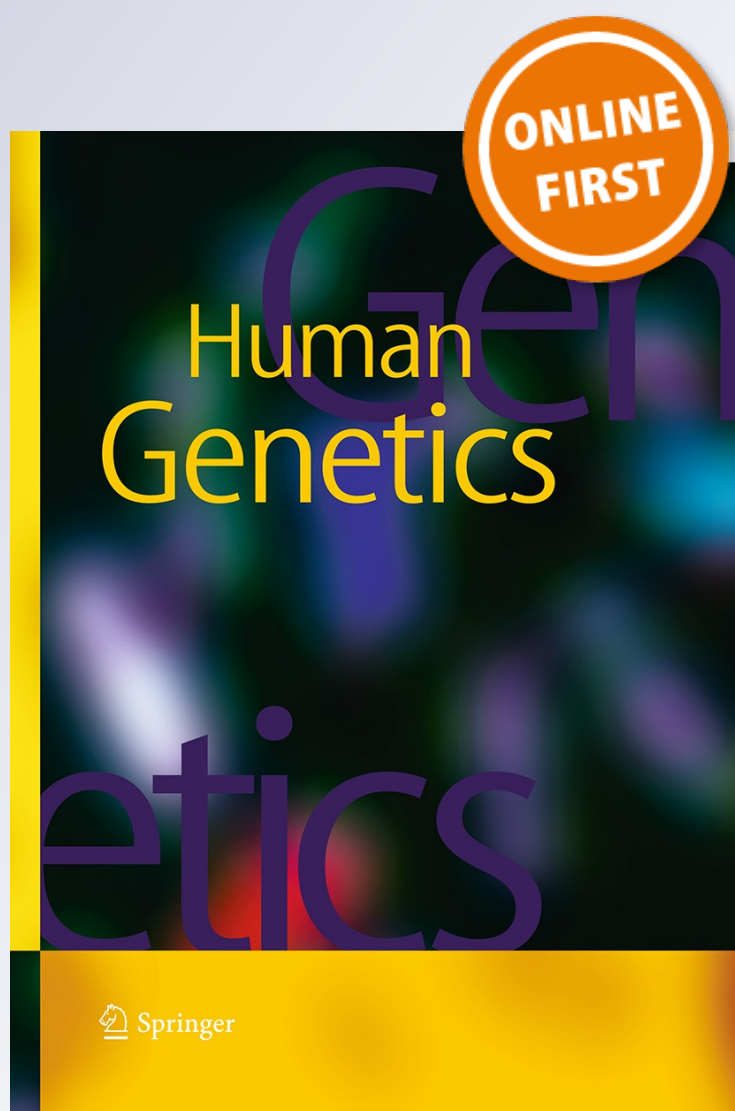
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Polymorphisms in the *MTHFR* gene influence embryo viability and the incidence of aneuploidy

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Abstract *MTHFR* is an important enzyme in the metabolism of folic acid and is crucial for reproductive function. Variation in the sequence of *MTHFR* has been implicated in subfertility, but definitive data are lacking. In the present study, a detailed analysis of two common *MTHFR* polymorphisms (c.677C>T and c.1298A>C) was performed. Additionally, for the first time, the frequencies of different *MTHFR* alleles were assessed in preimplantation embryos. Several striking discoveries were made. Firstly, results demonstrated that maternal *MTHFR* c.1298A>C genotype strongly influences the likelihood of a pregnancy occurring, with the 1298C allele being significantly overrepresented amongst women who have undergone several unsuccessful assisted reproductive treatments. Secondly, parental *MTHFR* genotypes were shown to affect the production of aneuploid embryos, indicating that *MTHFR* is one of the few known human genes with the capacity to modulate

rates of chromosome abnormality. Thirdly, an unusual deviation from Hardy–Weinberg equilibrium was noted for the c.677C>T polymorphism in subfertile patients, especially those who had experienced recurrent failure of embryo implantation or miscarriage, potentially explained by a rare case of heterozygote disadvantage. Finally, a dramatic impact of the *MTHFR* 677T allele on the capacity of chromosomally normal embryos to implant is described. Not only do these findings raise a series of interesting biological questions, but they also argue that testing of *MTHFR* could be of great clinical value, identifying patients at high risk of implantation failure and revealing the most viable embryos during in vitro fertilisation (IVF) cycles.

Introduction

Folic acid is an important B vitamin essential for human reproduction (Tamura and Picciano 2006). The processing of folic acid and other dietary folates is vital for many key processes such as amino acid metabolism, purine and pyrimidine synthesis, and methylation of nucleic acids, proteins and lipids (Wagner 1995). These folate-dependent functions are required for DNA synthesis and repair, control of gene expression and many other biological processes of fundamental importance for cell division and embryo development (Kim et al. 2009; Obican et al. 2010).

Folate deficiency (genetically determined or due to dietary restriction) results in a higher frequency of uracil misincorporation into DNA, disruption of nucleic acid integrity, slower DNA replication and an increased risk of chromosome breakage. Affected cells experience elevated rates of apoptosis and necrosis (Blount et al. 1997; Huang et al. 1999; Kimura et al. 2004). Insufficient folate or folic acid intake has also been shown to negatively affect specific

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reproductive functions; it has a detrimental effect on many processes involved in oocyte development, acquisition of endometrial receptivity, embryo implantation and also in the maintenance of pregnancy (Forges et al. 2007; Gmyrek et al. 2005; Hussein 2005; Thaler and Epel 2003). Several animal model studies have demonstrated that maternal folate deficiency prior to conception and during gestation has a negative effect on female fertility and foetal viability, emphasising the important role of folate during mammalian folliculogenesis and foetal development (Gueant et al. 2013; Kumar et al. 2013; Maloney et al. 2007).

Numerous variations in genes involved in folate metabolism have been identified. In some cases, these mutations and polymorphisms alter the efficiency of pathways involved in folate generation and processing. In terms of prevalence and impact, genetic variations affecting the 5,10-methylenetetrahydrofolate reductase gene (*MTHFR*) are among the most biologically important. *MTHFR* is a key enzyme that plays an important role in catalysing the conversion of 5,10-methylenetetrahydrofolate into 5-methylenetetrahydrofolate, the predominant circulating form of folate. This molecule provides the single carbon needed for the synthesis of nucleotides, the remethylation of homocysteine to methionine, the synthesis of S-adenosylmethionine and the methylation of DNA, proteins, neurotransmitters and phospholipids (Frosst et al. 1995; Yamada et al. 2005).

More than 20 DNA sequence variants and polymorphisms within the *MTHFR* gene have been described (Sibani et al. 2000). Two of the most investigated are single nucleotide polymorphisms (SNPs) at the mRNA positions 677 (rs1801133) and 1298 (rs1801131) (Frosst et al. 1995; van der Put et al. 1998). The well-characterised *MTHFR* c.677C>T transition, which results in an alanine to valine substitution (p.Ala222Val) in the predicted catalytic domain of *MTHFR*, renders the enzyme thermolabile and leads to a reduction in *MTHFR* activity. Homozygous and heterozygous individuals have in vitro *MTHFR* activity reduced by about 70 and 35 %, respectively (Frosst et al. 1995). Homozygosity for the 677T allele is associated with elevated circulating homocysteine in some individuals, predominantly those who have a low plasma folate level (Jacques et al. 1996). In these individuals, the level of plasma homocysteine can be lowered by folic acid supplementation (Malinow et al. 1997).

The other common polymorphism in the *MTHFR* gene, c.1298A>C transversion, results in a glutamate to alanine substitution (p.Glu429Ala) within a presumed regulatory domain of *MTHFR* (van der Put et al. 1998; Weisberg et al. 1998). The 1298C allele leads to decreased enzyme activity, although to a lesser extent than the 677T allele. Individuals who are homozygous for the 1298C allele have about a 40 % reduction in enzyme activity in vitro, but

do not appear to have higher plasma homocysteine levels than controls (Malinow et al. 1997; van der Put et al. 1998; Weisberg et al. 1998). However, individuals who are compound heterozygous for the 677T and the 1298C alleles (*MTHFR* c.677C/T plus c.1298A/C genotype) have a 40–50 % reduction in enzyme activity in vitro and a biochemical profile similar to that seen among 677T homozygotes, with increased homocysteine and decreased folate levels. The c.1298A>C polymorphism by itself may have clinically important effects under conditions of low folate intake or during times of high folate requirements, such as pregnancy and embryogenesis (van der Put et al. 1998).

Despite the fact that many studies have explored the relationship between *MTHFR* polymorphisms and aspects of human reproduction, the biochemical influence and clinical relevance of these polymorphisms are still debated. Some authors have reported an association of certain genotypes with an increased risk of miscarriage, a potential consequence of poor vascularization of the placental area of individuals carrying minor alleles (Cao et al. 2013; Kim et al. 2011; Nair et al. 2012; Nelen et al. 2000). Others have described a link between c.677C>T and c.1298A>C polymorphisms and the likelihood of aneuploid conceptions, pointing out the possible influence of *MTHFR* on chromosome non-disjunction and other processes involved in chromosome segregation (Hassold et al. 2001; Hobbs et al. 2000; James et al. 1999; Kim et al. 2011). More recent reports have explored the impact of these polymorphisms in patients undergoing IVF treatment, suggesting an influence of some *MTHFR* variants on embryo quality (assessed morphologically) and upon implantation potential (Laanpere et al. 2011; Soldo et al. 2012). However, these findings remain controversial, with other studies failing to detect evidence of the proposed associations (D'Elia et al. 2014; Marci et al. 2012; Poursadegh Zonouzi et al. 2012). To clarify the true situation, especially in the context of assisted reproductive cycles, a detailed evaluation of the relationship between *MTHFR* polymorphism and different reproductive outcomes, using highly accurate genotyping methods, is urgently required. In particular, there remains a paucity of data from the critical preimplantation stage of development, a time when a deficiency in folate biosynthetic pathways might be expected to have a range of negative effects, some with profound implications for embryo viability. The present study involved an exhaustive examination of the incidence of two of the most common *MTHFR* polymorphisms in a large population of infertile patients and, for the first time, in embryos during the first few days of life (prior to implantation). Results from this study may help to settle some of the present controversies about the influence of *MTHFR* on the first stages of pregnancy and provide answers to the following questions: firstly, does *MTHFR* have a significant influence on

the fertility of groups of patients with specific reproductive histories (e.g. patients with a history of recurrent miscarriages or patients suffering repetitive implantation failure after IVF treatment)? Secondly, do maternal and paternal *MTHFR* genotypes influence the frequency at which aneuploid embryos are produced? Finally, does the *MTHFR* genotype have an impact on the developmental competence of an embryo? It was hoped that this detailed analysis, including genotyping of embryos as well as adults, would help to define the aspects of human reproduction affected by inherited defects of the *MTHFR* gene.

Materials and methods

Ethics statement

Ethical approval and signed patient consent for research had been obtained for all patient samples used in this study. No embryos were biopsied specifically for the purpose of this study. The embryo DNA samples assessed consisted of surplus whole genome amplification products, surplus following routine aneuploidy screening. Ethical approval for this study was obtained from the North London REC 3 (10/H0709/26) and Western IRB (20060680 and 20131473).

Cohort selection

The initial study group consisted of 138 patients (92 females and 46 males) undergoing assisted reproductive treatment (ART) and having aneuploidy screening of their embryos for a variety of reasons including: (1) recurrent miscarriage, i.e. two or more failed clinical pregnancies ($n = 51$); (2) repetitive implantation failure, i.e. 3 or more IVF treatments including transfer of embryos to the uterus but no pregnancy ($n = 38$); (3) advanced maternal age, i.e. over 37 years of age ($n = 17$). Additionally a well-matched control population was assessed, composed of 161 fertile individuals (83 females, 78 males) that had previously achieved at least one successful pregnancy. Both groups were of varied ethnic origin, primarily European, but also including individuals of North African and South-east Asian descent. A second independent data set comprising 202 individuals (101 couples: 28 fertile couples, 62 subfertile couples and 11 couples with a history of recurrent miscarriage) was available for confirmatory analysis using an alternative methodology (see below, CarrierMap, Recombine, USA). In addition to the DNA samples from adults, 193 specimens from blastocyst stage embryos, previously screened for aneuploidy using microarray comparative genomic hybridisation (aCGH), were also available for analysis.

MTHFR genotyping

Genomic DNA was extracted from blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Amplification of the c.677C>T region of the *MTHFR* gene was performed using the primer pairs 5'-TGAA-GGAGAAGGTGTCTGCGGGA-3' (forward primer) and 5'-AGGACGGTGC GG TGAGAGTG-3' (reverse primer) described by Frosst et al. (1995). Amplification of the c.1298A>C region of the *MTHFR* gene was accomplished using the forward (5'-CTTTGGGGAGCTGAAGGACTACTAC-3') and reverse (5'-CACTTTGTGACCATTCCG-GTTTG-3') primers reported by van der Put et al. (1998). PCR amplifications (25 μ l volume) consisted of the following: 5 ng of genomic DNA in the case of patients and controls or 0.5 μ l of Sureplex-amplified DNA in the case of biopsied material from preimplantation embryos; 10 pmol each of the c.677C>T or c.1298A>C forward and reverse primers; 0.625 units of Perfect Taq DNA polymerase (5 prime GmbH, Hamburg, Germany); 0.2 mM of dNTPs (Thermo Scientific, Colchester, UK) in 1 \times PCR Buffer (5 prime GmbH, Hamburg, Germany). Amplification of the correct fragment was initially confirmed by uni-directional DNA sequencing followed by comparison and alignment to the NCBI Reference Sequence NG_013351.1.

PCR was followed by minisequencing as described by Zetterberg et al. (2002) with some modifications. Prior to the minisequencing reaction, 0.5 μ l of PCR product was purified using ExoSAP-IT (Affymetrix, High Wycombe, UK) following the manufacturer's instructions. Minisequencing was then conducted in a final volume of 5 μ l, consisting of 0.5 μ l of each of the purified *MTHFR* c.677C>T and c.1298A>C PCR products, 2.5 μ l SNaP-shot Multiplex Ready Reaction Mix (Applied Biosystems, UK) and 1 pmol of each of the minisequencing primers: 5'-T(20)GCGTGATGATGAAATC-G-3' (reverse primer) for *MTHFR* c.677C>T; 5'-GGAGCTGACCAGTGAAG-3' (forward primer) for c.1298A>C. The poly (T) sequence of the former was added to modify the electrophoretic mobility of the primer. The cycling protocol was 25 cycles of 96 °C/10 s, 50 °C/5 s and 60 °C/30 s. Minisequencing products were analysed by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems, UK). The reaction included 9.25 μ l Hi-Di Formamide (Applied Biosystems, UK), 0.25 μ l GeneScan-120LIZ Size standard (Applied Biosystems, UK) and 0.5 μ l of minisequencing product. Incubation at 95 °C for 3 min to denature the minisequencing product was performed prior to electrophoresis. Data were subsequently visualised and analysed using the GeneMapper software (Applied Biosystems, UK).

Embryo DNA samples consisted of trophectoderm biopsy specimens (~5 cells removed at the blastocyst

stage), which had been subjected to whole genome amplification (SurePlex, Rubicon, USA). The biopsies were performed for the purpose of routine preimplantation genetic screening (PGS) using microarray comparative genomic hybridisation (aCGH) (described below). To assess the polymorphisms, surplus SurePlex DNA was subjected to the same protocol as genomic DNA.

Confirmatory analysis on an independent population

An entirely independent data set was available from 202 individuals (101 couples) evaluated using an alternative, microarray-based, methodology (CarrierMap, Recombine, USA). The CarrierMap test is primarily used for preconception carrier screening, evaluating couples considering starting a family for the presence of >1700 mutations responsible for more than 250 different genetic conditions. However, a number of polymorphisms of potential relevance to reproduction are also assessed, including the *MTHFR* c.677C>T and c.1298A>C polymorphisms. The data were divided into three separate groups: 28 of the couples tested were considered to be fertile, having achieved at least one pregnancy without the help of any assisted reproductive treatments (average of 1.9 pregnancies per couple) and having had no more than one previous miscarriage; 62 couples had a history of infertility and had undergone treatment using IVF or intrauterine insemination (IUI); 11 couples had received a formal diagnosis of recurrent miscarriage.

Embryo aneuploidy testing

Embryo chromosome analysis was accomplished by the use of a well validated aCGH method following the protocol described by Alfarawati et al. (2011). Diagnosis of embryos was performed at the blastocyst stage and involved sampling and testing of 5 to 10 trophoctoderm cells. Briefly, cells were lysed and their DNA amplified using whole genome amplification (SurePlex, Rubicon, USA). Amplified sample and reference DNAs from chromosomally normal individuals were labelled with Cy3 and Cy5 fluorochromes, respectively, and then hybridized to the probes of a bacterial artificial chromosome (BAC) microarray (24Sure, Illumina, Cambridge, UK). Chromosome losses and gains were revealed by differences in the fluorescence intensity corresponding to sample and reference DNAs for BAC probes derived from the affected chromosome or chromosomal region. Although primarily employed for the purpose of detecting losses and gains of entire chromosomes, copy number changes affecting chromosomal segments of 6 Mb or greater are also accurately detected with this approach (Colls et al. 2012). Labelling of the amplified samples, hybridization to microarray slides,

post-hybridization washes and analyses were performed as described elsewhere (Alfarawati et al. 2011). Published values for the accuracy rate for aCGH are >95 % for biopsy specimens consisting of small numbers of trophoctoderm cells (Fragouli et al. 2011; Wells et al. 2014).

Embryo transfers

Fresh single embryo transfers of euploid blastocysts of good morphological quality according to Gardner's classification (Balaban et al. 2011) were performed in all cases.

Statistical analyses

MTHFR c.677C>T and c.1298A>C allele and genotype frequencies were determined for each of the three populations initially investigated (subfertile patients, fertile controls and preimplantation embryos) and compared using a Chi-squared goodness of fit test. Genotype frequencies in all groups were assessed for compliance with the Hardy-Weinberg equilibrium using GENEPOP v.4.2 software (Raymond and Rousset 1995; Rousset 2008). This software was also used to evaluate the existence of linkage disequilibrium (LD) with a goodness of fit G-test. Comparisons of patient genotypes with regards to sex, maternal age and reproductive history were also carried out using the same analysis. Similar analyses were carried out for the three populations with data obtained from Recombine (subfertile patients, fertile controls and patients with recurrent miscarriage). The proportion of aneuploid embryos (aneuploidy rate) produced by patients with specific *MTHFR* genotypes was assessed and compared using an ANCOVA test using maternal age as a covariant. Aneuploidy rates of different indication patient subgroups (recurrent miscarriage, repetitive implantation failure and maternal age) were compared using a *T* test. In the case of preimplantation embryos, comparisons of the genotype frequencies of chromosomally normal and aneuploid embryos, normal embryos with successful or failed implantation, and embryos from patients with a variety of referral reasons were also determined and compared using Chi-squared goodness of fit test.

Statistical significance was defined as $p < 0.05$ and all analyses were performed using the IBM SPSS Statistics Version 20 software (IBM Corporation, USA).

Results

MTHFR in subfertile patients

To assess the relationship between *MTHFR* polymorphisms and subfertility, allele and genotype frequencies for a group of patients undergoing assisted reproductive treatment

were determined and compared to a fertile control population. Analyses of patient subgroups with regards to sex and reproductive history (RM, RIF or AMA) were also performed.

A statistically significant increase in the frequency of the less common *MTHFR* 1298C allele was observed in subfertile patients compared to fertile controls ($p = 0.003$), leading to a higher prevalence of individuals homozygous for 1298C and a lower incidence of patients homozygous for the major allele (1298A) ($p = 0.01$ and $p = 0.02$, respectively) (Table 1; Fig. 1). No differences were observed for allele or genotype frequencies for the *MTHFR* c.677C>T polymorphism. Analysis of the second independent population of samples tested using CarrierMap yielded similar results, with the frequency of 1298C homozygotes doubled in subfertile couples (0.137 vs 0.007, χ^2 test, $p < 0.001$).

When data were subdivided by sex, the difference in the *MTHFR* c.1298A>C genotype frequency was clearly apparent in female patients. In comparison to controls, subfertile female patients displayed a significantly higher incidence

of *MTHFR* c.1298CC homozygotes and a lower frequency of *MTHFR* c.1298AA homozygotes ($p < 0.01$) (Table 2). In the male group a similar tendency was observed, but it was less pronounced and did not reach statistical significance. Within the fertile control group there were no differences in genotype frequencies between the two sexes. Examination of *MTHFR* c.677C>T polymorphism did not reveal any differences in the incidence of particular genotypes between males and females (Table 2).

Genotype distributions of both the *MTHFR* c.677C>T and c.1298A>C polymorphisms in the fertile control group were in Hardy–Weinberg equilibrium. However, a deviation from expected genotype frequencies was found for *MTHFR* c.677C>T in subfertile patients due to a deficit of heterozygotes ($p < 0.01$) (Fig. S1). Analysis of patient subgroups revealed that this was particularly apparent in couples with a history of failed implantation or miscarriage. Investigation of the independent set of samples tested using CarrierMap yielded concordant data, with genotype frequencies in the subfertile group and in patients with recurrent miscarriage differing significantly from those expected for a population in equilibrium ($p < 0.001$ and $p < 0.01$ respectively) (Fig. S2).

When both *MTHFR* polymorphisms were analysed in combination, no individuals carrying three or four mutant alleles (*MTHFR* c.677CT/c.1298CC, c.677TT/c.1298CA, c.677TT/c.1298CC) were detected in any of the populations studied, suggesting linkage disequilibrium between these two loci (Table S1). Based upon allele frequencies and protein function, it is likely that the ancestral *MTHFR* gene had a 677C/1298A haplotype. Mutation at the 677 nucleotide position later produced a 677T/1298A haplotype and, in an independent event, mutation at position 1298 produced a 677C/1298C haplotype. Consequently, 677T/1298C haplotypes can only be formed by recombination within the

Table 1 *MTHFR* allele frequencies for fertile control and subfertile groups

<i>MTHFR</i> allele	Allele frequency	
	Fertile control group $n = 161$	Subfertile group $n = 138$
677C	0.696	0.725
677T	0.304	0.275
1298A	0.727	0.647*
1298C	0.273	0.353*

χ^2 test was used to estimate whether the allelic frequencies differed significantly among the study groups

* Groups are significantly different to control, χ^2 test, $p < 0.05$

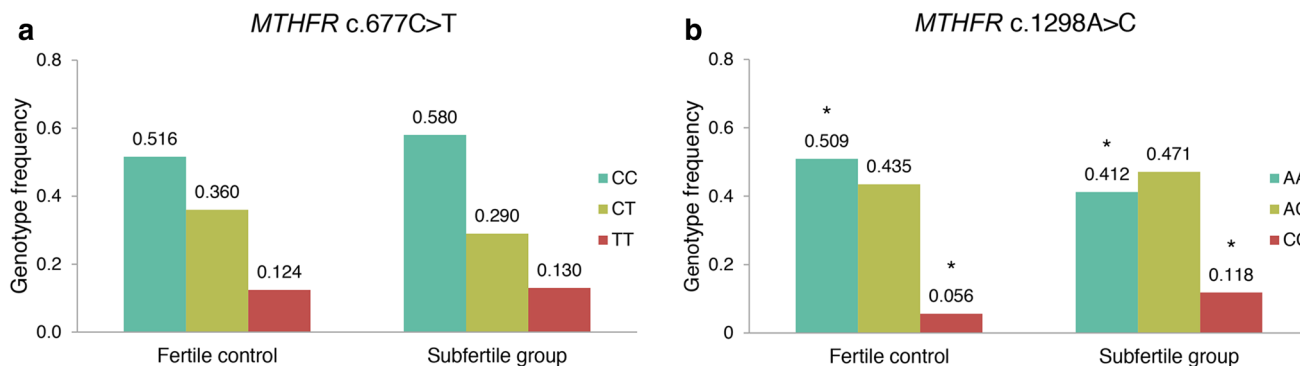


Fig. 1 Distribution of *MTHFR* c.677C>T and c.1298A>C genotypes in the study groups. **a** *MTHFR* c.677C>T genotype frequencies in subfertile and fertile control groups. **b** *MTHFR* c.1298A>C genotype frequencies in subfertile and fertile control groups. χ^2 test was used

to estimate whether the genotypic frequencies differed significantly among the study groups. Significantly different groups ($p < 0.05$) are highlighted with an asterisk

Table 2 Individual *MTHFR* c.677C>T and c.1298A>T genotype frequencies for fertile control and subfertile groups according to the sex of the patient

Genotype	Frequency		
	Fertile control group <i>n</i> = 161	Female subfertile group <i>n</i> = 92	Male subfertile group <i>n</i> = 46
677CC	0.516	0.620	0.500
677CT	0.360	0.272	0.326
677TT	0.124	0.109	0.174
1298AA	0.509	0.396*	0.444
1298AC	0.435	0.473	0.467
1298CC	0.056	0.132*	0.089

χ^2 test was used to estimate whether the genotypic frequencies differed significantly among the study groups

* Groups are significantly different to control, χ^2 test, $p < 0.05$

MTHFR gene, an extremely unlikely occurrence given the close proximity of the two polymorphisms. The existence of linkage disequilibrium was confirmed by G-test analysis ($p < 0.05$). The specific combination of 677T and 1298C alleles did not appear to have any additive effect in terms of impact on fertility in this patient population.

Patients were divided into groups according to their previous reproductive history, in an effort to determine whether differences in genotype frequencies with respect to the control population were related to subfertility in general or to particular reproductive problems. Analysis revealed that the significant excess of 1298C homozygotes observed in the subfertile group was primarily attributable to patients that had experienced repetitive implantation failure (RIF, i.e. three or more in vitro fertilisation treatments, including transfer of embryos to the uterus, but no pregnancy) relative to the control group ($p < 0.001$) (Table 3). The difference in the incidence of 1298C homozygotes amongst patients with RIF was equally apparent for both males and

females. No significant differences in genotype frequency were seen for any other category of patient [recurrent miscarriage (RM) or advanced maternal age (AMA)].

To summarise, results from this section showed an increase in the frequency of *MTHFR* c.1298C homozygotes in the group of subfertile patients compared to controls. More detailed analysis revealed that this difference was most apparent in female patients and primarily attributable to couples who had experienced repetitive failed IVF cycles.

Parental *MTHFR* genotype and embryo aneuploidy

To assess the influence of parental *MTHFR* alleles on embryo aneuploidy, the *MTHFR* genotypes of patients undergoing IVF were considered in relation to the proportion of chromosomally abnormal embryos they produced (aneuploidy rate). Factors such as gender or reproductive history were also explored.

A significantly higher aneuploidy rate was found in those patients presenting with at least one *MTHFR* minor allele (*MTHFR* 677T and/or *MTHFR* 1298C) compared to those patients with none (ANCOVA test, $p = 0.038$) (Table 4). Patients homozygous for the major alleles showed a mean embryo aneuploidy rate (\pm SEM) of 57.9 % (± 6.6), considerably lower than the 70.3 % (± 2.4) average for patients carrying a minor allele for at least one of the two *MTHFR* polymorphisms studied. When patients were divided according to their sex, it became clear that maternal genotype was associated with embryo aneuploidy ($p = 0.030$), whereas the paternal genotype did not have a significant effect.

When the embryo aneuploidy rates and *MTHFR* genotypes for patients with different indications (AMA, RIF or RM) were compared, results showed that in the case of AMA and RM patients the presence of one minor allele in at least one of the two *MTHFR* polymorphisms analysed

Table 3 Individual *MTHFR* c.677C>T and c.1298A>C genotype frequencies for fertile control group and different indication groups of subfertile patients

<i>MTHFR</i> genotype	Frequency			
	Fertile control group <i>n</i> = 161	RIF group <i>n</i> = 38	RM group <i>n</i> = 51	AMA group <i>n</i> = 17
677CC	0.516	0.525	0.588	0.588
677CT	0.360	0.275	0.275	0.353
677TT	0.124	0.200	0.137	0.059
1298AA	0.509	0.447	0.471	0.353
1298AC	0.435	0.316	0.451	0.588
1298CC	0.056	0.237*	0.078	0.059

χ^2 test was used to estimate whether the genotypic frequencies differed significantly among the study groups

RIF repetitive implantation failure, RM recurrent miscarriage, AMA advanced maternal age

* Groups are significantly different to control, χ^2 test, $p < 0.05$

Table 4 Mean embryo aneuploidy rates (mean \pm SEM) of patients with different *MTHFR* 677/1298 genotypes

	Mean embryo aneuploidy rate (%)		
	677CC/1298AA	Other genotypes (≥ 1 minor allele)	<i>p</i> value
Subfertile group	57.93 \pm 6.65	70.26 \pm 2.36	0.038*
Female subfertile group	52.64 \pm 11.02	71.22 \pm 2.91	0.030*
Male subfertile group	64.74 \pm 5.57	69.11 \pm 3.91	0.592
AMA group	58.14 \pm 9.41	75.00 \pm 2.96	0.045*
RM group	51.65 \pm 12.83	74.40 \pm 4.52	0.048*
RIF group	62.67 \pm 8.40	65.48 \pm 3.51	0.721

T test was used to assess whether the abnormality rates differed significantly among genotypes within the study groups

* Groups are significantly different, ANCOVA/*T* test, *p* < 0.05

was associated with a significantly higher level of affected embryos compared to those patients homozygous for the major alleles (*p* = 0.045 and *p* = 0.048, for AMA and RM, respectively). Again, these effects were only observed in relation to maternal genotype. No differences were found in the group of RIF patients (*p* = 0.72) (Table 4).

Results from this section show an influence of parental *MTHFR* genotype on embryo aneuploidy. The impact of *MTHFR* genotype on the production of aneuploid embryos was most pronounced for women of advanced reproductive age (>37 years) or those with a history of recurrent miscarriage.

The effect of embryonic *MTHFR* genotype

To assess the existence of linkage disequilibrium between the two polymorphisms studied and analyse the relationship between embryonic *MTHFR* genotype and embryo competence, the frequency and distribution of *MTHFR* c.677C>T and c.1298A>C alleles and genotypes were explored in preimplantation embryos at the blastocyst stage (5 days post-fertilisation).

MTHFR c.677C>T and c.1298A>C allele and genotype frequencies were calculated for the 193 embryos tested. No significant differences were found when embryo allele and genotype frequencies were compared to those seen in adults (Tables S2 and S3). When both *MTHFR* c.677C>T and c.1298A>C polymorphisms were analysed in combination, no embryos carrying 3 or 4 mutant alleles (677CT/1298CC or 677TT/1298CA) were detected, mirroring the findings in patients. No significant differences were observed when the genotype frequencies of euploid and aneuploid embryos were compared.

Conversely, when comparing the genotype frequencies of chromosomally normal embryos that successfully implanted (*n* = 19) with those euploid embryos that failed to implant (*n* = 27), significant differences were observed for *MTHFR* c.677C>T (*p* < 0.01). The incidence of embryos homozygous for the *MTHFR* 677 minor allele

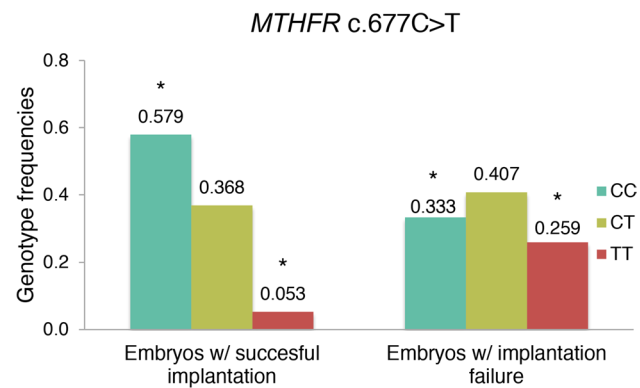


Fig. 2 Distribution of *MTHFR* c.677C>T genotypes in chromosomally normal embryos that successfully implanted compared to those that failed to implant. χ^2 test was used to estimate whether the genotypic frequencies differed significantly among the study groups. Significantly different groups (*p* < 0.05) are highlighted with an asterisk

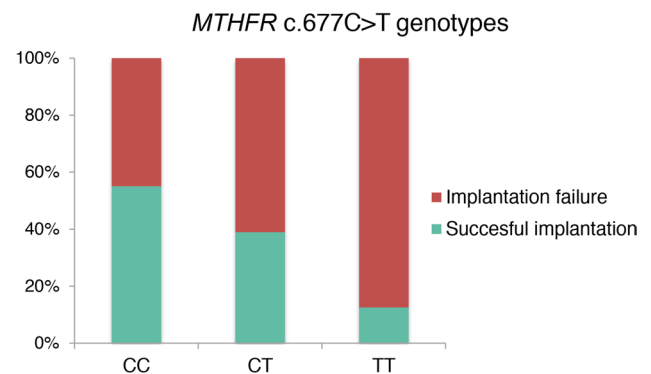


Fig. 3 Risk of implantation failure associated to embryonic *MTHFR* c.677C>T genotype. The frequency of implantation failure in embryos presenting different *MTHFR* c.677C>T genotypes is presented

(677T) was elevated in non-viable embryos compared to those that successfully formed on-going pregnancies (Fig. 2). One in four embryos experiencing failed implantation was homozygous for 677T, whereas only one of

the 19 embryos that produced a pregnancy was homozygous for the same allele. The risk of implantation failure was twofold higher for 677T homozygotes compared with 677C homozygotes (87.5 versus 45 %) (Fig. 3). Interestingly, significant differences in *MTHFR* c.677C>T genotype frequencies were also found between those embryos that failed to implant and the adult population composed of fertile and subfertile subjects ($p = 0.04$) (Fig. S3). No such differences were detected between adults and embryos that successfully implanted (Table S3).

A deviation from Hardy–Weinberg equilibrium, due to a deficit of heterozygote genotypes, was apparent for the total population of embryos ($p < 0.01$), resembling that seen for the samples from subfertile adults. Further division of the embryos into different categories (e.g. based upon patient indication or ability to implant) resulted in groups too small for meaningful statistical analysis. Nonetheless, it may be relevant that the embryos that successfully implanted ($n = 19$) had genotype frequencies in line with Hardy–Weinberg expectations, while those that failed to implant ($n = 27$) displayed an apparent increase in the proportion of homozygotes at the expense of heterozygotes. This was, however, not statistically significant due to the small size of the sample (Fig. S4). No significant differences in genotype frequencies were found in the case of the *MTHFR* c.1298A>C polymorphism.

Results from this section indicate that embryonic *MTHFR* genotype has a significant impact on viability. Indirect data suggest that c.677C>T heterozygotes are associated with increased risk of developmental failure occurring between fertilisation and the blastocyst stage, while 677T homozygotes succeed in reaching the final stages of preimplantation development, but have a reduced probability of producing a clinical pregnancy.

Discussion

MTHFR genotype and implantation failure

This study confirmed that the *MTHFR* c.1298A>C genotype has a strong influence on fertility. A prevalence of the 1298C allele and a corresponding increase in the frequency of *MTHFR* c.1298C homozygotes were observed for patients undergoing IVF treatment compared to fertile controls. This was later confirmed in an entirely independent population of patients, analysed using an alternative methodology. Analysis of different patient subgroups revealed that the increase in 1298C prevalence was most apparent in couples with a history of multiple unsuccessful IVF treatments. Amongst these patients the frequency of the 1298C allele was increased fourfold from 0.06 (seen in fertile controls) to 0.24. Women within this category

had received transfer of embryos to the uterus on at least three occasions, but without any pregnancy, indicating that the 1298C allele has a powerful impact on the ability of embryos to implant. The increase in 1298C frequency was principally observed in female patients, suggesting that reduced implantation rates may be related to an abnormal endometrial response or other maternal factors. Given the established reduction in *MTHFR* activity associated with homozygosity for the 1298C allele (Chango et al. 2000; van der Put et al. 1998; Weisberg et al. 1998), it can be inferred that individuals with compromised *MTHFR* activity are at increased risk of experiencing recurrent implantation failure. It is noteworthy that an elevated 1298C allele frequency was also observed in subfertile males, albeit to a lesser extent than seen females, suggesting that male factors related to this polymorphism might also influence the likelihood of implantation. No increase in the prevalence of the 1298C allele was detected in embryos that failed to implant compared to those that produced a viable pregnancy, arguing against the possibility of an effect at the level of the embryo.

The implantation of the blastocyst into the endometrium is a complex process that involves multiple molecular interactions between trophoblastic and endometrial cells, including coagulation and fibrinolysis processes at the embryo-maternal interface (Aflalo et al. 2004; Feng et al. 2000). It is conceivable that alteration of the functional activity of blood coagulation factors, related to diminished *MTHFR* activity, could affect the likelihood of implantation. There have been a number of publications reporting associations between inherited thrombophilic mutations/polymorphisms and recurrent unsuccessful IVF cycles (Azem et al. 2004; Ivanov et al. 2012; Coulam et al. 2006). It is undoubtedly true that appropriate coagulation processes, both maternal and placental, are of vital importance for pregnancy maintenance and are therefore of great relevance to miscarriage. This may be relevant to the distorted genotype frequencies of *MTHFR* polymorphisms observed in patients with recurrent pregnancy loss during this study. However, a role for coagulative processes at the time of implantation seems less likely. Perhaps more relevant in this regard are studies suggesting that folic acid concentration, modulated by *MTHFR*, has an important effect on trophoblast invasion, one of the very first steps of embryo implantation (Williams et al. 2011). Another possibility, potentially explaining increased rates of miscarriage and failure to implant, is that *MTHFR* variants influence levels of embryo aneuploidy (discussed below). Aneuploidy is extremely common in human preimplantation embryos and is believed to be the principal cause of implantation failure in both natural and assisted reproductive cycles (Fragouli et al. 2013; Hassold and Hunt 2001; Munne et al. 2006; Harton et al. 2013).

Unlike the c.1298A>C polymorphism, investigation of the other common *MTHFR* variant (c.677C>T) revealed no change in overall allele frequency for any of subfertile patient groups. However, a striking and unexpected discovery was that the two *MTHFR* c.677C>T alleles were not distributed in the expected proportions, resulting in a pronounced distortion of genotype frequencies (i.e. a deviation from Hardy–Weinberg equilibrium). Closer examination of the data revealed a deficit of *MTHFR* c.677C>T heterozygotes amongst patients with a history of recurrent implantation failure or miscarriage. Again, these data were subsequently confirmed by analysis of an independent set of samples using a different genotyping method. Interestingly, within these patient groups, there was a fourfold overrepresentation of couples in which the male and female had opposite homozygous genotypes (e.g. male homozygous for 677C and female homozygous for 677T or vice versa). Clearly, such couples can only produce heterozygous embryos, leading us to hypothesise that the two *MTHFR* c.677C>T alleles might be incompatible at a molecular level, leading to the formation of defective MTHFR dimers (essentially resulting in a heterozygote disadvantage) (Jacques et al. 1996; Yamada et al. 2001). An alternative possibility is that heterozygosity for c.677C>T is beneficial in terms of fertility and therefore less likely to be observed in patients undergoing fertility treatments. However, the mechanism by which the presence of any 677T alleles could be beneficial is unclear, especially considering that such alleles are known to be associated with diminished MTHFR function.

Investigation of preimplantation embryos demonstrated that deviation from expected c.677C>T genotype frequencies, mirroring those seen in the subfertile adults, can also be observed just a few days after conception. This suggests that the impact of the c.677C>T genotype must primarily be felt between fertilisation and the blastocyst stage. During this time, a period of 5 days, approximately half of all human embryos undergo developmental arrest and perish. It would be valuable to repeat the experiments described here on embryos that ceased developing during the first few days of life, to determine whether c.677C>T heterozygotes are at increased risk of developmental failure prior to blastocyst formation.

Parental MTHFR genotype and embryo aneuploidy

A significantly higher proportion of aneuploid embryos was found for patients carrying one or more alleles associated with reduced MTHFR activity compared to those with none. Patients with 677T and/or 1298C alleles showed a mean embryo aneuploidy rate 20 % higher than that of patients homozygous for the major (fully functional) *MTHFR* alleles. The association of *MTHFR* genotype with

embryo aneuploidy was more evident for female patients than males, indicating that the elevated rate of chromosome abnormality is primarily related to meiotic errors occurring during oogenesis. Results are suggestive of an influence in males as well, but an increased sample size would be needed to confirm this statistically. The impact of *MTHFR* genotype on the production of aneuploid embryos was most pronounced for women of advanced reproductive age (>37 years) or those with a history of recurrent miscarriage. This may be an indication that these patients are particularly sensitive to problems associated with compromised MTHFR function and that an increased incidence of embryo chromosomal abnormalities may be contributing to their difficulties achieving a viable pregnancy.

An influence of maternal *MTHFR* genotype on embryo viability was also suggested by Haggarty et al. (Haggarty et al. 2006). In their study, women homozygous for the 1298C allele were less likely to have a live birth after IVF compared to women homozygous for the more common 1298A allele. However, no chromosome analysis was carried out, so it is unclear whether the reduced viability was related to aneuploidy or other embryonic or maternal factors. Studies trying to establish an association between folic acid metabolism and preimplantation embryo development have shown that *MTHFR* is expressed in both human oocytes and embryos (Dobson et al. 2004, 2007). Folic acid is present in the follicular fluid and its supplementation has been associated with improved oocyte quality and the retrieval of larger numbers of mature eggs after ovarian stimulation, suggesting that MTHFR plays an important role in oocyte maturation and development (Boxmeer et al. 2008; Steegers-Theunissen et al. 1993; Szymanski and Kazdepka-Zieminska 2003). Moreover, during maturation, human oocytes express receptors involved in folic acid transport, while in *Xenopus* oocytes efficient movement of folate across their membrane has been demonstrated (Boxmeer et al. 2008; Dobson et al. 2004; Lo et al. 1991; Szymanski and Kazdepka-Zieminska 2003).

With regard to the specific question of chromosomal abnormality, several publications have suggested the potential for a relationship between maternal *MTHFR* gene polymorphisms and aneuploid pregnancy or birth. Some authors have reported an association between *MTHFR* mutations and the risk of conceiving a child with Down syndrome (Acacio et al. 2005; Hobbs et al. 2000; Hollis et al. 2013; James et al. 1999). The *MTHFR* c.1298A>C polymorphism has been linked to chromosomal aneuploidy leading to Turner Syndrome (Oliveira et al. 2008) and has also been proposed as an independent risk factor for spontaneous abortion with foetal chromosomal abnormality. Kim et al. (2011) reported a higher prevalence of heterozygosity and homozygosity involving the *MTHFR* 1298C allele in women with spontaneous abortions associated with foetal

aneuploidy compared to those with miscarriages of normal karyotype. However, some investigators have failed to confirm such a link (Boduroglu et al. 2004; Petersen et al. 2000). Hassold and co-workers (Hassold et al. 2001) observed a significant increase in the 677T allele polymorphism in mothers of trisomy 18 conceptuses but were unable to find associations with respect to other chromosomes.

Data from the current study support the notion that *MTHFR* mutations are associated with an increased risk of aneuploid pregnancy and that this is mostly attributable errors occurring during female meiosis. However, the underlying mechanism has not yet been elucidated. One hypothesis is that *MTHFR* mutations/polymorphisms lead to aberrant DNA methylation caused by abnormal folate metabolism and that this might increase the likelihood of meiotic aneuploidy (Hassold et al. 2001; James et al. 1999). In the absence of sufficient folic acid, intracellular homocysteine accumulates, methionine resynthesis is reduced and hence methylation reactions are compromised (Balaghi and Wagner 1993; Wainfan and Poirier 1992). Both clinical and experimental studies have shown that, in lymphocytes and liver cells, genomic DNA hypomethylation can be induced by deficiency of dietary folate, as well as other factors, and that this results in a variety of features such as abnormal gene expression, DNA strand breaks, chromosomal instability and aneuploidy (Crott et al. 2001; Ji et al. 1997; Pogribny et al. 1995; Wainfan and Poirier 1992).

To our knowledge, the current study is the first to look at the influence of maternal and paternal *MTHFR* genotypes on the production of aneuploid preimplantation embryos. The evaluation of such an early developmental stage is particularly important when considering chromosomal abnormality because most aneuploid embryos are lost around the time of implantation. In previous studies, chromosome analyses were performed during the first trimester of pregnancy, or even later. This is already far removed from the primary aneuploidy rate at conception.

Origin of *MTHFR* polymorphisms

The combined analysis of both c.677C>T and c.1298A>C polymorphisms in patients, controls and day-5 preimplantation embryos revealed linkage disequilibrium between the two loci. No individuals carrying three or four low-activity *MTHFR* alleles were found in any of the populations studied. These results are in agreement with several previous reports (Bae et al. 2007; Chango et al. 2000; Stegmann et al. 1999; van der Put et al. 1998; Weisberg et al. 1998). The fact that certain combinations of alleles were not detected indicates that the two polymorphisms arose independently on two different wild type alleles. The close proximity of these polymorphisms to one another (2.1 kb) is such that recombination between them is extremely

unlikely to occur (Friedman et al. 1999; Hanson et al. 2001; Weisberg et al. 1998).

Although concordant with most publications, our results contradict those of Isotalo et al. who found a considerable number of individuals homozygous for the *MTHFR* 677T allele and also heterozygous for the c.1298A>C polymorphism and proposed that the presence of 3 or 4 mutant alleles might compromise foetal viability (Isotalo and Donnelly 2000; Isotalo et al. 2000). Results obtained in the present study do not support this hypothesis. Combinations of 3–4 mutant alleles were not observed in any of the 193 preimplantation embryos analysed, suggesting that such combinations of mutant alleles are either very rare or non-existent. The most likely explanation for the conflicting results found by Isotalo and colleagues is inaccuracy of the method for examining the c.1298A>C polymorphism (Isotalo and Donnelly 2000; Zetterberg et al. 2007). The *MboII* RFLP method that they used has been criticised because of the potential for interference, and genotyping errors, caused by a silent polymorphism within the same exon of *MTHFR*. The problematic allele has a prevalence of approximately 5 % in the Canadian population that they studied (Weisberg et al. 1998).

Embryo *MTHFR* genotype and chromosome abnormalities

No significant differences in *MTHFR* c.677C>T and c.1298A>C genotype frequencies were seen when preimplantation embryos with and without chromosomal abnormalities were compared. This suggests that the increased frequency of aneuploidy observed in embryos derived from mothers carrying 677T and 1298C alleles occurs during meiosis, due to an affect in the oocyte or ovary rather than diminished *MTHFR* activity in the embryo itself. Complementary results were obtained by Bae et al. (2007) who explored the prevalence of *MTHFR* c.677C>T and c.1298A>C genotypes later in development, after a pregnancy had been established. Their analysis of miscarriages indicated that the distribution of *MTHFR* c.677C>T and c.1298A>C genotypes was the same regardless of whether the products of conception were euploid or aneuploid.

Embryo *MTHFR* genotype and implantation

A significantly higher risk of implantation failure was observed in euploid embryos homozygous for *MTHFR* 677T. This indicates that the embryonic *MTHFR* c.677C>T genotype influences the ability of an embryo to form a successful pregnancy. Indeed, data from the current study suggest that homozygosity for 677T could play a role in approximately 20 % of embryo implantation failures. Consequently, determination of the *MTHFR* genotype of IVF

embryos, prior to choosing which embryo will be transferred to the uterus, is likely to prove highly advantageous for fertility treatments (e.g. IVF), maximising the probability that a viable embryo will be transferred.

Defective folate metabolism has been shown to have negative effects on many essential processes that could impact embryogenesis and implantation, such as synthesis of nucleotide precursors for DNA synthesis and repair and cellular methylation reactions (Balaghi and Wagner 1993; Blount et al. 1997; De Cabo et al. 1995; Duthie 1999). Early embryo development involves crucial cell division and differentiation, requiring coordinated spatial and temporal changes in gene expression (Assou et al. 2011). Disruption of DNA methylation, a fundamental feature of transcriptional modulation, is likely to have serious consequences for the developing embryo (Razin and Shemer 1995). Other than affecting gene expression, the embryo *MTHFR* genotype could influence viability by introducing blood coagulation problems in the placenta, increasing the risk of miscarriage. Recent studies have shown that folic acid is potentially important in a number of crucial early stages of placental development, including extravillous trophoblast (EVT) invasion and angiogenesis (Williams et al. 2011).

An important consideration, which warrants further research, is the possibility that the impact of *MTHFR* deficiency on embryo implantation could be mitigated by dietary folic acid supplementation (e.g. 500 µg daily as recommended for women that have had a foetus with a neural tube defect) (WHO 2007). This may be particularly valuable for patients with a history of implantation failure following IVF treatment, especially if an adverse *MTHFR* genotype has been detected. Furthermore, the link between *MTHFR* deficiency and elevated aneuploidy risk raises the question of whether increased folic acid intake in female carriers of *MTHFR* 677T and 1298C alleles could help to reduce the incidence of chromosomally abnormal embryos, thereby leading to improved outcomes of conception achieved naturally or using ART.

Supplementation of embryo culture media, used during IVF treatments, should also be considered. We speculate that this might help to support homocysteine remethylation to methionine, assisting in the maintenance of the normal patterns of DNA methylation in embryos with reduced *MTHFR* activity. Currently, preimplantation embryos are routinely cultured in media that lack methyl donors. It would be valuable to determine whether folate-supplemented media could reduce the risk of implantation failure, miscarriage or aneuploidy. Consideration should also be given to the impact of folate supplementation on the incidence of imprinting disorders, associated with abnormal patterns of DNA methylation, which some studies suggest may be elevated in the conceptions of patients undergoing

infertility treatments (Cox et al. 2002; DeBaun et al. 2003; Orstavik et al. 2003).

In conclusion, a complex picture has emerged, in which *MTHFR* polymorphisms modulate reproductive success on several distinct levels. The data suggest that *MTHFR* genotype influences implantation, aneuploidy and the viability of euploid embryos. Multiple essential processes are affected, including meiosis, embryogenesis and initiation of pregnancy. Based upon the findings of this study, *MTHFR* potentially joins a rare group of human genes displaying an example of heterozygote disadvantage and another select group of genes with the ability to influence aneuploidy rates. Given the relative ease with which embryonic *MTHFR* genotype can be determined, the two polymorphisms may represent valuable new biomarkers for the assessment of embryo competence, enhancing IVF treatments by helping to reveal the embryos most likely to produce a viable pregnancy. Another exciting possibility is that cellular pathways dependent on *MTHFR* could be supported through maternal dietary supplementation or, for embryos produced using ART, via the addition of folate to culture medium. While this remains highly speculative, the potential benefits, in terms of reduced risks of implantation failure, aneuploidy, abnormal imprinting and miscarriage, argue that work to examine these possibilities should be encouraged as a matter of urgency.

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