Lack of Association between Genetic Polymorphisms in Enzymes Associated with Folate Metabolism and Unexplained Reduced Sperm Counts

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Abstract

Background: The metabolic pathway of folate is thought to influence DNA stability either by inducing single/double stranded breaks or by producing low levels of S-adenosyl-methionine leading to abnormal gene expression and chromosome segregation. Polymorphisms in the genes encoding enzymes in the folate metabolism pathway show distinct geographic and/or ethnic variations and in some cases have been linked to disease. Notably, the gene Methylene tetrahydrofolate reductase (MTHFR) in which the homozygous (TT) state of the polymorphism c.665C>T (p.A222V) is associated with reduced specific activity and increased thermolability of the enzyme causing mild hyperhomocysteinemia. Recently several studies have suggested that men carrying this polymorphism may be at increased risk to develop infertility.

Methodology/Principal Findings: We have tested this hypothesis in a case/control study of ethnic French individuals. We examined the incidence of polymorphisms in the genes MTHFR (R68Q, A222V and E429A), Methionine synthase reductase MTRR; (I22M and S175L) and Cystathionine beta-synthase (CBS; G307S). The case population consisted of DNA samples from men with unexplained azoospermia (n = 70) or oligozoospermia (n = 182) and the control population consisted of normospermic and fertile men (n = 114). We found no evidence of an association between the incidence of any of these variants and reduced sperm counts. In addition haplotype analysis did not reveal differences between the case and control populations.

Conclusions/Significance: We could find no evidence for an association between reduced sperm counts and polymorphisms in enzymes involved in folate metabolism in the French population.

Introduction

The metabolism of folate is key for the maintenance of genome integrity due to its role in DNA synthesis, repair and methylation [1,2]. Methylene tetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5, 10-methylene tetrahydrofolate to 5-methyltetrahydrofolate (MTHF), the predominant circulatory form of folate and carbon donor for the re-methylation of homocysteine to methionine. Thus, MTHFR is thought to participate in the provision of nucleotides essential for DNA synthesis and repair. Methionine, in its activated form, S-adenosyl methionine (SAM), is the methyl donor of many biologic transmethylation reactions [1,2]. A decreased pool of methionine may therefore also affect DNA methylation and this is supported by the observation that some MTHFR variants are associated with DNA hypomethylation [3].

Several polymorphisms have been described that result in amino acid changes, which could lead to altered MTHFR enzymatic activity [1,4]. A base change from C to T at nucleotide position 665 (also known as C677T) of the MTHFR gene results in the substitution of valine for alanine (p.A222V). Both heterozygous Ala/Val and homozygous Val/Val variants have reduced MTHFR enzyme activity compared with the homozygous Ala/Ala form, due to increased thermolability of the protein [5]. When compared to 665CC individuals, carriers of 665TT have ~34% residual MTHFR activity and 665CT individuals have ~71% residual MTHFR activity measured in vitro [6]. Individuals (particularly with a low folate status) carrying these variants can present with mild hyperhomocysteinemia [5].

A second polymorphism in MTHFR (c.A1286G; p.E429A) also results in reduced enzymatic activity in vitro, but by itself, it is...
not associated with higher plasma homocysteine (Hcy) or a lower plasma folate concentration [7]. However, combined heterozygosity with the c.655C>T polymorphism is associated with reduced MTHFR-specific activity, higher Hcy, and decreased plasma folate levels [7]. Both the c.1296A>C and c.665C>T polymorphism are associated with DNA hypomethylation [3]. A third MTHFR polymorphism leading to an arginine to glutamine change (c.203G>A; p.R68Q) has been described but the effect of this change on enzymatic activity is unknown.

Methylenetetrahydrofolate homocysteine methyltransferase (MTR) converts 5-methyltetrahydrofolate and homocysteine to tetrahydrofolate and methionine. Methionine synthase reductase (MTRR) plays a crucial role in maintaining the active state of MTR through the reductive methylation of cobalamin. Disturbances in the catalytic activity of MTRR could lead to higher levels of Hcy, and this can be a risk factor for neural tube defects (NTD) [8,9]. The most common polymorphism reported is the p.I22M polymorphism associated with DNA hypomethylation [3]. A third key enzyme in folate metabolism is cystathionine β-synthase (CBS). This polymorphism is an isoleucine to methionine change at position 22 (c.66A>G, p.I22M). Although the p.I22M polymorphism does not appear to alter the catalytic activity of the protein, the 66GG genotype is associated with a modest but significant decrease in plasma tHcy levels [10]. Other association studies have suggested that the p.I22M polymorphism is modest risk factor for Down syndrome [11] and NTD [9]. A second common MTRR polymorphism, c.524C>T (p.S175L), has been investigated in only a single study, which failed to detect an association between this polymorphism and NTDs [12].

A third key enzyme in folate metabolism is cystathionine β-synthase (CBS), which catalyzes irreversible cystathionine synthesis from homocysteine and serine. Disturbances in this process can lead to an increased cellular Hcy level and the most common type of inherited homocystinuria in the human is caused by a deficiency in CBS. Cystathionine is a substrate for cysteine synthesis, which is catalyzed by cystathioninase. The gene encoding cystathionine synthase (CBS) has been localized on chromosome 21 (21q22.3) in a region correlated with Down syndrome phenotype. A frequent mutation in the CBS gene in Caucasians, c.919G>A (p.G307S), is one of the most common causes of homocystinuria in patients of Celtic origin [13] and this mutation accounts for 71% of alleles in Irish homocystinuria patients [14]. This mutation has been described in individuals of French, Scottish, English and Irish Ancestry [13–13] and is probably specific for North-west European populations.

Recently several association studies have suggested that polymorphic variants in the MTHFR gene may be associated with reduced sperm counts in the human leading to male infertility in some populations [16–23]. Here, we describe an association study between 3 variants in MTHFR, two variants in MTRR and the p.G307S mutation in the CBS gene and reduced sperm counts in otherwise healthy individuals of French ethnic origin that sought treatment for infertility. We failed to detect an association between any of these variants and unexplained reduced sperm counts leading to male infertility. These data suggest that in our study population, genetic variants in enzymes involved in folate metabolism do not have a significant impact on sperm counts.

### Materials and Methods

#### Patient Recruitment

Patients presenting with idiopathic infertility or normospermic fertile donors were recruited from Tenon Hospital, Paris and Saint-Etienne Hospital, Saint-Etienne. All patients and controls were of French ethnic origin as determined by self-report of patients. Exclusion criteria included known genetic causes of infertility such as chromosome anomalies, Y chromosome AZF deletions [24] and presumed genetic risk factors for male infertility [Y chromosome gr/gr deletions; 25]. The final clinical breakdown of the study population was azoospermic (n = 70), oligozoospermic (<20 x 10⁶ sperm/ml; n = 182), normozoospermic and fertile (>20 x 10⁶ sperm/ml and father of at least one child; n = 114). Semen analysis was performed on at least two separate occasions.

#### Ethics Statement

All patients provided informed consent prior to participation in this study. The study was approved by the Comite Consultatif de

### Table 1. SNPs investigated in this study together with the PCR primers and detection method.

| Gene    | Chrom. | SNP    | NCBI A.N. | AA substitution | Base change | Detection method | PCR primers                              | AT  | FS (bp) | DFS (bp) |
|---------|--------|--------|-----------|----------------|-------------|-----------------|------------------------------------------|-----|---------|----------|
| MTHFR   | 1      | c.203G>A | rs2066472 | p.R68Q        | A/G         | PCR+Taql dig    | 5’CCA TGG GAA TCT GGT GAC AA3’ 5’ACC TGG CAT GAG TTT ACC TG3’ | 56 C | 101     | 48+53    |
|         |        | c.665C>T | rs1801133 | p.A222V       | C/T         | PCR+HinfI dig   | 5’CCA AAG GCC ACC CGG AAG3’ 5’GAA AGA TCC CGG GGA CGA TG3’ | 56 C | 180     | 75+105   |
|         |        | c.1286A>C | rs1801131 | p.E429A       | A/C         | PCR+Mbol dig    | 5’CTT TGG GGA GCT GAA GGA CTA CTA C3’ 5’CAC TTT GTG ACC ATT CCG GTT TG3’ | 56 C | 163     | 56+31+30 +28+18 |
| MTRR    | 5      | c.66A>G  | rs1801394 | p.I22M        | A/G         | PCR+Ndel dig    | 5’GCA AAG GCC ATC GCA GAA GAC3’ 5’TGG TAT TAG TGT CCT TT3’ | 56 C | 172     | 22+157   |
|         |        | c.524C>T | rs1352268 | p.S175L       | C/T         | PCR+Taql dig    | 5’GCT GGA TTG GTG GAC TCT G3’ 5’AGC AGC TCT GAC TTC ACA AGG3’ | 56 C | 133     | 101+32   |
| CBS     | 21     | c.919G>A | -          | p.G307S       | A/G         | PCR+Pvul dig    | 5’ATC ATT GGG GTG CAT CCCCA 3’ 5’ACC GTG GGG ATG TCG CAG3’ | 60 C | 113     | 92+21    |

Chrom: Chromosome.  
SNP: single nucleotide polymorphism.  
NCBI A.N: NCBI Accession Number.  
AA: Amino Acid.  
Dig: Digestion.  
AT: Annealing Temperature.  
FS: Fragment size.  
DFS: Digestion Fragment size.

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Table 2. The allelic frequencies and associations between reduced sperm counts and common polymorphisms in the MTHFR gene.

| Gene | SNP | AA substitution | Genotype | Cases | Controls | Odds Ratio (95%CI) | Chi² | p value |
|------|-----|-----------------|----------|-------|----------|-------------------|------|---------|
| MTHFR | c.203G>A | p.R68Q | GG | 69 | 175 | 113 | 99.12 | 1.286 | 0.2568 |
|       |      |               | AA | 0  | 2  | 0  | 0     |       |         |
|       |      |               | AG | 1  | 5  | 1  | 0.88 | 1.638 (0.101–26.615) | 0.123 | 1.261 | 0.7262 | 0.2614 |
|       |      |               | AA+AG | 1 | 7 | 1 | 0.88 | 1.638 (0.101–26.615) | 0.123 | 2.35 | 0.7262 | 0.1253 |
|       |      |               | A allele frequency | 1 | 9 | 1 | 0.88 | 1.638 (0.101–26.615) | 0.123 | 3.497 | 0.7262 | 0.0615 |
|       | c.665C>T | p.A222V | CC | 33 | 85 | 49 | 42.98 |       |         |
|       |      |               | CT | 31 | 70 | 52 | 45.62 | 0.885 (0.473–1.656) | 0.146 | 0.98 | 0.7028 | 0.3221 |
|       |      |               | TT | 6  | 25 | 13 | 11.4 | 0.685 (0.237–1.984) | 0.489 | 0.071 | 0.4845 | 0.7894 |
|       |      |               | CT+TT | 37 | 95 | 65 | 57.02 | 0.845 (0.465–1.537) | 0.304 | 0.506 | 0.5815 | 0.477 |
|       |      |               | T allele frequency | 43 | 120 | 78 | 34.21 | 0.819 (0.460–1.459) | 0.462 | 0.27 | 0.4966 | 0.6031 |
|       | c.1286A>C | p.E429A | AA | 34 | 97 | 54 | 47.79 |       |         |
|       |      |               | AC | 28 | 66 | 46 | 40.71 | 0.967 (0.512–1.827) | 0.011 | 0.769 | 0.917 | 0.3804 |
|       |      |               | CC | 7  | 18 | 13 | 11.5 | 0.855 (0.31–2.357) | 0.091 | 0.421 | 0.7623 | 0.5162 |
|       |      |               | AC+CC | 35 | 84 | 59 | 52.21 | 0.942 (0.517–1.715) | 0.038 | 0.938 | 0.8455 | 0.3328 |
|       |      |               | C allele frequency | 42 | 102 | 72 | 31.86 | 0.926 (0.522–1.643) | 0.068 | 1.075 | 0.7941 | 0.2999 |
Molecular analysis

Single-nucleotide polymorphism analysis. A total of 6 single-nucleotide polymorphism (SNPs) in key genes in folates metabolism where selected for this study. In this study, we used a combination of direct sequencing of PCR products and PCR/restriction fragment length polymorphism (RFLP) assays and genotyped the SNPs in well-defined ethnic French case and control populations. The methodology and the oligonucleotides used in the study are outlined in table 1.

Genotyping. A PCR protocol was applied in genotyping all SNPs. PCR was carried out in a volume of 25 μl containing 30 ng genomic DNA, 1.5 mM MgCl₂, 200 mM each deoxynucleotide triphosphate, 2 μM each primer, 0.5 U Taq DNA polymerase (Bioline, London, U.K.) and 10× reaction buffer. PCR consisted of an initial denaturation at 94°C for 10 min, followed by 33 cycles of 94°C for 30 s, annealing temperature (table 1) for 30 s, and 72°C for 30 s, with a 7-min 72°C final extension. The PCR amplicons were digested by those restriction endonucleases corresponding to their respective SNPs (New England Biolabs, Beverly, Mass.). Digestion products were electrophoresed on a 3% agarose gel. In addition, the allelic status of 96 DNA samples was confirmed by direct sequencing of PCR products to validate the results obtained by RFLP-PCR.

Statistical analysis

In order to assess a possible distortion in allele frequencies between cases and controls, for the different polymorphisms tested, we performed a chi-square test with one degree of freedom for both allelic and genotypic distributions between the groups of cases and controls. Further, we tested if certain allelic combinations could be associated with an increased risk of infertility in any of the genes. For that, we reconstructed haplotypes from unphased genotypic data using the accelerated Expectation Maximization algorithm implemented in Haploview v3.1. Association testing for the haplotypes as well as the measure of linkage disequilibrium (D') between all pairs of SNPs was performed using the same software. p value was determined using a χ² test for the distribution of haplotype alleles between the cases and controls. Significant associations were defined by a p-value below 0.05. Haplotypes occurring at less than 1% frequency were excluded from the analysis.

Results

We analysed 3 MTHFR common variants (c.203G>A; p.R68Q; c.665C>T; p.A222V and c.1286A>G; p.E429A), 2 MTRR polymorphisms (c.66A>G; p.I22M and c.524C>T; p.S175L) and one CBS mutation (c.919G>A; p.G307S). We compared the distributions of the MTHFR and MTRR genotypes between the cases and controls (tables 2 and 3). The MTHFR genotypes distribution for the c.665C>T (p.A222V) and c.1286A>G (p.E429A) polymorphism were in Hardy-Weinberg equilibrium (respectively p = 0.20 and p = 0.22), but not for the c.203G>A (p.R68Q) polymorphism (p = 0.002). For this polymorphism, genotypic distributions was in Hardy-Weinberg equilibrium for cases with azoospermia (p = 1) but not for cases with oligozoospermia (p = 0.0002). The MTRR genotypes distribution for the c.524C>T (p.S175L) polymorphism was in Hardy-Weinberg equilibrium (p = 0.091) but not for the c.66A>G (p.I22M) polymorphism (p = 0.015). The lack of Hardy-Weinberg equilibrium for some of the alleles may be due to the small sample size.

| Gene | SNP | AA substitution | Genotype | Cases | Controls | Odds Ratio (95% CI) | Chi² | p value |
|------|-----|-----------------|----------|-------|----------|--------------------|------|---------|
|      |     |                 |          | Azoospermic | Oligozoospermic |                    |      |         |
|      |     |                 |          | Azoo | Oligo | Azoo | Oligo | Azoo | Oligo |
|      |     |                 |          | n | % | n | % | n | % | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 32 | 28.79 | 61 | 35.26 | 42 | 37.94 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 19 | 28.79 | 61 | 35.26 | 42 | 37.94 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 8 | 12.12 | 19 | 10.98 | 12 | 10.81 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 47 | 71.21 | 112 | 64.74 | 69 | 62.16 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 55 | 41.67 | 131 | 37.86 | 81 | 36.49 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 29 | 41.43 | 61 | 35.33 | 47 | 58.57 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 30 | 42.86 | 78 | 43.87 | 35 | 57.14 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 11 | 15.71 | 40 | 22.35 | 16 | 14.42 | Azoospermic | Oligozoospermic | Case | Control |    |    |
|      |     |                 |          | 41 | 58.57 | 118 | 37.86 | 76 | 62.14 | Azoospermic | Oligozoospermic | Case | Control |    |    |
these markers has been noted elsewhere and this could be a signature of either, genetic drift, non-random mating patterns or an indication of selection acting on specific genotypes. If the latter hypothesis is correct we do not have evidence that this is due to an affect of reduced sperm counts in the male. For all the polymorphisms we did not observe any statistically significant association with reduced sperm counts in the French population. There was no association between the two phenotypes (azoospermia or oligozoospermia). The Bonferroni correction was not applied since significance was not observed with all markers between the case and control cohorts.

The MTHFR c.665C and c.1286C polymorphic sites are only 2.1 kb apart and are in strong linkage disequilibrium (see figure 1). It has been suggested that these polymorphisms are the result of independent founder effects in which each variant evolved on a separate wild-type allele.

We did not identify any individual carrying the CBS c.919G>A (p.G307S) mutation.

**Haplotype analysis**

Haplotypes for the MTHFR and MTRR genes were reconstructed using the accelerated Expectation Maximization algorithm implemented in Haploview v3.1. We identified three haplotypes that characterize 93% of all haplotypic diversity in the MTHFR gene. There was no statistical significant difference in the distribution of these haplotypes between case and control cohorts. The analysis of the MTRR gene revealed 4 major haplotypes. There was no difference in the distribution of the haplotypes between the case and control groups (tables 4 and 5).

**Discussion**

Changes in folate status could affect spermatogenesis in two ways: 1) by causing DNA hypomethylation and thereby disrupting gene expression and 2) inducing uracil misincorporation during DNA synthesis leading to errors in DNA repair, strand breakage and chromosomal anomalies. There is considerable experimental evidence that key enzymes in the folate metabolism are necessary for male spermatogenesis. Mice that lack the Mthfr gene exhibit hyperhomocystenemia, significantly decreased S-adenosylmethionine levels, global DNA hypomethylation and developmental retardation with severe neuropathology [26]. These mice also showed delayed maturation of the external genitalia but appeared to be fertile [26]. However, extensive backcrossing of these mice to a BALB/c background resulted in spermatogenic failure during early postnatal development and resultant complete male infertility [27]. Fertility could be restored in a subset of Mthfr−/− mice by supplementing the diet with betaine, a choline directive that can serve as an alternative methyl donor for the remethylation of methionine.

![Figure 1. Haplotype organisation of the MTHFR gene in the CEPH pedigrees.](image-url)
Table 4. Associations between reduced sperm counts and the most common MTHFR haplotypes.

| Haplotype | Frequency | Cases (alleles) | Controls (alleles) | Chi² | p   |
|-----------|-----------|----------------|-------------------|------|-----|
|           | n (%)     | n (%)          | n (%)             |      |     |
| GCA       | 0.400     | 201 40.26      | 87 38.07          | 0.217| 0.6410|
| GTA       | 0.290     | 144 28.78      | 65 28.33          | 0.0020| 0.9627|
| GCC       | 0.261     | 127 25.48      | 60 26.4           | 0.114| 0.7360|
| GTC       | 0.034     | 13 2.68        | 11 5.0            | 2.647| 0.1037|

Table 5. Associations between reduced sperm counts and the most common MTRR haplotypes.

| Haplotype | Frequency | Cases (alleles) | Controls (alleles) | Chi² | p   |
|-----------|-----------|----------------|-------------------|------|-----|
|           | n (%)     | n (%)          | n (%)             |      |     |
| GC        | 0.352     | 165 33.02      | 78 34.12          | 0.032| 0.8580|
| GT        | 0.270     | 125 24.98      | 61 26.84          | 0.199| 0.6556|
| AC        | 0.229     | 109 21.78      | 49 21.58          | 0.021| 0.8860|
| AT        | 0.149     | 73 14.62       | 30 13.07          | 0.393| 0.5310|

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Author Contributions

Conceived and designed the experiments: KM AB. Performed the experiments: CR SCB CC KM. Analyzed the data: CR CC LBB KM. Contributed reagents/materials/analysis tools: CR SCB IAS JP IB JM JPS. Wrote the paper: CR KM AB.
### Table 6. Summary of published associations between the MTHFR C667T variant and unexplained male infertility.

| Infertile | MTHFR 665 genotype (%) | Controls | MTHFR 665 genotype (%) | Odds Ratio (CI) 95% CI | Chi² Population |
|-----------|-------------------------|----------|------------------------|-------------------------|-----------------|
| Bezold et al 2001 | 255 CC: 114 (44.7) | 200 CC: 92 (46) | 0.843 (0.565–1.258) | p = 0.4 | Not defined |
| Stuppia et al. 2003 | 93 CC: 37 (39.8) | 105 CC: 33 (31.4) | 0.767 (0.403–1.460) | p = 0.4193 | Caucasian |
| Singh et al. 2005 | 151 CC: 105 (69.5) | 200 CC: 163 (81.5) | 1.678 (1.008–2.795) | p = 0.04 | Indian |
| Park et al. 2005 | 373 CC: 105 (28.15) | 396 CC: 145 (36.62) | 1.42 (1.03–1.95) | p = 0.0319 | Korean |
| Lee et al. 2006 | 360 CC: 115 (31.94) | 325 CC: 118 (36.31) | 1.21 (0.88–1.67) | p = 0.2287 | Korean |
| A et al. 2007 | 355 CC: 130 (36.6) | 252 CC: 128 (50.8) | 1.72 (1.07–2.76) | p = 0.023 | Chinese |
| Dhillon et al. 2007 | 179 CC: 81 (45.25) | 200 CC: 70 (35) | 1.5 (0.97–2.33) | p = 0.076 | Indian |
| This study 2008 | 250 CC: 118 (47.2) | 114 CC: 49 (42.98) | 0.907 (0.503–1.294) | p = 0.3718 | Caucasian |
| | CT: 101 (40.4) | CT: 52 (45.62) | 0.807 (0.503–1.294) | p = 0.9789 | (French) |
| | CT: 31 (12.4) | CT: 13 (11.40) | 0.990 (0.478–2.051) | p = 0.4539 | |
| | CT: 132 (52.8) | CT: 65 (57.02) | 0.843 (0.540–1.317) | p = 0.4539 | |

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