Association of the *MTHFR* A1298C Variant with Unexplained Severe Male Infertility

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Abstract

The methylenetetrahydrofolate reductase (*MTHFR*) gene is one of the main regulatory enzymes involved in folate metabolism, DNA synthesis and remethylation reactions. The influence of MTHFR variants on male infertility is not completely understood. The objective of this study was to analyze the distribution of the *MTHFR* C677T and A1298C variants using PCR-Restriction Fragment Length Polymorphism (RFLP) in a case group consisting of 344 men with unexplained reduced sperm counts compared to 617 ancestry-matched fertile or normozoospermic controls. The Chi square test was used to analyze the genotype distributions of MTHFR polymorphisms. Our data indicated a lack of association of the C677T variant with infertility. However, the homozygous (C/C) A1298C polymorphism of the *MTHFR* gene was present at a statistically high significance in severe oligozoospermia group compared with controls (OR = 3.372, 95% confidence interval CI = 1.27–8.238; p = 0.01431). The genotype distribution of the A1298C variants showed significant deviation from the expected Hardy-Weinberg equilibrium, suggesting that purifying selection may be acting on the 1298CC genotype. Further studies are necessary to determine the influence of the environment, especially the consumption of diet folate on sperm counts of men with different MTHFR variants.

Citation: Eloualid A, Abidi O, Charif M, El houate B, Benrahma H, et al. (2012) Association of the *MTHFR* A1298C Variant with Unexplained Severe Male Infertility. PLoS ONE 7(3): e34111. doi:10.1371/journal.pone.0034111

Editor: Alejandro Lucia, Universidad Europea de Madrid, Spain

Received October 5, 2011; Accepted February 23, 2012; Published March 23, 2012

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Funding: These authors have no support or funding to report.

Competing Interests: Noureddine Louanjli is employed by In Vitro Fecundation Center, 40 Rue Prince Moulay Abdeelah 20 000, Casablanca, Morocco. No other authors have any conflicts to declare. This does not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Infertility is estimated to affect 10–15% of couples, and roughly half of these cases are due to the male factor [1]. Spermatogenic failure is the most common form of male infertility; however, in most cases the etiology remains unknown. Genetic abnormalities are thought to account for 15%–30% of male factor infertility and these can include Y chromosome microdeletions, chromosomal aberrations and rare single-gene defects [2–5]. Deleterious gene polymorphisms in key genes involved in testicular function, in combination with environmental insults, may be responsible for the reduced sperm numbers and poor sperm quality that are observed in many infertile men.

A possible candidate for genetic susceptibility to spermatogenic failure is the gene 5,10-Methylenetetrahydrofolate reductase (*MTHFR*). *MTHFR* is an important regulatory enzyme in folate and homocysteine metabolism, which is necessary for a number of key biological cellular mechanisms [6,7]. This regulatory enzyme catalyzes the reduction of 5,10-methylenetetrahydrofolate to produce 5- methyltetrahydrofolate, which is the methyl donor for the remethylation of homocysteine to methionine. Subsequently, methionine provides the methyl group for the formation of S-adenosylmethionine, the methyl donor for DNA methylation [8]. Methylation anomalies of sperm DNA has been linked to male infertility [9]. Reduced enzymatic activity due to *MTHFR* polymorphisms is associated with hyperhomocysteinemia that is considered as a risk factor for many diseases, including infertility [10]. Moreover, the activity of *MTHFR* is much higher in testis than in other major organs in the adult mouse, suggesting that it might play an important role in testicular function [11]. Two single nucleotide polymorphisms (SNPs) in the *MTHFR* gene, C677T (A→V) [12] and A1298C (E→A) [13,14], are individually associated with a reduction in the biochemical activity of the enzyme. The *MTHFR* C677T variant decreases *MTHFR* activity and increases the homocysteine level. Similarly the *MTHFR* A1298C variant also reduces enzyme activity but to a lesser degree than C677T [12–15]. Some studies have shown a significant statistical correlation between *MTHFR* polymorphisms and human male infertility [16,17,18,19,20], whereas others did not find any such association [21,22,23,24,25].

In the present study, we investigated the frequency of the C677T and A1298C polymorphisms in the *MTHFR* gene in men with unexplained reduced sperm counts compared with ancestry-matched fertile and/or normozoospermic individuals of Moroccan origin. We observed an association of reduced sperm counts with the *MTHFR* 1298C variant.

Materials and Methods

Subjects

We recruited a total of 344 idiopathic infertile Moroccan patients, consisting of 110 men with azoospermia, 89 men with...
severe oligozoospermia, 58 men with oligozoospermia and 87 men with asthenozoospermia and/or teratozoospermia. All were aged from 25 to 50 years and all underwent an andrological work-up, performed in clinics specializing in male infertility, which included medical history, physical examination, hormonal estimation (FSH, LH, and testosterone) and semen analysis. Patients with known causes of infertility including genetic factors (chromosomal abnormalities and microdeletions in the AZF region of the Y chromosome), lifestyle factors (eg. Smoking, alcoholism, occupation) and clinical factors (varicocele, cryptorchidism) were excluded from the study group. The control group consisted of 450 fertile men who had more than one child and 240 normozoospermic men, all aged from 30 to 55 years. We obtained written informed consent from each subject and the study protocol was approved by the Committee on Research Ethics of Institut Pasteur du Maroc.

Molecular analysis
Genomic DNA was extracted from peripheral leukocytes using the standard phenol–chloroform method.

Genotyping of SNP C677T in the MTHFR gene: The C677T (rs1801133) mutation was analyzed by polymerase chain reaction (PCR) of genomic DNA using the following primer pairs: 5’-TGAAGAGAAAGTGCTCGGGGA-3’ (forward) and 5’-AGGACCGGTTCGGGAGATG -3’ (reverse). These primers generate a 198 bp fragment. PCR amplification was carried out in a total volume of 10 μL containing approximately 50 ng of genomic DNA, 200 μM dNTPs, 10 pmol of each primer, 1.5 mmol/L MgCl2, 0.5 U Taq polymerase and 1 μL of 10× PCR buffer. The PCR reaction profile was: predenaturation at 94°C for 5 min followed by denaturation at 94°C for 30 s, annealing at 38°C for 30 s and extension at 72°C for 30 s for 35 cycles, with a final extra extension at 72°C for 7 min. PCR amplicons were digested with restriction enzyme HinfI. Then the products of digestion were electrophoresed on a 3% agarose gel stained with ethidium bromide and visualized using ultraviolet illumination. The wild type homozygote (CC), heterozygote (CT) and homozygous (TT) in the control group were compared with each other using Hardy-Weinberg equilibrium. The genotype and allele frequencies obtained for the MTHFR A1298C polymorphism are presented in table 2. We observed that the control cohort and a combination of control and infertile men showed highly significant Hardy-Weinberg disequilibrium for the MTHFR A1298C polymorphism, (p<1×10^-4 and p<1×10^-4 respectively; Tables 3 and 4). The MTHFR 1298C variant showed Hardy-Weinberg equilibrium in the infertile group. This suggests that natural selection is acting against the 1298CC genotype. This is supported by the statistically significant difference in frequencies of this variant between the case and control cohorts. Overall the homozygote CC had an elevated frequency in infertile men (4.94%) compared to the control samples (2.46%). Moreover, this genotype (CC) showed highest frequencies in the severe oligozoospermia group (OR = 3.37, 95% CI = 1.27–8.24; p = 0.01431).

We also investigated possible associations of different combinations of both polymorphisms between controls and patients (Table 3). The haplotype CCAA showed a slightly significant difference (p = 0.03078) between controls and all infertility patients (OR = 1.589, 95% CI = 1.039–1.353), and the haplotype CCCA showed significantly significant frequencies between the control group and a combination of all patients (OR = 3.313, 95% CI = 1.488–7.659; p = 0.003880), between control and severe oligozoospermia group (OR = 6.687, 95% CI = 2.461–17.72; p = 0.0000449) and between controls and all azoospermia, severe oligozoospermia and oligozoospermia group respectively (OR = 3.326, 95% CI = 1.4–8.035; p = 0.007307).

Discussion
Folates are group B vitamins that play essential roles in the synthesis of nucleic acids and in epigenetic regulation of gene expression through remethylation of homocysteine into methionine [26]. Changes in folate status could negatively impact on spermatogenesis by causing DNA hypomethylation and thereby disrupting gene expression or by 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 [19,24]. Folate deficiency is associated with cardiovascular and obstetrical disorders and neurodegeneration [26,27,28]. MTHFR gene encodes key regulatory enzyme involved in folate metabolism, and genetic variants of this gene may predispose some men to reduced sperm counts [22,29]. The C677T variant is associated with a reduction in
Table 1. Distribution of C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR) gene.

| Genotype     | Controls (n = 690) | Azoospermia (1) (n = 110) | Severe Oligozoospermia (2) (n = 89) | Oligozoospermia (3) (n = 58) | Total (1+2+3) (n = 257) | Total (1+2+3+4) (n = 344) |
|--------------|--------------------|---------------------------|-----------------------------------|---------------------------|-------------------------|---------------------------|
|              | N (% )             | N (%)                     | N (%)                             | N (%)                     | N (%)                   | N (%)                     |
| CC           | 351 (50.87)        | 33 (30.90)                | 34 (37.78)                        | 152 (51.54)               | 47 (46.12)              | 199 (58.23)               |
| CT           | 286 (41.45)        | 30 (27.27)                | 21 (23.58)                        | 88 (30.41)                | 125 (48.78)             | 205 (59.61)               |
| TT           | 53 (7.68)          | 8 (7.27)                  | 3 (3.35)                          | 17 (6.14)                 | 20 (7.75)               | 37 (10.76)                |
| CT+TT        | 339 (49.13)        | 45 (40.91)                | 36 (40.45)                        | 106 (40.66)               | 145 (56.51)             | 250 (72.61)               |
| C            | 988 (71.58)        | 167 (73.91)               | 136 (76.40)                       | 89 (76.72)                | 302 (76.26)             | 523 (76.02)               |
| T            | 392 (28.42)        | 53 (24.09)                | 42 (23.60)                        | 27 (23.28)                | 122 (23.74)             | 245 (23.98)               |

| Odds Ratio  | 95% CI*     | p value* |
|--------------|------------|----------|
| CC           | CT         | TT       | CT+TT   | C         | T         |
| Odds Ratio   | 1          | 2         | 3        | 1+2+3     | 1+2+3+4   | 1         | 2         | 3         | 1+2+3     | 1+2+3+4   |
| Controls     | vs. (1)    | (2)       | (3)      | (1+2+3)   | (1+2+3+4) | vs. (1)    | (2)       | (3)      | (1+2+3)   | (1+2+3+4) |
| N (%)        | (50.87)    | (41.45)   | (7.68)   | (49.13)   | (71.58)   | (50.87)    | (41.45)   | (7.68)   | (49.13)   | (71.58)   |
| N (%)        | (30.90)    | (33.64)   | (37.78)  | (40.91)   | (73.91)   | (30.90)    | (33.64)   | (37.78)  | (40.91)   | (73.91)   |
| N (%)        | (37.78)    | (33.64)   | (30.90)  | (37.78)   | (33.64)   | (37.78)    | (33.64)   | (30.90)  | (37.78)   | (33.64)   |
| N (%)        | (51.54)    | (40.41)   | (37.78)  | (40.41)   | (37.78)   | (51.54)    | (40.41)   | (37.78)  | (40.41)   | (37.78)   |
| N (%)        | (46.12)    | (60.41)   | (30.90)  | (60.41)   | (30.90)   | (46.12)    | (60.41)   | (30.90)  | (60.41)   | (30.90)   |
| N (%)        | (58.23)    | (76.40)   | (76.72)  | (76.40)   | (76.72)   | (58.23)    | (76.40)   | (76.72)  | (76.40)   | (76.72)   |
| N (%)        | (23.74)    | (23.28)   | (23.28)  | (23.28)   | (23.28)   | (23.74)    | (23.28)   | (23.28)  | (23.28)   | (23.28)   |
| Odds Ratio   | 0.7163     | 0.7186    | 0.802    | 0.7358    | 1.045     | 0.8064     | 0.7421    | 0.9653   | 0.869     | 0.8515     |
| 95% CI       | (0.4653–1.09) | (0.4469–1.139) | (0.4529–1.394) | (0.5444–0.9909) | (0.6619–1.641) | (0.616–1–0.52) | (0.4718–1.481) | (0.1042–1.261) | (0.4279–1.231) | (0.4279–1.231) |
| p value      | 0.4635     | 0.7186    | 0.6559   | 0.802     | 1.045     | 0.8064     | 0.7421    | 0.9653   | 0.869     | 0.8515     |
| Odds Ratio   | 0.7312     | 0.7155    | 0.7155   | 0.7155    | 0.7546    | 0.1337     | 0.1529    | 0.3185  | 0.0281    | 0.0400     |
| 95% CI       | (0.4201–1.258) | (0.345–0.9558) | (0.345–0.9558) | (0.345–0.9558) | (0.3611–1.38) | (0.2134–0.0483) | (0.1502–0.052) | (0.081–0.049) | (0.0385–0.0129) |
| p value      | 0.2841     | 0.0281    | 0.0281   | 0.0281    | 0.0401    | 0.2134     | 0.1502    | 0.081   | 0.0385    | 0.0129     |

CC, wild type homozygote; CT, heterozygote; TT, mutant homozygote; OR, odds ratio; CI, confidence interval.
Asth, Asthenozoospermia; Tera, Teratozoospermia.
* Controls vs. (1), (2), (1+2+3), (1+2+3+4).
doi:10.1371/journal.pone.0034111.t001
### Table 2. Distribution of A1298C polymorphism in methylenetetrahydrofolate reductase (MTHFR) gene.

| Genotype | Controls (n = 690) (n = 110) | Azoospermia (1) (n = 89) | Severe Oligozoospermia (2) (n = 58) | Total (1+2) (n = 257) | Asthen/and/or Teratozoospermia (4) (n = 87) | Total (1+2+3+4) (n = 344) |
|----------|-----------------------------|--------------------------|-----------------------------------|-----------------------|--------------------------------------------|--------------------------|
|          | N (%)                       | N (%)                    | N (%)                             | N (%)                 | N (%)                                      | N (%)                    |
| AA       | 370 (53.62)                 | 49 (60.91)               | 38 (53.06)                        | 154 (59.02)           | 51 (58.62)                                 | 205 (59.92)              |
| AC       | 303 (43.45)                 | 39 (35.45)               | 33 (37.08)                        | 122 (35.47)           | 31 (35.68)                                 | 91 (35.41)               |
| CC       | 17 (2.46)                   | 5 (7.86)                 | 1 (1.72)                          | 12 (5.75)             | 17 (4.94)                                  | 1 (0.1184)               |
| AG/CC    | 320 (46.38)                 | 40 (44.94)               | 20 (44.48)                        | 36 (41.38)            | 139 (40.41)                                | 139 (41.38)              |
| A        | 1043 (75.58)                | 173 (78.64)              | 131 (73.68)                       | 95 (81.90)            | 133 (76.44)                                | 133 (72.33)              |
| C        | 337 (24.42)                 | 47 (21.16)               | 47 (26.40)                        | 21 (18.10)            | 115 (22.37)                                | 115 (22.37)              |

| Odds Ratio (95%CI)* | p value* |
|---------------------|----------|
| AA                  |          |
| AC                  |          |
| CC                  |          |
| AG/CC               |          |
| A                   |          |
| C                   |          |

AA, wild type homozygote; AC, heterozygote; CC, mutant homozygote; OR, odds ratio; CI, confidence interval.

*Controls vs. (1+2)/3 or (1+2+3)/4.

Asth, Asthenozoospermia; Tera, Teratozoospermia.

doi:10.1371/journal.pone.0034111.t002
*MTHFR* activity by 30% in heterozygotes (CT) and 70% in homozygotes (TT) [13]. This polymorphism has been reported to result in mild hyperhomocysteinemia particularly in patients with low folate intake [30]. Our data indicate a lack of association of the C677T variant with infertility. This is in contrast with the reports where the C677T variant was associated with infertility [17,21,22,29,31]. A study of 77 subfertile men of Dutch ancestry found no significant difference in the frequency of the CC/CT/TT genotypes between the case and the control group, implying a lack of association between for *MTHFR* C677T and infertility, [32]. Another study comprising of 93 infertile and 105 fertile and/or normospermic individuals of Italian ancestry did not find any association between the C677T allele and male infertility [23]. Recently, a meta-analysis was performed in a total of 10 case–control studies, including 2275 cases and 1958 controls; this meta-analysis supports that *MTHFR* C677T polymorphism is capable of causing male infertility susceptibility in Asians, but not in Caucasians [33]. These contradictory results from studies on different populations suggest that the role of C677T in susceptibility to male infertility may depend on environmental factors such as levels of dietary folate uptake [29].

The *MTHFR* A1298C variant results in a glutamine to alanine change at codon 429 (exon 7) and is found in a regulatory region of the *MTHFR* enzyme. This variant results in a decrease *MTHFR* activity, which is more pronounced in the homozygous (CC) than in the heterozygous (AC) or wild type (AA) genotype [14]. Although the *MTHFR* p.429A variant is not thermolabile, in vitro studies have shown that this variant has approximately 65% of wild-type enzymatic activity compared to that of 40% of wildtype conferred by the C677T variant [13]. Here, we observed a deviation from the Hardy-Weinberg equilibrium for the *MTHFR* 1298 C genotype in the control population. Although a number of factors may be responsible for the observed deviation from the Hardy-Weinberg expectation, we suggest that our data are consistent with purifying selection impacting on the 1298CC genotype. Indeed, we observed a strong association between the 1298CC genotype and severe oligozoospermia (OR = 3.372, 95% CI = 1.27–8.238; p = 0.01431) as compared to the control group. There is growing evidence to indicate that the 1298C variant may be a genetic risk factor for male infertility. A recent study on 151 men with idiopathic infertility and 140 healthy fertile controls of Indian origin concluded that the *MTHFR* 1298CC genotype is a genetic risk factor for idiopathic male infertility in an Indian population [35]. Meta-analyses of the published data concerning *MTHFR* variants and infertility is inconclusive. A study of 1633 cases and 1735 controls from seven case control studies identified the 1298C allele as a genetic risk factor for infertility, whereas a more comprehensive meta-analyses of 3,850 cases and 4,085 controls from twelve published case–control studies did not observe an association of the 1298C genotype with male infertility, although an association with the *MTHFR* 677T allele was detected [36,37]. In both of these studies the genetic association was observed in specific phenotypic subgroups and/or in association with specific ethnic groups. Genetic association following phenotype or ethnic stratification was also reported by Wu et al. [33]. In conclusion our data suggest a link between the *MTHFR* variant 1298C and unexplained reduced sperm counts, at least in the Moroccan population. Further studies are necessary to determine the influence of the environment, especially the consumption of dietary folate, on sperm counts of men with different *MTHFR* variants. Since a significant proportion of men with severe oligozoospermia have been reported to have methylation anomalies in their mature sperm [9,38], the role of *MTHFR* status, dietary folate and methylation profiles and their co-relationship in human sperm also merits further investigation.

**Table 3.** Hardy-Weinberg distribution for *MTHFR* 1298 genotypes in infertile and control populations.

| Allele X Allele Y Observed frequency % HW Expected frequency % Chi² P value |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| AA             | 1150           | 0              | 575            | 55.61          | 600            | 58.00          | 1.023          |
| AC             | 425            | 425            | 425            | 41.10          | 375            | 36.31          | 6.533          |
| CC             | 0              | 68             | 34             | 3.29           | 59             | 5.68           | 10.436         |
| Total          | 1575           | 493            | 1034           | 100.00         | 1034           | 100.00         | 1034           |
|                |                |                |                |                |                |                |                |

**Table 4.** Hardy-Weinberg distribution for *MTHFR* 1298 genotypes in control population.

| Allele X Allele Y Observed frequency % HW expected frequency % Chi² P value |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| AA             | 740            | 0              | 370            | 53.62          | 394            | 57.12          | 1.479          |
| AC             | 303            | 303            | 303            | 43.91          | 255            | 36.91          | 9.158          |
| CC             | 0              | 34             | 17             | 2.46           | 41             | 5.96           | 14.172         |
| Total          | 1043           | 337            | 690            | 100.00         | 690            | 100.00         | 24.809         |

P value
Table 5. Association between the combined MTHFR alleles and infertility.

| Geno-type    | N (%)       | N (%)       | N (%)       | N (%)       | N (%)       | N (%)       | Odds Ratio (95%CI)* | p value*                |
|--------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------------|-------------------------|
| Controls     | (n=690)     | Azoopermia  | (1) (n=110) | Severe Oligozoospermia | (2) (n=89) | Oligozoospermia | (3) (n=58) | Total (1+2+3) | (n=257) | Asth and/or Téra | (4) (n=87) | Total (1+2+3+4) | (n=344) | 1 | 2 | 3 | 1+2+3 | 4 | 1+2+3+4 | 1 | 2 | 3 | 1+2+3 | 4 | 1+2+3+4 |
| CCAA         | 164 (23.76) | 36 (24.72)  | 22 (34.48)  | 78 (30.35)  | 26 (29.89)  | 104 (30.23) | 1.559 (1.001–2.402) | 0.05786 0.9474          |
| CCAC         | 186 (26.96) | 26 (28.09)  | 25 (22.41)  | 64 (24.90)  | 17 (19.54)  | 81 (23.55)  | 0.8389 (0.5164–1.333) | 0.5376 0.9206           |
| CCCC         | 10 (1.45)   | 3 (2.73)    | 8 (9.99)    | 1 (1.72)    | 12 (4.67)   | 4 (4.60)    | 1.905 (0.4179–6.691)  | 1.0608 0.6647           |
| CTA A        | 174 (25.22) | 24 (21.82)  | 20 (23.86)  | 59 (26.96)  | 22 (25.29)  | 81 (35.35)  | 0.6278 (0.502–2.331)  | 0.1853 0.09785          |
| CTA C        | 113 (16.38) | 12 (10.91)  | 8 (9.99)    | 6 (10.34)   | 26 (10.12)  | 14 (11.63)  | 0.6296 (0.3191–1.149) | 0.8983 0.003880          |
| CTC C        | 0 (0)       | 1 (0.91)    | 0 (0)       | 1 (0.91)    | 0 (0)       | 1 (1.15)    | 0.297 (0.091–0.91)   | 0.2103                  |
| TTA A        | 35 (5.07)   | 7 (6.36)    | 6 (6.36)    | 3 (5.17)    | 16 (6.23)   | 3 (4.50)    | 1.271 (0.512–2.386)  | 0.7386 0.6807           |
| TTA C        | 8 (1.16)    | 1 (0.91)    | 0 (0)       | 1 (0.91)    | 0 (0)       | 1 (0.91)    | 1.023 (0.0346–4.938) | 0.7983 0.6437           |
| TCC C        | 0 (0)       | 0 (0)       | 0 (0)       | 0 (0)       | 0 (0)       | 0 (0)       | 0.288 (0.01108–1.564) | 0.8579 0.6210           |

OR, odds ratio; CI, confidence interval.
*Controls vs. (1+2),(3),(1+2+3),(4),(1+2+3+4).
doi:10.1371/journal.pone.0034111.t005
Author Contributions
Conceived and designed the experiments: AE OA HR. Performed the experiments: AE OA MG. Analyzed the data: AE KM HR. Contributed reagents/materials/analysis tools: AE BE HB NL EC MA AB. Wrote the paper: AE KM.

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