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**Original article**

# Association between A1298C Variants of the MTHFR Gene and Male Infertility in the Indian Population: A Case-Control Study

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**Abstract:**

Background: Methylenetetrahydrofolate reductase (MTHFR) is a crucial enzyme involved in folate metabolism, and its reduced activity can result in irregular sperm production, ultimately leading to male infertility. According to previous studies there is a connection between MTHFR gene polymorphisms and male infertility, but the impact remains unclear due to various factors. This study investigated the potential association between A1298C variations in the MTHFR gene and infertility in males among Indian population.

Methods: A total of 127 infertile men were selected as the case group, and an equal number of controls were subjected to molecular genetic analysis. The genotype SNPs were analyzed using PCR-RFLP. The groups were statistically compared. Statistical significance was set at $p<0.05$.

Results: Statistically significant difference were detected with a P value of 0.031 ($p < 0.05$) which indicated an association between the occurrence of the A1298C polymorphism and male infertility.

Conclusion: There was a significant association between the MTHFR A-1298C gene variant and an increased likelihood of male infertility

**Keywords:** Folate, Polymorphism, MTHFR, A1298C, Male Infertility

## Introduction:

Infertility has become a significant global health concern. Infertility is the lack of ability to reproduce within 1-2 years of unprotected, regular sexual intercourse (1). Male infertility is a disorder with multiple causes that revolves around life patterns, genetics, environmental factors and medical history (2). Production of sperm is mediated by complex gene expression processes. Genes on the Y chromosome and autosomal genes regulate spermatogenesis at various stages, and their abnormal expression can result in infertility (3). Many genetic studies have shown association of abnormal congenital genetic disorders and polymorphisms with male infertility (4). Men with azoospermia have a 20% chance of developing chromosomal abnormalities, which have been established as one of the most prevalent causes of male infertility (5). Folate, its cofactors, and vitamins B2, B6, and B12 keep the deoxynucleotide at a constant level; if this process is hampered, uracil may be retained in the DNA backbone instead of thymine. This occurrence is similar to a mutational alteration that eventually leads to uracil binding to adenine, the typical base partner of thymine, in the DNA double helix (6). Abnormal methylation can activate latent transposons which may lead to chromosomal breakage and genomic instability and ultimately leading to a sick



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phenotype (6). Folate deficiency is relatively common and the resulting hyperhomocysteinemia is linked to many illnesses, including male infertility (7). MTHFR is an important regulator of folate metabolism and is essential for cell health. MTHFR role is to convert 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate and a methyl donor, which are needed for methionine synthesis from homocysteine. Methionine, in turn, makes available the methyl group to produce S-adenosylmethionine, which is involved in a variety of biological processes such as DNA, RNA, and histone methylation (7). The MTHFR gene's reduced activity has been linked to multiple disorders, such as irregular sperm production, which can ultimately result in male infertility (8) (9).

Although certain studies had implicated a connection between MTHFR anomalies and male infertility among Caucasians and Asian populations, the precise impact of MTHFR polymorphisms on male infertility remains unclear. Notably, there have been limited reports on this topic among other human races, particularly Africans (10). The relationship between the gene-polymorphisms in MTHFR and infertility in males has been the subject of conflicting research outcomes. This is due to various factors, including the selection process for research populations, differences in genetics, and variations in environmental conditions (11). The MTHFR gene is composed of 11-exons present in the short arm of chromosome 1 (1p36.3) (12, 13). There are mainly three kinds of variations in the DNA sequence (at a single position ) of the MTHFR gene termed- Single Nucleotide Polymorphisms (SNPs) that consequently impact these enzymes activities (14). These consist of C677-T, A1289-C, G1793-A (15,16). Polymorphism of the MTHFR A1298-C gene that leads to mutation of cytosine- adenine in exon 7 points of the gene where alanine replaces glutamate (Glu429Ala) (17).

In this research, we have conducted an investigation into the potential association between A1298C variations in the MTHFR gene and infertility in males among the Indian population.

**Methods:**

Patients and controls:

A total of 127 infertile men were selected as the case group which underwent investigation for molecular genetic analysis. Concurrently, an equal number of controls were subjected to molecular genetic analysis. Formal extended family studies were carried out for individuals presenting with a normal karyotype. The recruitment of patients was carried out at Wardha Test Tube Baby Center, Acharya Vinoba Hospital, Sawangi, Wardha, Maharashtra. The inclusion criteria included infertile male patients with a normal karyotype, healthy female partners, primary infertility, abnormal semen parameters (oligozo-ospermia, azoospermia, etc.), normal sexual and ejaculatory function without obstruction, varicocele, and no history of infection or diseases causing infertility. Conversely, the exclusion criteria covered patients with an abnormal karyotype, normal semen parameters, partners with infertility issues, secondary infertility, obstructive azoospermia, abnormal sexual and ejaculatory function, and a history or presence of diseases/infections causing infertility. All participants in the study signed informed written consent and the ethical committee of our Institute gave the approval for this study.

DNA extraction and PCR-RFLP analysis:

Screening for MTHFR A1298C polymorphism PCR amplification:

Singh et al. described primers were used to screen for the A1298/C mutation in exon 7 of the MTHFR gene. A total reaction amount of 20µl was used for PCR amplification, which included 2µl of 100ng/µl of DNA, 0.2µl each of reverse and forward primers at a final concentration of 0.1M, 10µl of master mix, and 7.6µl of milliQ water. The PCR was done as follows: firstly, denaturation is done at 94°C for 4 minutes, then continue denaturation for 30 cycles at 94°C for 30 seconds, followed by annealing which is done at 62°C for 30 seconds, and then extension took place at 72°C for 40 seconds. The final extension occurred at 72°C for 5 minutes and kept at 4°C. Following the completion of the heat cycle, the samples were PCR products. The products of amplification were assessed and analyzed on agarose gels and then using Mbo II restriction enzymes, they were subjected to enzymatic digestion. Following viewing with a gel documentation system, the distinct band structures corresponding to the mutations were recorded.

Statistical analysis:

The gene counting approach was used to determine gene frequencies, and the chi square test was employed to calculate the deviation from observed frequencies. Using 2x2 contingency tables, the odds



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ratio (OR) for the genotype and homozygous and heterozygous frequencies of A1298C in the MTHFR gene was calculated. A genotypic test was done to assess the potential link between the single nucleotide polymorphism and male infertility. All analyses were conducted using SPSS 26.0 for Windows Student Version (SPSS Inc., Chicago, USA). A 0.05 as P value was considered significant. The mean and standard deviation with maximum and minimum ranges, were utilized for the quantitative assessment of physiological parameters.

**Results:**

Descriptive statistical data for physiological measures, including minimum value, mean, minimum value and the values of standard deviation for the variables of age (in years), height in feet and inches, weight in kilograms, and BMI (Body Mass Index) (kg/m2), are presented in the table 1.

| | Cases (N=127) | | | | Control (N=127) | | | |
|---|---|---|---|---|---|---|---|---|
| **Variables** | **Minimum** | **Maximum** | **Mean** | **Std. Deviation** | **Minimum** | **Maximum** | **Mean** | **Std. Deviation** |
| Age (in Years) | 26 | 49 | 35.66 | 5.331 | 25 | 39 | 32.13 | 2.859 |
| Height (Feet, inch) | 5.1 | 6.1 | 5.665 | 0.2249 | 5 | 6.11 | 5.63 | 0.2464 |
| Weight (Kg) | 58 | 90 | 72.51 | 6.488 | 55 | 110 | 70.09 | 7.224 |
| BMI ($Kg/m^2$) | 19.21 | 37.09 | 24.38 | 2.61 | 18.87 | 35.19 | 23.79 | 2.41 |

Table 1. Descriptive statistics Physiological variables (Mean) Age, Height, weight, BMI. (Cases &Control)

The information presented in Table 1 displays the age values for a sample of 127 individuals, comprised of 127 cases and 127 controls. The average age for the case group was 35.66 years, with 5.331 of standard deviation, while the average age for the control group was 32.13 years, with 2.859 standard deviation. The maximum age in the case group was 49 years and minimum age recorded is 26 years, whereas in control group, maximum age is 39 years and minimum are 25 years. The height values for the case group had a mean of 5.665 feet, with 0.22 feet standard deviation, while the control group had 5.63 feet mean and 0.2464 feet standard deviation. The maximum height for the case group was 6.1 feet, with a minimum of 5.1 feet, compared to a maximum of 6.11 feet and a minimum of 5 feet for the control group. The mean weight values for the case group were 72.51 kilograms, with a 6.488 standard deviation, and a maximum of 90 kilograms and a minimum of 58 kilograms. The control group had a mean weight of 70.09 kilograms, with a standard deviation of 7.224, and a maximum of 110 kilograms



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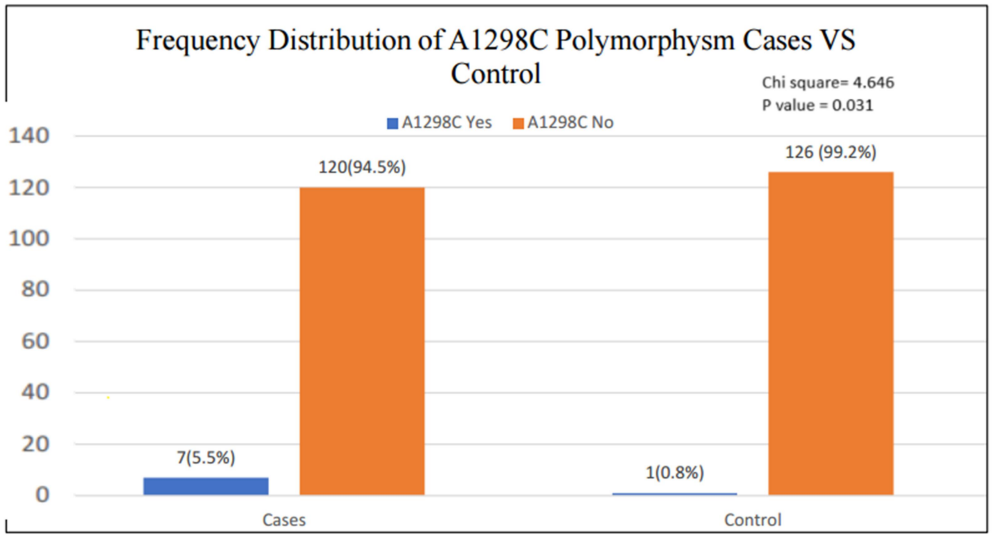
and a minimum of 55 kilograms. The body mass index (BMI) values for the case group had a mean of 24.38, with 2.61 standard deviation, and a maximum of 37.09. The control group had a mean BMI of 23.79, with 2.41 standard deviation, and a maximum of 35.19. The minimum BMI for the case group was 19.21, whereas for control group, minimum BMI is18.87.

MTHFR A1298C genotype analysis: Table 2 shows MTHFR A1298C analysis between Cases and Controls. While Table 3 shows genotype frequencies of A1298C Variants of methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms in Cases and Controls.

| | | A1298C | | Total | Chi Square Test | P-value | Odd Ratio | CI |
|---|---|---|---|---|---|---|---|---|
| | | No | Yes | | | | | |
| Group | Cases | 120 (94.5%) | 7(5.5%) | 127 | 4.646 | 0.031* | 7.35 | 0.89 to 60.63 |
| | Control | 126(99.2%) | 1(0.8%) | 127 | | | | |
| Total | | 245 | 8 | 254 | | | | |

Table 2. MTHFR A1298C analysis-Cases and Controls

The odds ratio analysis of cases and controls with a record of 7 (5.5%) for the A1298C mutant in cases compared to 1 (0.8%) in controls has yielded significant results with 0.031 p value ($p < 0.05$). This indicated a close association between the occurrence of the polymorphism in A1298C and male infertility. The odds ratio value of O.R = 7.35, with a confidence interval of (0.89 to 60.33), demonstrated that the odds of the A1298C polymorphism occurring in the cases were 7.35 times higher than the odds of it occurring in the control group. Graph 1 represents frequency distribution of A1298C Polymorphism among Cases vs Controls.




Graph 1. Frequency distribution of A1298C Polymorphism -Cases vs Controls



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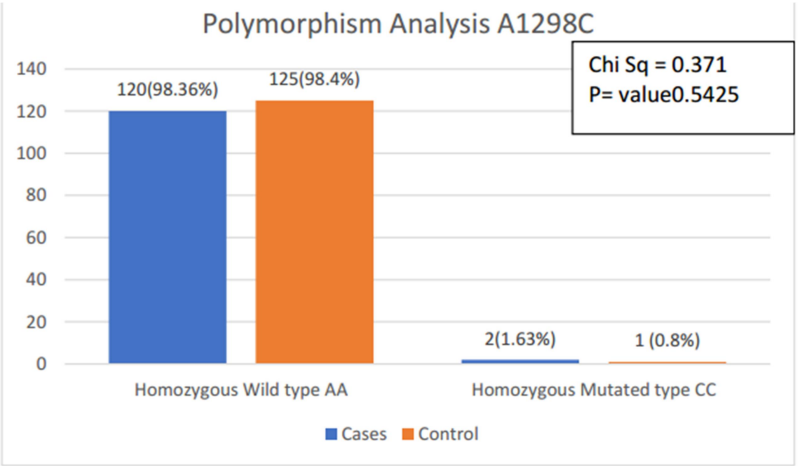
| **Odd ratio** | | AA | CC | Chi Square Test | P-value | Odd Ratio | CI |
|---|---|---|---|---|---|---|---|
| **Group (A1298C)** | **Cases** | 120(98.36) | 2 (1.63%) | 0.371 | 0.5425 | 2.08 | 0.19 to 23.28 |
| | **Control** | 125 (98.4%) | 1(0.8%) | | | | |
| **Total** | | 245 | 3 | | | | |
| **Odd ratio** | | AA | AC | Chi Square Test | P-value | Odd Ratio | CI |
| **Group (A1298C)** | **Cases** | 120(96%) | 5 (4%) | 2.76 | 0.09 | 5.2 | 0.59 to 45.23 |
| | **Control** | 125 (99.21%) | 1(0.79%) | | | | |
| **Total** | | 245 | 6 | | | | |

Table 3. Genotype frequencies of A1298C Variants of methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms in fertile and infertile men

The frequency of the mutation in A1298C with CC genotype (homozygous) was observed in two cases (1.64%) and one control (0.80%), resulting in insignificant findings with a P-value of 0.5425 and an odds ratio value is 2.08 (0.19-23.28). For the A1298C mutation with a heterozygous (AC) genotype, the frequency was noted in five cases (4%) and one control (0.79%), again yielding insignificant results where P-value is 0.09 and an odds ratio is 5.2 (0.59-45.23).




Graph 2. Frequency distribution of A1298C Polymorphism (Wild type AA and mutate type CC -Cases vs Controls



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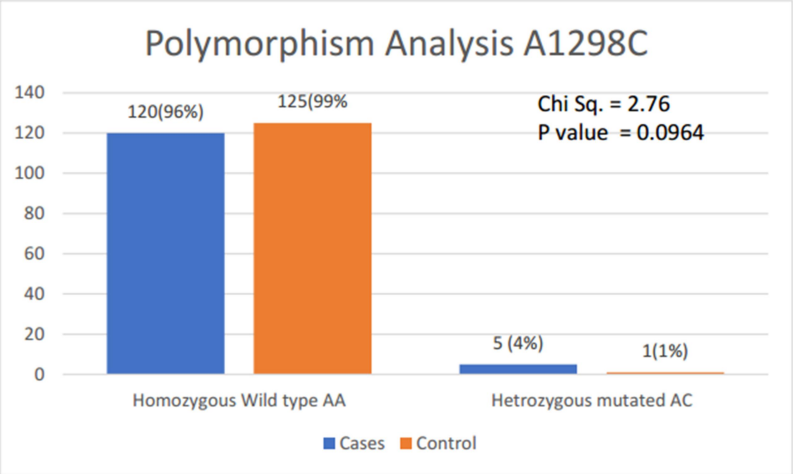


Graph 3. Frequency distribution of A1298C Polymorphism (Wild type AA and mutate type AC -Cases vs Controls)

**Discussion:**

In our research, we observed a significant correlation of the MTHFR A-1298C gene variant with an increased risk of male infertility, as prevalence is 5.5% in the test group whereas 0.8% in the control group. The odds ratio for this association is 7.35, with a confidence interval of 95% that ranges from 0.89 to 60.63, and a significance level of 0.031. Some studies suggest that the A-1298C variation may serve as one of the genetic risk factors causing male infertility. As a recent study, which included 50 males with oligozoospermia and azoospermia (non-obstructive), and a control group of 50 fertile individuals of Indian origin, showed that the presence of MTHFR A-1298C mutations constitutes a genetic risk factor for causing male infertility in the Indian population (18). Similarly, a study by Singh et al. (2010) concluded a significant link between A-1298C gene mutation and infertility in males (19). Several investigators have also reported a significant relationship between A-1298C MTHFR variations and in infertile patients from South Korea, Morocco, Brazil, and India (18-21). Although Study by Kim SY et al and Mfady DS et al reported no significant relation between A-1298C gene mutation and male infertility ($p>0.05$) (21,22). In India, Dhillon et al. (2007), in a study, found no correlation between this gene mutation and male infertility (23). Our findings are consistent with those of Singh et al. (2010) and Balunathan et al. (2021), who reported a statistically significant association between the A-1298C mutation and male infertility ($p<0.05$). According to Shen et al.'s (2011) meta-analysis, the presence of the A-1298C polymorphism has association with an increased risk of male infertility, particularly azoospermia. The frequency of the C-1298 allele was found to be substantially correlated with male infertility risk in that study. An analysis subgroup of the individuals reported that the homozygote comparison and recessive model of MTHFR 1298C were associated with a significantly elevated risk of azoospermia.

The potential association between the MTHFR A1298C polymorphism and male infertility warrants further investigation. It is essential to conduct comprehensive research on a large population to gain a more knowledge of the factors that contribute to male infertility.

**Conclusion:**

We aimed to explore the genetic elements that contribute to primary male infertility using cytogenetic analysis. A total of 254 men were subjected to molecular analysis for polymorphisms in genes encoding 5,10-methylenetetrahydrofolate reductase (MTHFR), with a focus on the A-1298C variant. In our study, we observed a prevalence of 5.5% for the A-1298C gene polymorphism in the test group and 0.8% in the control group. Our analysis of gene frequencies using the Pearson chi-square test revealed a significant association between the MTHFR A-1298C gene variant and an increased likelihood of male infertility, which is consistent with the findings of Singh et al. (2010) and Balunathan et al. (2021).

**Limitations of the Study:**
This study was limited by its small sample size. To address this issue, future research should consider increasing the sample size.

**Declaration of Conflict of Interest:**
The authors confirm that they have no financial or personal relationships that may have influenced the study findings

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