

## Study Protocol

### “Correlation of MTHFR Gene Polymorphism with Male Infertility”

**Comment [S1]:** It is better to determine the SNP in the title (C677T)

#### Abstract:

**Background:** Folate is present in Vitamin B, which is not present in the body, and one has to consume it from outside. Its functions are to help in the production of RBCs, helps in the production of genes, DNA, RNA, and protein, helps in the metabolism of homocysteine, and is also the main component which is required for the process of methylation. For the metabolism of the folate group, the MTHFR gene is responsible. When there is a problem occurs in the MTHFR gene such as SNP, then it can create a problem with many allied metabolisms and other biological reactions or processes in the body of human and deficiency of folate in the body may lead to affect various processes such as it can increase the homocysteine level in the body and can cause hyperhomocysteinemia, which has been linked with many diseases along with causing male infertility.

**Comment [S2]:** It is better to replace by more scientific definition like (Folate is the natural form of vitamin B)

**Comment [S3]:** It is better to be replaced by (it is one of the essential vitamins)

**Comment [S4]:** It is better to mentioned as name (Methylenetetrahydrofolate reductase (MTHFR) for the first time in text

**Comment [S5]:** It is better to mention as Single nucleotide polymorphisms (SNPs).

**Comment [S6]:** Human body

#### Objectives

1. To identify nucleotide polymorphism in cases and control group.
2. To correlate polymorphism status with male infertility.
3. To correlate MTHFR polymorphism with abnormal semen parameters.
4. To correlate BMI with male infertility.

**Methodology:** For this study, we will use various collective methods to conduct the study which includes the recording of treatment history and the indications of infertile patients visiting WARDHA TEST TUBE CENTRE and counselling them. After that semen collection and analysis will be done along with blood collection, DNA extraction, DNA quantification with nanodrop and agarose gel, PCR, and RFLP. We have also made use of PubMed for getting related articles.

**Comment [S7]:** Why the recording is restricted to treatment history only?, data may include medical history, presence of any chronic diseases, history of any familial or genetic disorders, history of any thromboembolic events...

**Results and conclusion:** We have done many searches on PubMed and NCBI sites (<http://www.ncbi.nlm.nih.gov/pubmed/>) with keyword MTHFR gene, polymorphism in MTHFR gene, methylation, folate methylation, methylenetetrahydrofolate reductase, methylenetetrahydrofolate reductase, and primer for MTHFR gene and retrieved many articles related to primers for MTHFR gene and will design our primer for forward and reverse annealing with the help of Amplify software (Engels, 1993). With the help of our study, we will analyze the MTHFR genomic sequence along with analysis of RFLP, in which we will design an amplicon of 513bps in which we will find restriction site for Hinf I and will check that if polymorphism is there then it will cut the DNA amplicon into two and will confirm it with electrophoresis.

**Comment [S8]:** for

**Keywords:** MTHFR, SNPs, polymorphism, male infertility, folate metabolism, methylenetetrahydrofolate reductase, homocysteine, MTHFR gene;

## INTRODUCTION:

To maintain progeny, reproduction and fertility are foremost. As claimed by WHO, infertility is expounded as ineptitude to propagate through sexual reproduction unescorted by using any contraception.<sup>(1)(2)</sup> Worldwide, 15% of couples are agonizing over infertility, which means 48.5 million couples.<sup>(2)</sup> In 20-30% of infertility cases are of males exclusively and come up with 50% of cases all in all.<sup>(3)</sup> Despite the facts that, customary semen scrutiny has been arraigned as not verifiable assessment of sperm concomitant rooted on its substandard divination of fertility when juxtaposed to further worldly-wised tried out such as sperm penetration, capacitation, acrosome reaction, and, more freshly, by sperm chromatin structure assay (SCSA) for the discernment of DNA integrity.<sup>(4)</sup>

Still the process of non-success formation of spermatogonia is the main event that matters in these causes of infertility.<sup>(5)</sup>

In the process of formation of spermatogonia more or less 10% of genes are responsible.<sup>(6)</sup> Out of this, it has been put forward that, genetic deformities contemplated for male factor infertility (15%-30%), which incorporate Y-chromosome microdeletions (YCM), translocation, chromosomal anomaly, and single gene mutation which may cause azoospermia or aspermia in males.<sup>(7)</sup>

In these times many studies are going on to analyze the role of mutation in folate-related enzyme genes on male infertility and the main gene which is in suspect is the MTHFR gene and they reckon that the main effect on male infertility is due to a single nucleotide polymorphism in that MTHFR gene.

## SINGLE NUCLEOTIDE POLYMORPHISM

The most customary and natural mutation in the human genome are SNPs, which represents single nucleotide alteration in the genome and differ between individuals within populations.<sup>(8)</sup> Single nucleotide polymorphism make-up take around 90% of human genetic mutation and it take place once in take around 100–300 base pairs. These SNPs can act as biological markers which are associated with many genetic diseases. SNPs are participated in causing many diseases and some SNPs do not play any role in causing diseases. Alike in SNP in MTHFR gene can be responsible for male factor infertility by causing mutation in folate related enzyme gene which take part in a crucial role in the synthesis of DNA and its methylation.<sup>(9)</sup>

## ROLE OF MTHFR GENE IN MALE FACTOR INFERTILITY

The MTHFR gene which is on chromosome one commands for the formation of enzyme methylenetetrahydrofolate reductase which reduces 5,10- methylenetetrahydrofolate to 5-methylenetetrahydrofolate.<sup>(10)</sup> Folates group in the enzyme take part in the production of genes, proteins, and also in the process of methylation reaction.<sup>(11)</sup> Disturbance in this gene can cause accumulation of homocysteine which can cause hyperhomocysteinemia<sup>(12)</sup> which can decrease the normal level of folate inside the body<sup>(13)</sup> and responsible for increased reductive oxidative stress<sup>(13)</sup> and can damage sperms or the whole process of formation of sperms that is spermatogenesis and this is how it can be responsible for the male factor infertility.<sup>(14)(15)</sup>

In this study we will compare the genetic status (single nucleotide polymorphism), BMI, hormonal level i.e., Serum testosterone, LH, FSH, reductive oxidative stress, patient's sperm count, and morphology of sperm of male partners visiting Wardha Test Tube Baby Centre.

**Comment [S9]:** These parameters did not mentioned in your methods

This comparison will be done with the control who had a healthy baby without any infertility treatment.

**Comment [S10]:** It is better to mention that if they are volunteers or seeking any medical advice.

## HYPOTHESIS

The reason for the increase in male infertility may be due genetic factor like single nucleotide polymorphism.

## RESEARCH QUESTION

**STUDY TYPE :-** Case control study

**POPULATION:-** Infertile men attending WARDHA TEST TUBE BABY CENTRE, AVBRH, Sawangi (Meghe), Wardha.

**Comment [S11]:** It is better to add India

**INTERVENTION:-** NA

**COMPARISON:-** Nucleotide polymorphism in patient and control group will be compared.

**OUTCOME:-** Outcome may provide insights about *MTHFR* nucleotide polymorphism in study group and its correlation with male infertility.

## AIM AND OBJECTIVES

### AIM:

To identify *MTHFR* gene polymorphism and to correlate its role in male infertility.

### OBJECTIVES

1. To identify nucleotide polymorphism in cases and control group.
2. To correlate polymorphism status with male infertility.
3. To correlate *MTHFR* polymorphism with abnormal semen parameters.
4. To correlate BMI with male infertility.

**Comment [S12]:** Why you did not mention the type of polymorphism you will searched for (*MTHFR* C677T)?

**Comment [S13]:** In your methods or study design you did not mention any data about BMI!

## MATERIALS AND METHODS

**Study Design** – Case control study

**Study duration**– June 2019-June 2022

**Study population** – Male partner from couple attending WARDHA TEST TUBE BABY CENTRE AT AVBRH

**Place of study**– WARDHA TEST TUBE BABY CENTRE AT AVBRH

**Sample size** –

95 infertile male

**Sample size formula** –

$$N = \frac{\chi^2 * N * p(1-p)}{C^2(N-1) + \chi^2 p(1-p)}$$

$$C^2(N-1) + \chi^2 p(1-p)$$

$$\text{Total population} = N=120$$

$\chi^2$  = Chi-square value for 1 degrees at some desired probability level. This is 3.84 at 5%

**Comment [S14]:** India

level of significance.

P=50% proportion

Q=100-p

=50

C= Confidence interval of the one choice(95% CI)

=0.05

$N = \frac{3.84 * 120 * 0.5 * 0.5}{(0.05)^2}$

$= \frac{3.84 * 120 * 0.5 * 0.5}{(0.05)^2}$

=91.61

Total Sample Size required in this study will be 95.

**Comment [S15]:** You must mention number of patients and number of controls

## INCLUSION CRITERIA AND EXCLUSION CRITERIA:

### INCLUSION CRITERIA

1. A normal 46 chromosome with XY karyotype
2. Absence of Y chromosome microdeletions.
3. Lack of hypogonadotropic hypogonadism.
4. Normal sexual and ejaculatory function without obstruction, varicocele.
5. No history of infection or diseases that could be responsible for infertility

**Comment [S16]:** You must mention this is primary or secondary infertility, you can mention it in inclusion or exclusion criteria

**Comment [S17]:** You did not mention anything about hormonal assessment of your patients how to prove this criteria of inclusion

### EXCLUSION CRITERIA

1. Men with mono or bilateral cryptorchidism.
2. Iatrogenic infertility
3. Previous history of testicular trauma
4. Obstructive azoospermia
5. Abnormal sexual and ejaculatory function

### METHODOLOGY

This investigation will be undertaken in Wardha test tube baby center A.V.B.R.H. Sawangi (Meghe), Wardha. Pertinent data on the demographics and therapeutic history as well as the manifestations will be set down. Counseling of all entrants for research work will be done. The groove convention is in our setup is as follows: History of the patient will be taken

### SEMEN COLLECTION AND ANALYSIS

Semen will be collected succeeding 3-5 days of ejaculatory abstinence in a germ free plastic receptacle by exercising self-masturbation or coitus. Semen samples will be collected in the laboratory room in a dry, clean, biologically inert container. In the case of an Oligozoospermic with <15 million/ml of sperms or an Azoospermia patient, three semen samples will be collected. The collected sample will be analyzed as per WHO guidelines.

Patient and control samples will be employed in the investigation of the targeted genotyping study included participants of Wardha Test Tube Baby Centre. The infertile couple incorporated in the research will be looked for out and out andrological investigation workup for couple infertility. Based on the detailed andrological investigation along with case history

and physical inspection, semen scrutiny, scrotal USG, hormone investigation, karyotyping, and Y chromosome micro-deletion screening, all infertile forebears will be defined as 'idiopathic'. Patients with any type of cryptorchidism, obstructive azoospermia, varicocele, previous testicular trauma, recurring infection will be not included in the research.

In wholly, normozoospermic controls (>15 million sperms/ml), severe oligozoospermic (<5 million sperms/ml), non-obstructive azoospermic will be incorporated in the follow-up inquiry. Along with moderately oligozoospermic (5-10 million sperms/ml) will be scrutinized, along with all oligozoospermic patients will be delineated one by one.

### DNA EXTRACTION

A blood sample will be collected at room temperature in an EDTA tube and kept at room temperature and processed for extraction of genomic DNA in no more than 2 hours of blood draw. As per the manufacturer's guideline pure genomic DNA will be drawn out from unbridged blood using QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). To yield an exceedingly strenuous DNA, on the membrane the elute is reloaded which is accommodated with the DNA and incubated for 5 mins at room temperature. Again centrifuge for 2 mins at 4,500 g. Then transfer it into 200µl of distilled water and eventually stockpiled at -20oC till analysis.

In our research, we will extract genomic DNA from whole blood but we can also isolate genomic DNA from saliva and biopsies employing phenol extraction base DNA purification or by using ethanol and many DNA preparation kits are available commercially.

**Comment [S18]:** What do you mean by unbridged? Anticoagulated blood!!

**Comment [S19]:** It is better to use (stored) for blood samples

**Comment [S20]:** You may mention that the extraction process is manual or automated

**Comment [S21]:** If you didn't use these samples or steps, you don't need to mention it

### DNA QUANTIFICATION BY NANODROP

On the end of the fiber optic cable, we will pipette out 1µl of each sample and with the help of Nanodrop 1,000 spectrometer, we will quantify nucleic acid-based on absorbance. Two examinations were performed on the same sample with each sample by setting pathlength 0.2mm to compute the absorbance and resulted in the ng/µl. Two values come by each sample will be averaged. Quality and concentration can be checked via this device. Absorbance will be measured at 260nm and 280nm to take the ratio and check the purity of DNA (normal value is ≈1.8). A ratio of 260:280 lesser than reference may stipulate the existence of proteins, phenol, etc., as contaminants. And another optical density ratio will be considered at 260:230 nm which is the secondary nucleic acid purity measurement ratio to check co-purified contaminants like salts.

### DNA QUANTIFICATION ON AGAROSE GEL

In 0.6% agarose gel electrophoresis carried out at 100mV, we will scrutinized the quality and clarity of DNA and the amount will be reckoned using MassRuler High Range Forward and Reverse DNA Ladder as a marker. By considering the quantification results of nanodrop, we will take a solution containing 30ng of DNA. To gain a DNA concentration of 30ng/µl, whenever necessary we will keep mixing eluted DNA and distilled water. 1µl of DNA sample mixed with bromophenol blue loading dye (6X) will be loaded in agarose gel well. We will mix tris-acetate-EDTA buffer (1X) with 0.6% of agarose gel and ethidium bromide (EtBr) which will trace the path of DNA in the gel and makes us easy to observe it under UV light. We will run DNA marker fragments of familiar length at the same time as the sample.

### POLYMERASE CHAIN REACTION

We will design primers with standard formula (Caracausi, Piovesan, Vitale, &Pelleri, 2017; Sharrocks, 1994). 2 U of Platinum Taq DNA Polymerase (Invitrogen; Thermo Fisher Scientific), PCR buffer (10X) 5µl, MgCl<sub>2</sub> (final 1.5 mM) 50mM, dNTPs mix (final 0.2 mM)

10 mM, each primer 0.2 $\mu$ M and 150 ng of genomic DNA template will be in each 50 $\mu$ l PCR reaction. We will denature the mixture for 2mins at 94oC and then perform 35 cycles of PCR in a thermocycler (GenePro TC-E-48D, Bioer Technology, Hangzhou, China) by meeting the following required conditions: at 940C we will denature for 30 secs, at 61oC annealing for 30 s, and then augmentation for 30 secs at 72oC. The concluding augmentation cycle will be for 7mins at 72oC.

Comment [S22]: 94 degree centigrade

We will load 3 $\mu$ l of PCR products in 2% agarose gel made along with EtBr for electrophoresis at 100mV to precisely authenticate the size of the amplicon which is 513bp with the help of the required marker.

### RESTRICTION FRAGMENT LENGTH POLYMORPHISM

We will use AnzaTM 71 Hinf I (Thermo Scientific; Thermo Fisher Scientific) for RFLP reaction. To digest 0.2-1 $\mu$ g of PCR product in 15mins we will require 20 U (1  $\mu$ l) of this enzyme. We will prepare each 20 $\mu$ l of brew reaction with Red buffer (10X), 20 U (1  $\mu$ l) of Hinf I enzyme, and 100ng of PCR product. We will let the reaction incubate for 15min at 37oC in a thermocycler then enzymes will get inactivated by heating for 20 mins at 80oC by manufacturer's edict. If mutation will present in the MTHFR gene, in this, T is there in place of C which will allow Hinf I to show restriction activity on DNA fragment of 513bp which we will amplify previously via PCR and divide it into two fragments of 146bp and 367bp.

Comment [S23]: MTHFR C677T

We will load the RFLP sample 15 $\mu$ l in 2% agarose gel made along with EtBr for electrophoresis at 100mV to precisely clear authentication of different genotypes with the help of the required marker GeneRuler DNA Ladder Mix.

### EXPECTED OUTCOME

We have done many searches on PubMed and NCBI sites (<http://www.ncbi.nlm.nih.gov/pubmed/>) with keyword MTHFR gene, polymorphism in MTHFR gene, methylation, folate methylation, methylenetetrahydrofolate reductase, methylenetetrahydrofolate reductase, and primer for MTHFR gene and retrieved many articles related to primers for MTHFR gene and will design our primer fro forward and reverse annealing with the help of Amplify software (Engels, 1993). With the help of our study, we will analyze the MTHFR genomic sequence along with analysis of RFLP, in which we will design an amplicon of 513bps in which we will find restriction site for Hinf I and will check that if polymorphism is there then it will cut the DNA amplicon into two and will confirm it with electrophoresis.

### DISCUSSION:

The foremost aim of this study is to identify MTHFR gene polymorphism and to correlate its role in male infertility. Male infertility is idiopathic and various factors are affecting it and most likely genetical factors. Through this study, we will get to know how SNP in the MTHFR gene contributing to male infertility and to what extent. Through this study, we will also get to know that exactly which metabolic reactions are getting affected and how it is related to male infertility and production of sperm and quality of semen.

Comment [S24]: MTHFR C677T

As per a review article published on June 09, 2017, in which they correlate the effect of vitamin B12 on semen standards and sperm physiology. They select an article published between September 1961 to March 2017 on vitamin B12 and semen standards. And they concluded that there is the pragmatic effect of Vitamin B12 on semen standards by improving motility of sperm, its morphology, and count also. They also conclude that Vitamin B12 reduces DNA damage and its fragmentation. And they stated that these pragmatic changes in sperm physiology along with semen quality are due to the role of vitamin B12 in metabolism

occur in almost every cell of the human body due to which it also works on increasing efficacy of reproductive organ, lessen the accumulation of homocysteine.<sup>(12)</sup>

A meta-analysis published by Huifengjin, in which they world-widely evaluate the covariate-dependent consequences of the MTHFR SNP rs1801133 on blood Hcy on corroboration based. In this meta-analysis, they conclude that their findings will help doctors to elucidate the rs1801133 data via genetic testing for their patients. It is also useful for research people who are receiving genetic data from mercantile recourses unaccompanied by elucidating its clinical significance.<sup>(14)</sup>

In 2019, one study is done to analyzed polymorphism in MTHFR C677T by using efficacious restriction enzyme-based modus operandi by ameliorating an antecedent convention. They retrieved all possible articles which have proper PCR-RFLP method to analyze MTHFR C677T polymorphism from PubMed by using a combination of Boolean operator and MeSH terms. And they concluded that, their authentic PCR-RFLP policy which is particularly made for analyzing perfect concerning to PCR primers and gel analysis and proved to be the best technique than techniques used by Frosst et al (1995) and techniques used by others for PCR-RFLP to find out MTHFR C677T Polymorphism genotyping.<sup>(15)</sup>

There is one cohort study done by appraising 172 candidates for checking polymorphism for an alliance with oligozoospermia and azoospermia of European descent and pinpointed some SNPs which are notably analogous to oligozoospermia and azoospermia. And reported that there are many SNPs are responsible for the failure of spermatogenesis but they fail to find out that particular one and concluded that need to work more on the larger genome-wide studies to emphatically proving which SNPs are most affecting male infertility and spermatogenesis.<sup>(16)</sup>

In 2017, NATURE journal published one meta-analysis on the relationship between rising male infertility and polymorphism in DNA methyltransferase gene in epigenetic marking. In this meta-analysis, they particularly look into the consortium between male infertility and SNPs in DNA methyltransferase (DNMT) genes. They took two groups of people, one group with infertility problems and another group as the control group. They bespeak a crucially soaring threat to rising infertility accompanied by anomalous semen criterion in alliance accompanied by the heterozygous genotype of variant rs4804490. The decreased threat of rising male infertility in alliance with the AA genotype of variant rs4804490 with anomalous semen criterion. In alliance with the homozygous genotype of variant rs2424909. And their outcome suggests that variants in distinct DNMT correspondences with rising male infertility.<sup>(17-18)</sup> A number of related studies were reviewed <sup>(19-25)</sup>.

## SCOPE

This study may help us identify MTHFR gene polymorphism in infertile male subjects.

Comment [S25]: MTHFR C677T

## LIMITATIONS

1. One of the factors for assessing male infertility is a hormonal assay in my study because of the cost many of the patients may not be willing for investigation.
2. Because of male dominance in the country male patients do not consider them as a patient.

Comment [S26]: ???

### 3. COMPETING INTERESTS DISCLAIMER:

- 4.
5. Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers

Comment [S27]: predominantly used products

Comment [S28]:

of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

Comment [S29]: by the personal efforts

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