Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(1), 1490-1496 | ISSN:2251-6727

Molecular Study of *MTHFR* C677T and *MTR* A2756G Polymorphisms in Alcohol and Tobacco Users from Eastern Uttar Pradesh, India

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(Received: 05 November 2025 Revised: 15 December 2025 Accepted: 31 January 2026)

KEYWORDS

MTHFR C677T, *MTR* A2756G, folate metabolism, homocysteine, alcohol, to- bacco, PCR-RFLP, Eastern Uttar Pradesh

ABSTRACT:

Background: Folate-mediated one-carbon metabolism supports nucleotide biosynthesis and methylation reactions. Functional variants in folate-pathway genes such as *methylenete- trahydrofolate reductase* (*MTHFR*) C677T and *methionine synthase* (*MTR*) A2756G can alter enzyme activity, affect homocysteine remethylation, and modulate methylation po- tential. Alcohol consumption and tobacco use may further perturb this pathway through nutritional and oxidative-stress mechanisms.

Methods: We analyzed an Eastern Uttar Pradesh cohort of $N = 981$ adults (age 18– 88 years). Controls were pooled RANDOM and CONTROL individuals ($N = 621$) and cases were pooled substance users ($N = 360$) comprising TOBACCO ($N = 232$), ALCOHOL ($N = 30$), and ALCOHOL TOBACCO ($N = 98$). Genotypes were available from PCR-RFLP assays. We computed genotype and minor-allele frequencies, tested Hardy–Weinberg equilibrium (HWE) in controls, performed chi-square association tests, reported odds ratios (ORs) with 95% confidence intervals (CIs), and fitted additive logistic regression adjusted for age and sex.

Results: In controls, minor-allele frequencies were $f_T = 0.107$ for *MTHFR* C677T and $f_G = 0.099$ for *MTR* A2756G. Pooled cases showed higher minor-allele frequencies ($f_T = 0.140$ and $f_G = 0.136$, respectively). Allelic association tests indicated increased odds for the minor allele in cases (*MTHFR*: $OR_T = 1.36$ [95% CI 1.03–1.79], $p = 0.035$; *MTR*: $OR_G = 1.43$ [1.08–1.90], $p = 0.015$). HWE held in controls for *MTHFR* ($p = 0.227$) but deviated for *MTR* ($p = 0.008$). In age/sex-adjusted additive logistic regression, *MTHFR* remained associated (adjusted OR per T allele = 1.43, $p = 0.015$) whereas *MTR* attenuated (adjusted OR per G allele = 1.24, $p = 0.144$). Subgroup analyses suggested strongest signals in alcohol-only users (small *N*).

Conclusions: Variant alleles in one-carbon metabolism genes were modestly enriched among substance users in this Eastern UP dataset, with strongest signals in the alcohol- only subgroup. Replication with biomarker-informed phenotyping and quantitative exposure measures is needed to confirm and interpret these findings.

1. Introduction

Folate is an essential micronutrient that supports one-carbon transfer reactions required for (i) *de novo* nucleotide synthesis and (ii) methylation of DNA, RNA, proteins, and lipids through S-adenosylmethionine (SAM). Perturbations in folate availability or one-carbon flux can lead to uracil misincorporation, DNA strand breaks, altered methylation profiles, and chromosomal instability, which are relevant to cancers and other chronic disorders [1].

A central branch of one-carbon metabolism involves remethylation of homocysteine to methionine. *MTHFR* catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor used by *MTR* (methionine synthase) to convert homocysteine to methionine.

Methionine is then converted to SAM, the main cellular methyl donor. Genetic variants that reduce *MTHFR* or *MTR* activity can therefore shift homocysteine metabolism and methylation potential, especially under nutritional stress.

Two widely investigated polymorphisms are *MTHFR* C677T (rs1801133) and *MTR* A2756G (rs1805087). The *MTHFR* 677T allele produces a thermolabile enzyme with reduced activity, and has been proposed as a genetic risk modifier in conditions where folate and homocysteine are relevant [2, 3]. The *MTR* A2756G

Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(1), 1490-1496 | ISSN:2251-6727

polymorphism has also been studied in relation to homocysteine and methylation-related outcomes [4].

Alcohol consumption and tobacco use are major exposures that may interact with one- carbon metabolism. Alcohol can impair folate absorption and utilization, while tobacco expo- sure is linked to oxidative stress and altered nutritional status. Co-use of alcohol and tobacco is common, and epidemiologic work has described the burden and co-occurrence patterns of these exposures [5, 6, 7, 8]. These considerations motivate profiling folate-pathway variants in substance-user populations and comparing them to non-user controls.

1.1 Objectives

This study aims to:

1. estimate genotype and minor-allele frequencies of *MTHFR* C677T and *MTR* A2756G in an Eastern Uttar Pradesh cohort;
2. evaluate Hardy–Weinberg equilibrium (HWE) in controls;
3. compare genotype/allele distributions between substance users and controls using chi- square tests and odds ratios; and
4. quantify adjusted associations using logistic regression controlling for age and sex.

2. Materials and Methods

2.1. Study Setting and Participants

The analysis uses the user-provided dataset from Eastern Uttar Pradesh (Jaunpur region). Blood samples were collected after obtaining written informed consent as per the study protocol. The dataset includes N = 981 adult participants (age 18–88 years) drawn from six locations (Table 3). Participants were labeled by exposure group as RANDOM, CONTROL, TOBACCO, ALCOHOL, or ALCOHOL TOBACCO.

Table 1: Summary of PCR-RFLP genotyping details (from the study protocol)

Gene (SNP)	PCR primers (5’→3’)	Enzyme	Amplicon gel	RFLP gel
MTHFR C677T	F: TGAAGGAGAAGGTGTCT- GCGGGA; R: AGGACGGTGCGGTGAGAG	HinfI	2%	3%
MTR A2756G	F: TGTTCCCAGCTGTTAGAT- GAAAATC; R: GATCCAAAGCCTTTTACACTC- CTC	HaeIII	2%	4%

Case–Control Definition (used in this paper). Controls were pooled RANDOM and CONTROL participants (N = 621). Cases were pooled substance users (N = 360): TOBACCO (N = 232), ALCOHOL (N = 30), and ALCOHOL TOBACCO (N = 98).

The study synopsis describes an initial sampling plan of approximately 500 randomly selected individuals and 200 case samples, with blood collected in EDTA vacutainers for molecular analysis. The dataset provided for this manuscript contains a larger final sample (N = 981), enabling more precise frequency estimates and subgroup comparisons.

2.2. Blood Collection and DNA Extraction

Peripheral venous blood (approximately 3 mL per participant) was collected in EDTA-coated vacutainers after written informed consent. Genomic DNA was extracted from whole blood using a standard method [9]. Briefly, erythrocytes were lysed, leukocytes were pelleted and lysed, proteins were removed by salt/organic extraction, and DNA was precipitated with ethanol, washed, and resuspended in TE buffer. DNA integrity was checked by agarose gel electrophoresis prior to genotyping.

2.3. PCR-RFLP Genotyping

***MTHFR* C677T.** The *MTHFR* C677T locus was amplified by PCR using the primer pair 5’-TGAAGGAGAAGGTGTCTGCGGGA-3’ and 5’-AGGACGGTGCGGTGAGAG-3’. PCR amplicons were confirmed on 2% agarose gel, then digested with *HinfI* restriction enzyme and resolved on 3% agarose gel to assign CC/CT/TT genotypes based on the restriction-fragment pattern [2].

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JCHR (2026) 16(1), 1490-1496 | ISSN:2251-6727

***MTR* A2756G.** The *MTR* A2756G locus was amplified by PCR using the primer pair 5'-TGTTCCCAGCTGTTAGATGAAAATC-3' and 5'-GATCCAAAGCCTTTTACACTCCTC-3'.

PCR amplicons were confirmed on 2% agarose gel, then digested with *HaeIII* and resolved on 4% agarose gel to infer AA/AG/GG genotypes from fragment patterns.

Genotype coding and allele definition. For *MTHFR* C677T, genotypes are CC/CT/TT and the minor allele is T. For *MTR* A2756G, genotypes are reported here as AA/AG/GG and the minor allele is G.

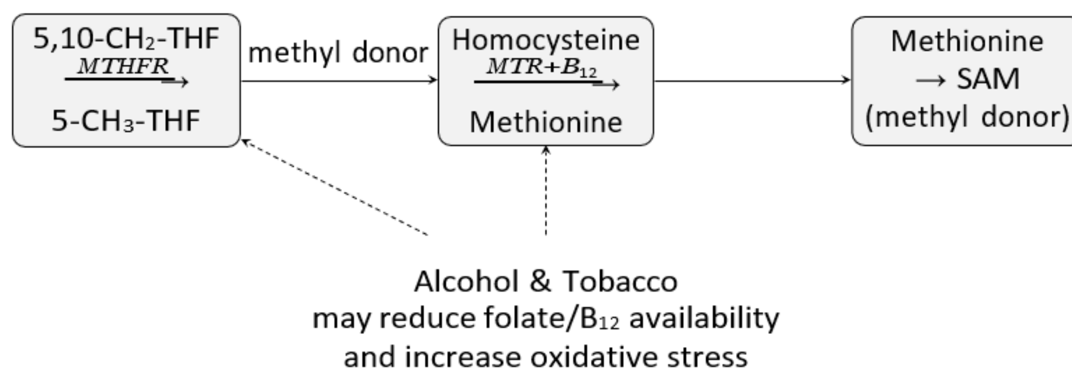

Figure 1: Simplified schematic of one-carbon metabolism highlighting *MTHFR* and *MTR*.

Note on the spreadsheet encoding: In the provided dataset, the *MTR* A2756G geno- types were encoded as CC/CT/TT for analysis convenience. For scientific reporting, we mapped those to AA/AG/GG (wild-type homozygous, heterozygous, variant homozygous) and report the minor allele as G. This relabeling does not change counts or statistical results; it only corrects the genotype/allele notation.

2.4. Statistical Analysis

We summarized age and sex by exposure group. The dataset had 0% missingness for age, sex, place, and both genotypes. Pooled cases vs controls were compared using Welch's *t*-test (age) and chi-square test (sex). For each locus, genotype counts and minor-allele frequency were computed: f_T for *MTHFR* and f_G for *MTR*. HWE in controls was tested using a chi-square goodness-of-fit test with 1 degree of freedom.

Association analyses included:

- 2 × 3 chi-square tests comparing genotype distributions in cases vs controls;
- 2 × 2 chi-square tests comparing allele counts (minor vs major allele);
- odds ratios (OR) with 95% confidence intervals under allelic, dominant (heterozygous+variant homozygous vs wild-type), and recessive (variant homozygous vs others) models.

Additive logistic regression was fitted with genotype dosage (0/1/2) as predictor and case status as outcome, adjusted for age and sex (male vs female). Significance was assessed at $\alpha = 0.05$.

3. Results

3.1. Cohort Composition and Demographics

The dataset contained 292 RANDOM, 329 CONTROL, 232 TOBACCO, 30 ALCOHOL, and 98 ALCOHOL

TOBACCO participants (total $N = 981$).

Controls (pooled RANDOM+CONTROL) were younger on average than pooled cases (Welch *t*- test: $p = 7.63e-03$) and were predominantly female, whereas pooled cases were predominantly male (chi-square: $p = 3.38e-119$). Group-wise summaries are reported in Table 2.

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JCHR (2026) 16(1), 1490-1496 | ISSN:2251-6727

Table 2: Participant characteristics by exposure group (from the provided dataset). Values are mean ± SD for age and count (%) for gender

Group	N	Age (years)	Gender (M/F)
RANDOM	292	40.77 ± 16.09	87/205
CONTROL	329	41.98 ± 16.20	67/262
TOBACCO	232	42.29 ± 15.78	213/19
ALCOHOL	30	35.13 ± 13.97	28/2
ALCOHOL TOBACCO	98	43.16 ± 14.54	97/1
Controls (RANDOM+CONTROL)	**621**	**41.41 ± 16.15**	**154/467**
Cases (all users)	**360**	**42.02 ± 15.47**	**338/22**

Table 3: Distribution of participants by sampling location and exposure group

Place	Alc	Alc+Tob	Ctrl	Rand	Tob	Total
Badshahpur	8	33	79	84	41	245
Belwa	0	0	88	74	0	162
Dhasekhar	1	17	68	38	99	223
Madanpur	3	17	48	50	56	174
Purev	18	30	46	46	36	176
Raypur	0	1	0	0	0	1
Total	**30**	**98**	**329**	**292**	**232**	**981**

3.2. Genotype Frequencies, Minor-Allele Frequencies, and HWE

Genotype distributions and minor-allele frequencies are shown in Table 4. In controls, minor- allele frequencies were $f_T = 0.107$ for *MTHFR* C677T and $f_G = 0.099$ for *MTR* A2756G. In pooled cases, f_T increased to 0.140 and f_G increased to 0.136.

HWE tests in controls supported equilibrium for *MTHFR* (HWE $p = 0.227$). For *MTR*, controls deviated from HWE (HWE $p = 0.008$), which can arise from genotyping error, popula- tion structure, or other factors; this warrants quality-control review and cautious interpretation of *MTR* associations.

3.3. Association Analysis

Allelic tests (minor vs major allele) indicated increased odds for the minor allele among pooled cases (*MTHFR*: $OR_T = 1.36$, $p = 0.035$; *MTR*: $OR_G = 1.43$, $p = 0.015$; Table 4). Genotype-

distribution tests (2 × 3) were less significant (*MTHFR* $p = 0.104$; *MTR* $p = 0.060$), consistent with modest, approximately additive effects.

3.4. Subgroup Analysis by Exposure Type

Table 5 reports allelic ORs comparing each exposure subgroup to controls. Tobacco-only users showed modest enrichment without statistical significance. Alcohol-only users showed stronger enrichment for both loci; however, this subgroup is small ($N = 30$) and effect estimates should be interpreted with caution. Alcohol+tobacco users showed intermediate patterns with borderline.

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JCHR (2026) 16(1), 1490-1496 | ISSN:2251-6727

Table 4: Genotype and allele frequencies (controls vs pooled substance users). Genotype asso- ciation p-values are from 2×3 chi-square tests

Gene (SNP)	Group	WT	Het	Var	Minor-allele freq.
MTHFR C677T (p = 0.104)	Controls	CC: 494 (79.5%)	CT: 123 (19.8%)	TT: 4 (0.6%)	f_T = 10.7%
	Cases	CC: 264 (73.3%)	CT: 92 (25.6%)	TT: 4 (1.1%)	f_T = 14.0%
MTR A2756G (p = 0.060)	Controls	AA: 509 (82.0%)	AG: 100 (16.1%)	GG: 12 (1.9%)	f_G = 9.9%
	Cases	AA: 279 (77.5%)	AG: 64 (17.8%)	GG: 17 (4.7%)	f_G = 13.6%

Allelic association (minor vs major allele), cases vs controls: *MTHFR* ORT = 1.36 (95% CI 1.03–1.79), p = 0.035; *MTR* ORG = 1.43 (95% CI 1.08–1.90), p = 0.015.

HWE in controls: *MTHFR* p = 0.227; *MTR* p = 0.008.

Table 5: Allelic association (minor vs major allele) by exposure subgroup vs pooled controls

Subgroup vs Controls	Gene	OR (95% CI)	p-value
TOBACCO vs Controls	*MTHFR* (T)	1.20 (0.88–1.63)	0.250
TOBACCO vs Controls	*MTR* (G)	1.25 (0.90–1.74)	0.185
ALCOHOL vs Controls	*MTHFR* (T)	2.59 (1.42–4.73)	0.002
ALCOHOL vs Controls	*MTR* (G)	2.75 (1.52–4.98)	0.001
ALCOHOL TOBACCO vs Controls	*MTHFR* (T)	1.47 (0.99–2.18)	0.055
ALCOHOL TOBACCO vs Controls	*MTR* (G)	1.41 (0.91–2.17)	0.123

significance for *MTHFR*.

3.5. Adjusted Logistic Regression

In additive models adjusted for age and sex (Table 6), *MTHFR* C677T showed a significant per-allele effect for pooled cases vs controls (adjusted OR per T allele = 1.43, p = 0.015). The *MTR* A2756G per-allele effect attenuated after adjustment (adjusted OR per G allele = 1.24, p = 0.144). Subgroup models showed strong signals for alcohol-only users for both genes, consistent with the unadjusted subgroup analysis but limited by sample size.

4. Discussion

4.1. Summary of Findings

This paper reports genotype and allele frequencies of *MTHFR* C677T and *MTR* A2756G in a dataset from Eastern Uttar Pradesh and compares substance users to controls. We found that pooled substance users had higher minor-allele frequencies for both loci. *MTHFR* showed a significant association with substance-use status in both unadjusted allelic tests and age/sex-adjusted regression. *MTR* showed an unadjusted allelic association but was not significant after adjustment, and controls deviated from HWE for *MTR*.

Table 6: Additive logistic regression (genotype dosage 0/1/2) adjusted for age and sex.

Outcome	Gene	Adj.	OR per minor allele	95% CI	p-value	N
Any user vs Control	*MTHFR* (T)		1.43	(1.07–1.91)	0.015	981
Any user vs Control	*MTR* (G)		1.24	(0.93–1.66)	0.144	981
Tobacco vs Control	*MTHFR* (T)		1.27	(0.91–1.76)	0.165	853
Tobacco vs Control	*MTR* (G)		1.17	(0.83–1.66)	0.367	853

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Outcome	Gene	Adj.	OR per minor allele	95% CI	*p*-value	N
Alcohol vs Control	*MTHFR* (T)		2.57	(1.36–4.87)	0.004	651
Alcohol vs Control	*MTR* (G)		2.11	(1.11–4.00)	0.022	651
Alcohol+Tobacco vs Control	*MTHFR* (T)		1.58	(1.05–2.37)	0.029	719
Alcohol+Tobacco vs Control	*MTR* (G)		1.44	(0.93–2.23)	0.102	719

4.2. Biological Interpretation

The *MTHFR* 677T allele reduces enzyme activity and can lower 5-methyltetrahydrofolate avail- ability, thereby impairing remethylation of homocysteine and reducing methylation potential [2, 3]. Alcohol exposure can compromise folate status via reduced absorption and altered metabolism, and tobacco exposure is associated with oxidative stress and inflammatory pro- cesses. These mechanisms provide a plausible gene–environment context in which one-carbon metabolism variants may be enriched in exposed populations or may modulate exposure-linked biochemical phenotypes. Importantly, this paper evaluates association with exposure category (user vs control), not disease endpoints; biomarker and outcome-linked studies are needed for causal inference.

4.3. Limitations

Important limitations include: (i) exposure status is categorical and lacks intensity/duration measures; (ii) strong sex imbalance in user groups (especially alcohol+tobacco) limits stable stratified inference; (iii) the alcohol-only subgroup is small ($N = 30$); (iv) HWE deviation for *MTR* in controls suggests possible genotyping or sampling issues; and (v) nutritional biomark- ers (folate, vitamin B_{12}, homocysteine) and clinical endpoints are not available, preventing mechanistic and clinical interpretation.

4.4. Future Work

Future studies should incorporate quantitative exposure phenotyping, dietary and biomarker measurements (folate/B_{12}/homocysteine), and replication in independent cohorts. Extending the design to clinical outcomes (for example cardiovascular disease and cancer) would enable direct assessment of gene–environment risk relationships.

5. Conclusion

In the provided Eastern Uttar Pradesh dataset, the *MTHFR* C677T variant allele is modestly enriched among alcohol/tobacco users and remains significant after adjustment for age and sex.

MTR A2756G shows enrichment in unadjusted analyses but not in adjusted pooled models, and control HWE deviation motivates cautious interpretation. Overall, these results encourage more detailed, biomarker-informed studies of one-carbon metabolism polymorphisms under alcohol and tobacco exposures.

Declarations

Ethics Approval and Consent to Participate: Blood collection studies require written informed consent and institutional ethics approval.

Availability of Data and Materials: This manuscript is based on the user-provided dataset (Excel sheet) and synopsis document.

Competing Interests: The authors declare no competing interests.

Funding: Not specified.

Author Contributions: Apoorva Singh: data collection / experiments / analysis / drafting. Dr. Rajesh Sharma: supervision, study design guidance, editing and approval of the final manuscript. (Modify as appropriate.)

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