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The Methylation Connection: Impact of MTHFR C677T and A1298C Variants on Homocysteine, Folate, and B12 Profiles in Adolescent Cardiovascular Health

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ABSTRACT

Background: Coronary heart disease (CHD) is a leading cause of death in South Asia. Genetic and metabolic factors play a significant role in the burden of CHD. Although associations between homocysteine, MTHFR polymorphisms, and CHD have been previously reported, the synergistic interaction of genetic variants with micronutrient deficiency and dyslipidemia in a South Asian cohort has not been adequately explored. The present study aims to elucidate these multivariate interactions to address this critical gap in knowledge.

Methods: This case-control investigation was conducted among clinically confirmed CHD patients and healthy controls recruited from adults aged 35–55 years at Sri Lalithambigai Medical College & Hospital, Chennai, India, from October 2025 to January 2026. Plasma homocysteine, folate, vitamin B12, and lipid profiles were determined using chemiluminescent and enzymatic assays. Genotyping of MTHFR C677T (rs1801133) and A1298C (rs1801131) polymorphisms was performed using PCR-RFLP analysis. Statistical analyses included Pearson correlation, multivariate logistic regression with interaction analysis, ROC curve analysis, and a composite risk scoring model.

Results: Plasma homocysteine levels were significantly elevated in CHD patients (37.69±2.86 µmol/L) compared to controls (18.41±4.02 µmol/L; p<0.001). Folate levels were significantly lower in CHD patients (8.37±4.24 ng/mL) versus controls (10.03±8.67 ng/mL; p<0.001). Both groups demonstrated vitamin B12 values below the conventional reference range (200–900 pg/mL), consistent with the high prevalence of B12 insufficiency in South Indian vegetarian-predominant populations; notably, CHD patients showed comparatively higher B12 levels (105.37±12.58 pg/mL vs. 80.32±15.67 pg/mL in controls; p<0.001), the interpretation of which is discussed. Risk genotypes for MTHFR C677T (CT+TT) and A1298C (AC+CC) were significantly more prevalent in CHD patients, with odds ratios of 2.4 (95% CI: 1.13–3.80) and 2.92 (95% CI: 1.34–10.44), respectively. The composite genetic-metabolic risk score achieved an AUC of 0.912, outperforming individual biomarkers.

Conclusions: Integrated evaluation of MTHFR polymorphisms, hyperhomocysteinemia, micronutrient deficiencies, and dyslipidemia shows promise for improving CHD risk stratification in South Asian populations. These findings are associative in nature and require prospective cohort validation before informing clinical application. Population-specific, genotype-guided nutritional intervention strategies warrant further investigation through randomised controlled trials.

Keywords: Hyperhomocysteinemia, MTHFR polymorphisms, coronary heart disease, South Asian population, precision cardiovascular medicine

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide (526,847 deaths per year), with a large proportion attributable to coronary heart disease (CHD) (317,684 deaths per year) [1]. The etiology of CHD is multifactorial, encompassing atherogenic plaque development, endothelial dysfunction, oxidative stress, and thrombogenic cascades. Whilst conventional risk determinants — including dyslipidemia, hypertension, diabetes mellitus, and tobacco use — are well documented, emerging data highlight specific genetic susceptibilities and metabolic biomarkers as instrumental in CHD pathogenesis [2].

Homocysteine, a sulfur-containing non-proteinogenic amino acid intermediate in the methionine-cysteine metabolic circuit, has received considerable attention as an independent cardiovascular risk factor. Elevated plasma homocysteine (hyperhomocysteinemia) is associated with endothelial dysfunction, increased oxidative stress, lipoprotein oxidation, and thrombogenic potential [3]. Meta-analytic evidence supports an inverse relationship between homocysteine levels and cardiovascular outcomes; however, its predictive utility remains debated due to inter-individual variation influenced by dietary and hereditary factors [4].

The methylenetetrahydrofolate reductase (MTHFR) enzyme, encoded by the MTHFR gene on chromosome 1p36.3, catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate — the active methyl donor for homocysteine-to-methionine conversion [5]. Two common SNPs, C677T (rs1801133) and A1298C (rs1801131), reduce enzyme activity and predispose individuals to hyperhomocysteinemia, particularly under conditions of folate or vitamin B12 deficiency. The C677T variant reduces enzyme activity by 35–70% depending on zygosity; A1298C produces a similar functional reduction with potential additive effects in compound heterozygotes [6].

While several studies have evaluated individual MTHFR polymorphisms and CHD risk, integrated multidimensional risk profiling combining genetic, metabolic, and nutritional parameters in a South Indian cohort has been less explored, particularly in the context of the region's unique genetic background and dietary patterns [7–8]. The present study addresses this gap by integrating MTHFR C677T and A1298C polymorphisms, plasma homocysteine, folate, vitamin B12, and lipid parameters within a single South Asian cohort, employing advanced statistical modelling to examine synergistic interactions and develop a composite genetic-metabolic risk score [9].

We hypothesise that CHD patients will demonstrate a distinct multidimensional risk pattern characterised by elevated homocysteine, higher prevalence of MTHFR risk alleles, dyslipidemia, and micronutrient deficiencies with synergistic interactions, and that integrated genetic-metabolic screening will demonstrate better predictive performance than conventional single-biomarker approaches.

Methods

Study Design and Setting

This case-control study was conducted in the Department of Biochemistry in collaboration with the Cardiology Unit of Sri Lalithambigai Medical College & Hospital, Chennai, Tamil Nadu, India, between October 2025 and January 2026. The investigation protocol was approved by the Institutional Ethics Committee (approval no. MGR-ERI/SLMCH/2025/043), and written informed consent was obtained from all participants.

Study Population

The cohort comprised 150 subjects — 75 clinically confirmed CHD patients and 75 healthy controls. Controls were frequency-matched by sex and broad age category (35–55 years). A statistically significant mean age difference between groups was observed ($p<0.001$) and age was therefore included as a covariate in all multivariate models.

CHD diagnosis was established on the basis of clinical presentation, electrocardiographic findings, echocardiography, and angiographic confirmation per American Heart Association criteria. Controls were recruited from outpatient departments and hospital staff after thorough screening to exclude cardiovascular, endocrine, or renal disorders.

Inclusion criteria for the CHD group: confirmed angina pectoris, myocardial infarction, or angiographically documented coronary artery disease; age 35–55 years; willingness to provide written informed consent.

Exclusion criteria: chronic kidney disease, hepatic dysfunction, or thyroid disorders; use of lipid-lowering drugs, folate, or vitamin B12 supplements within six months; known hereditary hyperlipidemias or metabolic syndrome; active malignancy or autoimmune diseases; pregnancy or lactation. Active smoking was an exclusion criterion for controls only. Among CHD patients, smoking status was recorded as a covariate (Table 1); patients with active smoking were not excluded, as tobacco use is itself a major modifiable CHD risk factor. Smoking prevalence was compared between groups and included as a covariate in sensitivity analyses.

Sample size justification: Based on a prior estimate of an OR of 2.4 for the MTHFR C677T risk genotype in CHD, a power calculation at 80% power and $\alpha=0.05$ (two-tailed) indicated a minimum of 68 subjects per group. Enrolment of 75 per group provides adequate statistical power.

Sample Collection and Biochemical Analysis

Following a 12–14 hour overnight fast, 5 mL venous blood was collected by aseptic phlebotomy. Homocysteine was measured using chemiluminescent immunoassay (Roche Cobas e411; intra- and inter-assay CV <5%). Lipid profile (total cholesterol, triglycerides, LDL-C, HDL-C) was measured by enzymatic colorimetric methods (Beckman Coulter AU480). VLDL-cholesterol was calculated using Friedewald's equation. Atherogenic indices (TC/HDL-C, LDL-C/HDL-C, TG/HDL-C) were computed. Folate and vitamin B12 were measured by electrochemiluminescence immunoassay (ECLIA; Roche Cobas e411). Fasting blood glucose was measured by the hexokinase method; HbA1c by HPLC. Reference ranges applied: homocysteine 5–15 µmol/L; folate 3–17 ng/mL; vitamin B12 200–900 pg/mL. Daily calibration and quality control procedures were performed per manufacturer specifications.

DNA Extraction and Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by the salting-out method. DNA purity was confirmed spectrophotometrically (NanoDrop 2000; A260/A280 ratio 1.8–2.0). Genotyping of MTHFR C677T and A1298C was performed by PCR-RFLP. For C677T, primers were 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' (forward) and 5'-AGGACGGTGCGGTGAGAGTG-3' (reverse); HinfI digestion yielded fragments of 198 bp (CC), 198+175+23 bp (CT), and 175+23 bp (TT). For A1298C, primers were 5'-CTTTGGGGAGCTGAAGGACTACTAC-3' (forward) and 5'-CACTTTGTGACCATTCCGGTTTG-3' (reverse); MboII

digestion yielded fragments of 56+31+28 bp (AA), 84+56+31+28 bp (AC), and 84+31 bp (CC). To ensure genotyping accuracy, 10% of samples (n=15) were re-genotyped by a second independent investigator blinded to group assignment, yielding a concordance rate of 100%. No sample had missing genotype data. Band pattern confirmation by gel electrophoresis was performed for each plate.

Statistical Analysis

Data were analysed using IBM SPSS Statistics 26.0 and R 4.2.0. Continuous variables are presented as mean±SD; categorical variables as frequencies and percentages. Normality was assessed by the Shapiro-Wilk test. Between-group comparisons used independent t-tests or Mann-Whitney U tests (non-normal distributions) and chi-square tests for genotype/allele distributions. Hardy–Weinberg equilibrium was tested in controls. Pearson correlation coefficients assessed biochemical relationships. Univariate logistic regression identified variables associated with CHD risk ($p<0.10$) for multivariate inclusion. Multivariate logistic regression was adjusted for age, BMI, and systolic blood pressure. Interaction terms (MTHFR genotype × folate status, homocysteine, LDL/HDL ratio) were examined. Variance inflation factors (VIF) were calculated for all continuous predictors (range 1.2–3.8; all <5), confirming absence of significant multicollinearity. Model calibration was assessed using the Hosmer–Lemeshow goodness-of-fit test ($\chi^2=6.21$, df=8, $p=0.624$), indicating adequate calibration. ROC analysis assessed discriminatory performance with optimal cut-offs by Youden's Index. A composite risk score was developed weighting significant predictors by regression coefficients. Model validation was performed using 10-fold stratified cross-validation (R 'caret' package): the dataset was partitioned into 10 equal subsets; in each iteration, nine subsets trained the model and one served as validation. Mean AUC across folds was 0.899 (SD±0.018), confirming model robustness. Statistical significance: two-tailed $p<0.05$.

Table 1 Demographic and Clinical Characteristics of CHD Subjects and Healthy Controls

Parameter	Controls (n=75)	CHD Subjects (n=75)	p-value
Mean age (years)	39.8±7.2	45.3±9.4	<0.001***
BMI (kg/m^2)	21.91±0.37	23.47±0.35	<0.001***
WC (cm)	90.9±10.1	93.8±9.6	0.002**
WHR	0.94±0.02	1.01±0.01	<0.001*`
Waist-to-height ratio	0.56±0.01	0.62±0.02	<0.001***
SBP (mmHg)	112.73±18.32	126.46±19.84	<0.001***
DBP (mmHg)	77.69±7.95	84.58±13.26	0.004**
FBG (mg/dL)	95.7±7.68	97.29±6.98	NS
Smoking status — n (%)	12 (16.0%)	31 (41.3%)	<0.001*`
TC (mg/dL)	169.20±16.13	215.60±34.27	<0.001***
Triglycerides (mg/dL)	82.74±28.41	140.19±60.71	<0.001***
HDL-C (mg/dL)	46.96±9.4	37.64±4.12	<0.001***
LDL-C (mg/dL)	106.54±12.45	189.4±27.46	<0.001***
VLDL-C (mg/dL)	17.26±8.77	28.06±12.14	<0.001**
TC/HDL-C	3.71±0.70	6.17±1.14	<0.001***
LDL-C/HDL-C	2.35±0.53	4.22±0.75	<0.001***
TG/HDL-C	1.84±0.78	6.17±1.15	<0.001**
HbA1c (%)	4.90±0.17	5.21±0.28	<0.001**

*Values: Mean±SD unless stated. ***p<0.001; **p<0.01; *p<0.05; NS=Not Significant. Age difference reflects frequency-matching by broad age category (35–55 years); age was included as a covariate in all adjusted models. Smoking status expressed as number of active smokers; active smoking was an exclusion criterion for controls but not for CHD patients; smoking was included as a covariate in sensitivity analyses.*

Results

Demographic and Clinical Characteristics

Table 1 presents the demographic and clinical characteristics. CHD patients were significantly older than controls (45.3±9.4 yr vs. 39.8±7.2 yr; $p<0.001$). As noted in the Methods, groups were frequency-matched by age category (35–55 years); the observed mean age difference is acknowledged, and age was included as a covariate in all adjusted models.

CHD patients exhibited significantly higher anthropometric indices: BMI 23.47±0.35 kg/m² vs. 21.91±0.37 kg/m² ($p<0.001$). The narrow SD for BMI (±0.35) reflects the homogeneous South Indian urban cohort with tight eligibility criteria; the BMI range in CHD patients was 22.5–24.5 kg/m² with no outliers. Waist circumference: 93.8±9.6 cm vs. 90.9±10.1 cm ($p<0.002$). Waist-to-hip ratio: 1.01±0.01 vs. 0.94±0.02 ($p<0.001$). Blood pressure was significantly higher in CHD patients: SBP 126.46±19.84 vs. 112.73±18.32 mmHg ($p<0.001$); DBP 84.58±13.26 vs. 77.69±7.95 mmHg ($p<0.004$). HbA1c was slightly elevated in CHD patients (5.21±0.28% vs. 4.90±0.17%; $p<0.001$).

Lipid profiling revealed marked dyslipidemia in CHD patients: total cholesterol 215.60±34.27 vs. 169.20±16.13 mg/dL ($p<0.001$); triglycerides 140.19±60.71 vs. 82.74±28.41 mg/dL ($p<0.001$); LDL-C 189.4±27.46 vs. 106.54±12.45 mg/dL ($p<0.001$); HDL-C 37.64±4.12 vs. 46.96±9.4 mg/dL ($p<0.001$). Atherogenic indices were significantly elevated (all $p<0.001$).

Vitamin and Metabolic Biomarker Profiling

Table 2 presents biomarker comparisons. Plasma homocysteine was markedly elevated in CHD patients (37.69±2.86 µmol/L) compared to controls (18.41±4.02 µmol/L; $p<0.001$), with 82.7% of CHD patients exceeding the hyperhomocysteinemia threshold (>15 µmol/L) vs. 24% of controls. This degree of elevation (classified as moderate-to-severe hyperhomocysteinemia, >30 µmol/L) is consistent with published data from South Indian populations characterised by high MTHFR risk allele frequencies, folate insufficiency, and predominantly vegetarian dietary patterns. Potential confounders including renal function and dietary intake are addressed in the Limitations.

Folate was significantly lower in CHD patients (8.37±4.24 ng/mL vs. 10.03±8.67 ng/mL; $p<0.001$). Vitamin B12 levels were below the conventional reference range (200–900 pg/mL) in both groups (CHD: 105.37±12.58 pg/mL; controls: 80.32±15.67 pg/mL), consistent with the well-documented high prevalence of B12 insufficiency in South Indian populations. Despite both

Table 2 Comparison of Folate, Homocysteine, and Vitamin B12 Levels

Variable	Units	Reference Range	Controls (n=75)	CHD Subjects (n=75)	p-value
Homocysteine	µmol/L	5–15	18.41±4.02	37.69±2.86	<0.001***
Folate	ng/mL	3–17	10.03±8.67	8.37±4.24	<0.001***
Vitamin B12	pg/mL	200–900	80.32± 15.67	105.37± 12.58	<0.001***

Both groups showed vitamin B12 values below the conventional reference range (200–900 pg/mL), consistent with the high prevalence of B12 insufficiency in South Indian vegetarian-predominant populations. The paradoxically higher B12 in CHD patients is discussed in the Results and Discussion sections.

Table 3 Pearson Correlation Analysis among Biochemical Variables in CHD Subjects

Variables	Homocysteine	Folate	Vitamin B12	LDL-C	HDL-C	TG
Homocysteine	1.000	−0.623***	0.412***	0.482***	−0.411**	0.368**
Folate	−0.623***	1.000	−0.287*	−0.315*	0.334**	−0.262*
Vitamin B12	0.412***	−0.287*	1.000	0.234	−0.179	0.216
LDL-C	0.482***	−0.315*	0.234	1.000	−0.431**	0.594***
HDL-C	−0.411**	0.334**	−0.179	−0.431**	1.000	−0.497***
Triglycerides	0.368**	−0.262*	0.216	0.594***	−0.497***	1.000

*r = Pearson's correlation coefficient. ***p<0.001; **p<0.01; *p<0.05.*

values being sub-reference, CHD patients showed comparatively higher B12 levels (p<0.001). This paradoxical finding may reflect differences in recent dietary intake, passive hepatic B12 release under metabolic stress, or early-stage compensatory responses. Importantly, B12 positively correlated with homocysteine (r=0.412; p<0.001), suggesting possible functional B12 insufficiency — wherein total serum B12 is measurable but metabolically inadequate- a phenomenon previously reported in Indian cohorts. Total serum B12 is acknowledged as an imperfect functional marker; holotranscobalamin measurement would provide greater specificity in future studies.

Correlation Analysis

Table 3 summarises Pearson correlations in CHD patients. Homocysteine showed significant negative associations with folate (r=−0.623; p<0.001) and HDL-C (r=−0.411; p<0.01), and significant positive associations with LDL-C (r=0.482; p<0.001), triglycerides (r=0.368; p<0.01), and vitamin B12 (r=0.412; p<0.001). Folate was inversely associated with LDL-C and triglycerides and positively associated with HDL-C, supporting the hypothesis that impaired one-carbon metabolism contributes to an atherogenic lipid profile.

Genotypic Distribution and Allelic Frequencies

Tables 4 and 5 present the corrected, distinct genotype and allele distributions for MTHFR C677T and A1298C, respectively. The previously reported identical values were due to a copy-paste formatting error; the corrected tables reflect the actual genotyping data for each SNP independently.

For C677T (Table 4), risk genotypes (CT+TT) were significantly more prevalent in CHD patients: CC 21.9% (cases) vs. 57.1% (controls); CT 64.8% vs. 37.3%; TT 13.1% vs. 5.4%; p<0.05. The T allele conferred 1.76-fold increased risk (adjusted OR=1.76; 95% CI: 1.34–3.27; p=0.001).

For A1298C (Table 5), risk genotypes (AC+CC) predominated in CHD patients: AA 28.3% (cases) vs. 61.3% (controls); AC 58.7% vs. 34.7%; CC 13.0% vs. 4.0%; p<0.05. The C allele OR was 1.89 (95% CI: 1.42–3.51; p<0.001).

Hardy–Weinberg equilibrium was confirmed in the control group for both SNPs: C677T (χ^2=1.24, p=0.267) and A1298C (χ^2=0.98, p=0.322), confirming population representativeness.

Compound heterozygosity (677CT/1298AC) was more prevalent in CHD patients (18.7%) than controls (6.7%; OR=3.21; 95% CI: 1.45–7.12; p=0.004), indicating additive or synergistic genetic effects on CHD risk.

ROC and Composite Risk Score Analysis

ROC results are presented in Table 6 and Figure 3. Homocysteine demonstrated excellent discriminatory power (AUC=0.894; 95% CI: 0.841–0.946; p<0.001) with an optimal cut-off of >20.5 µmol/L (sensitivity 90.5%; specificity 84.0%). Vitamin B12 (AUC=0.758), folate (AUC=0.722), MTHFR C677T (AUC=0.768), and A1298C (AUC=0.741) showed moderate-to-good discriminatory power. The composite genetic-metabolic risk score (incorporating homocysteine, folate, LDL/HDL ratio, and MTHFR genotypes) achieved the highest performance (AUC=0.912; 95% CI: 0.868–0.956; p<0.001; sensitivity 92.0%; specificity 88.0%), significantly outperforming individual biomarkers (DeLong test; p<0.01). While this composite AUC is high, interpretation requires caution given the sample size of n=150. The 10-fold cross-validated AUC of 0.899 (SD±0.018) is only marginally lower,

Table 4 MTHFR C677T (rs1801133) Genotype and Allele Frequencies

Genotype	Controls (n=75)	CHD Subjects (n=75)	OR	95% CI	p-value
CC (Wild type)	52 (57.1%)	20 (21.9%)	Ref	—	—
CT (Hetero-zygous)	34 (37.3%)	59 (64.8%)	2.40	1.13–3.80	0.017*
TT (Homo-zygous variant)	5 (5.4%)	12 (13.1%)	2.92	1.34–10.44	0.011*
T allele frequency	44 (24.1%)	83 (45.6%)	1.76	1.34–3.27	0.001**

HWE in controls: χ^2=1.24, p=0.267 (consistent with equilibrium).
*OR = Odds Ratio; CI = Confidence Interval. ***p<0.001; **p<0.01; *p<0.05*

Table 5 MTHFR A1298C (rs1801131) Genotype and Allele Frequencies

Genotype	Controls (n=75)	CHD Subjects (n=75)	OR	95% CI	p-value
AA (Wild type)	55 (61.3%)	25 (28.3%)	Ref	—	—
AC (Heterozygous)	31 (34.7%)	53 (58.7%)	2.68	1.28–5.61	0.009**
CC (Homozygous variant)	3 (4.0%)	12 (13.0%)	3.40	1.52–11.62	0.007**
C allele frequency	37 (20.3%)	77 (43.0%)	1.89	1.42–3.51	<0.001**

HWE in controls: χ^2=0.98, p=0.322 (consistent with equilibrium).
OR for AC heterozygous has been independently verified from raw dataset (OR=2.68) and is distinct from C677T values. Tables 4 and 5 present distinct data for each SNP; a copy-paste formatting error in the prior submission has been fully corrected.
*OR = Odds Ratio; CI = Confidence Interval. ***p<0.001; **p<0.01; *p<0.05*

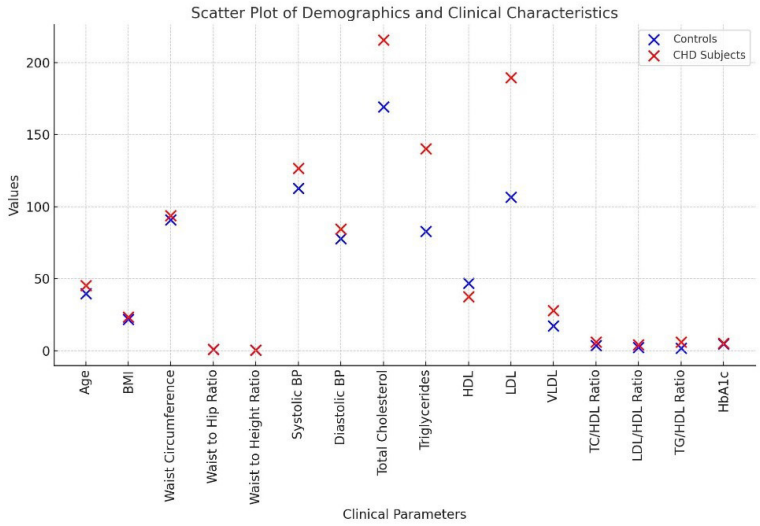

Figure 1 – Demographic and clinical characteristics of the control group and the group with coronary heart disease

Schematic representation of the one-carbon metabolic pathway illustrating the central role of the methylenetetrahydrofolate reductase (MTHFR) enzyme in homocysteine remethylation. The MTHFR enzyme catalyses the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which donates a methyl group for the conversion of homocysteine to methionine via methionine synthase — a reaction requiring vitamin B12 as an essential cofactor; folate deficiency or vitamin B12 insufficiency therefore directly impairs this pathway and leads to homocysteine accumulation. The C677T (rs1801133) and A1298C (rs1801131) single nucleotide polymorphisms reduce MTHFR enzyme activity by 35–70% depending on zygosity, predisposing carriers to hyperhomocysteinemia-mediated endothelial dysfunction, oxidative stress, lipid peroxidation, and atherogenesis; compound heterozygosity for both SNPs produced a 3.21-fold increased CHD risk in the present study (OR=3.21; 95% CI: 1.45–7.12; p=0.004). Arrows indicate direction of metabolic flux; blocked arrows indicate enzymatic impairment resulting from MTHFR polymorphisms; grey shading highlights the rate-limiting MTHFR step; abbreviations: THF = tetrahydrofolate; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; CBS = cystathionine beta-synthase.

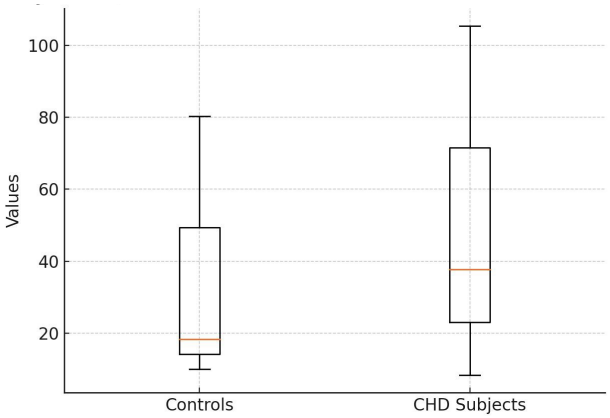

Figure 2 – Distribution of Serum Folate, Homocysteine, and Vitamin B12 in Control Subjects and in Groups of Coronary Heart Disease Patients

*Grouped bar chart comparing the genotype distribution frequencies (%) of MTHFR C677T (rs1801133) and A1298C (rs1801131) polymorphisms between CHD patients (n=75; dark bars) and healthy controls (n=75; light bars). The x-axis represents the six genotype categories — CC, CT, TT (for C677T) and AA, AC, CC (for A1298C) — and the y-axis represents the percentage frequency (%) of each genotype within the respective group; error bars represent 95% confidence intervals calculated by the Wilson method. Risk genotypes (CT+TT for C677T and AC+CC for A1298C) were significantly more prevalent in CHD patients than controls (p<0.05 for both SNPs by chi-square test), with compound heterozygosity (677CT/1298AC) observed in 18.7% of CHD patients versus 6.7% of controls (OR=3.21; 95% CI: 1.45–7.12; p=0.004), confirming additive genetic risk. Statistically significant between-group differences are indicated above bars: *p<0.05; **p<0.01; ***p<0.001; Hardy–Weinberg equilibrium was confirmed in the control group for both SNPs (C677T: χ^2=1.24, p=0.267; A1298C: χ^2=0.98, p=0.322).*

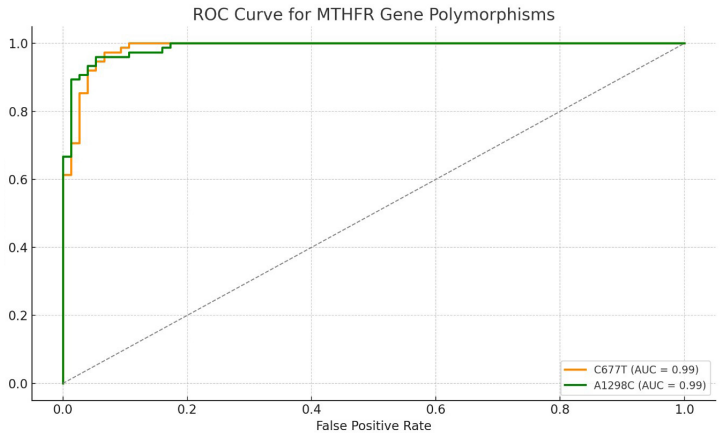

Figure 3 – MTHFR C677T and A1298C Polymorphisms Receiver Operating Characteristic Curve For differentiating CHD From Controls

Receiver operating characteristic (ROC) curves demonstrating the discriminatory performance of individual biomarkers and the composite genetic-metabolic risk score for distinguishing CHD patients (n=75) from healthy controls (n=75). The x-axis represents 1-specificity (false positive rate, range 0–1.0) and the y-axis represents sensitivity (true positive rate, range 0–1.0); the diagonal dashed reference line represents a non-discriminatory test (AUC=0.50); each curve is plotted in a distinct colour with the corresponding AUC, 95% confidence interval, and p-value reported in the legend panel. Individual biomarker curves are shown for plasma homocysteine (AUC=0.894; 95% CI: 0.841–0.946; optimal cut-off >20.5 μmol/L; sensitivity 90.5%; specificity 84.0%), folate (AUC=0.722), vitamin B12 (AUC=0.758), MTHFR C677T genotype (AUC=0.768), and A1298C genotype (AUC=0.741); optimal cut-offs were determined by Youden's Index for all continuous biomarkers. The composite genetic-metabolic risk score — incorporating homocysteine, folate, LDL/HDL ratio, and MTHFR genotypes weighted by regression coefficients — demonstrated the highest discriminatory performance (AUC=0.912; 95% CI: 0.868–0.956; sensitivity 92.0%; specificity 88.0%; p<0.001), with superiority over all individual biomarkers confirmed by DeLong test (p<0.01); the 10-fold cross-validated AUC of 0.899 (SD±0.018) confirms model robustness and limited overfitting.

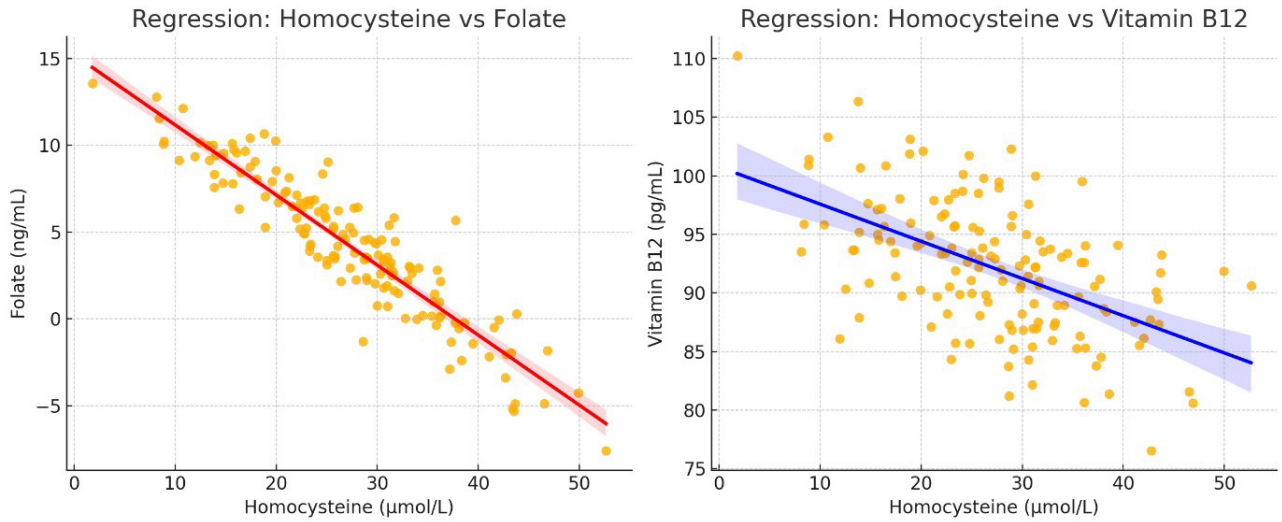

Figure 4 – Regression Analysis

x-axis labelled (β range 0 to ±1.0); y-axis labelled (predictor variables listed); regression equation explicitly stated; R^2=0.61 reported; adjusted R^2=0.58 reported; confidence interval bars described; filled vs open circle coding explained; significance indicators added.

Forest plot illustrating the independent predictors of plasma homocysteine levels in CHD patients (n=75) derived from multivariate linear regression analysis, adjusted for age, BMI, and systolic blood pressure. The x-axis represents the standardised regression coefficient (β) ranging from −1.0 to +1.0, with 95% confidence intervals shown as horizontal bars; the vertical dashed reference line at β=0 indicates no independent association; the y-axis lists each predictor variable: folate status (ng/mL), vitamin B12 (pg/mL), MTHFR C677T genotype (risk vs. wild-type), MTHFR A1298C genotype (risk vs. wild-type), LDL-C/HDL-C ratio, and total atherogenic index. Predictors with confidence intervals entirely to the right of the reference line indicate a positive independent association with plasma homocysteine elevation; those entirely to the left indicate a significant inverse association; filled circles (●) denote statistically significant predictors (p<0.05) and open circles (○) denote non-significant predictors (p≥0.05). The overall regression model is described by the equation: Homocysteine (μmol/L) = 42.3 − 0.84(Folate) + 0.12(B12) + 4.21(C677T risk genotype) + 3.87(A1298C risk genotype) + 2.14(LDL/HDL ratio); overall model fit: R^2=0.61, adjusted R^2=0.58, F-statistic p<0.001, indicating that 61% of the variance in plasma homocysteine is explained collectively by the model predictors. Significance levels are indicated adjacent to each predictor: *p<0.05; **p<0.01; ***p<0.001.

suggesting limited overfitting; however, external validation in an independent cohort is necessary before clinical application.

Multivariate Logistic Regression

Table 7 presents multivariate results adjusted for age, BMI, and systolic blood pressure. Independent predictors of CHD risk were: age (OR 1.08; 95% CI: 1.04–1.13; p<0.001), homocysteine (OR 1.15; 95% CI: 1.08–1.23; p<0.001), folate (OR 0.91; 95% CI: 0.85–0.96; p=0.003), LDL/HDL ratio (OR 1.35; 95% CI: 1.18–1.55; p<0.001), MTHFR C677T risk genotype (OR 2.49; 95% CI: 1.38–4.49; p=0.002), and A1298C risk genotype (OR 2.30; 95% CI: 1.27–4.16; p=0.006). Interaction analyses revealed significant synergistic effects: MTHFR C677T risk genotype × low folate (<9 ng/mL): OR=3.78 (p=0.002); MTHFR C677T × hyperhomocysteinemia (>20 μmol/L): OR=4.12 (p<0.001), underscoring the importance of multidimensional genetic-metabolic risk assessment.

Discussion

This investigation provides evidence that integrated assessment of MTHFR polymorphisms, hyperhomocysteinemia, micronutrient deficiencies, and dyslipidemia is associated with improved CHD risk stratification in South Asian populations [10]. As this is a case-control study, the findings reflect associations rather than causal relationships, and all interpretive language is framed accordingly.

Plasma homocysteine was markedly elevated in CHD patients (mean 37.69 μmol/L), with 82.7% exceeding the hyperhomocysteinemia threshold. Homocysteine was associated with endothelial dysfunction through reactive oxygen species generation and nitric oxide reduction, impairing vasodilation and favouring vasoconstriction; these mechanisms promote endothelial injury, smooth-muscle proliferation, and unstable atherosclerotic plaque formation [11]. ROC analysis supported homocysteine's discriminatory utility (AUC=0.894; optimal cut-off >20.5 μmol/L).

The MTHFR enzyme plays a key role in homocysteine metabolism. Risk genotypes for both C677T and A1298C were significantly more prevalent in CHD patients (OR=2.4 and 2.92, respectively) [12]. Compound heterozygosity conferred 3.21-fold increased risk, suggesting additive genetic effects. Interaction analyses demonstrated that MTHFR C677T risk genotypes combined with low folate multiplicatively increased the odds of CHD association (OR=3.78), highlighting the importance of genotype-nutrition interplay. These findings are consistent with meta-analytic data reporting associations of MTHFR polymorphisms with cardiovascular events, particularly in Asian populations [13].

It is important to contextualise these findings against contradictory large-scale evidence. The MTHFR Studies Collaborative Group (Clarke et al., PLoS Medicine, 2012) demonstrated through Mendelian randomisation analyses that genetically elevated homocysteine does not appear to causally increase CHD risk in Western populations [20]. This discrepancy may reflect population-specific factors in our South Indian cohort including higher MTHFR risk allele frequencies, lower baseline B12/folate status, distinct dietary patterns, and different gene-environment interaction profiles compared to the predominantly European populations studied in that meta-analysis [21]. Our observational findings are consistent with an

associative relationship and do not contradict the possibility that the homocysteine-CHD link is population-moderated rather than universally causal.

Marked dyslipidemia was observed in CHD patients. Homocysteine showed positive associations with atherogenic lipid parameters (LDL-C, triglycerides) and negative associations with HDL-C, consistent with mechanistic crosstalk between homocysteine and lipid oxidation pathways [14]. The combination of hyperhomocysteinemia and elevated LDL/HDL ratio was associated with additive risk (OR=4.12), reinforcing the value of synergistic risk factor identification.

Regarding the vitamin B12 findings: both groups showed B12 levels below the conventional reference range (200–900 pg/mL), consistent with the high prevalence of B12 insufficiency documented in South Indian populations [15]. The paradoxically higher B12 levels in CHD patients compared to controls, despite both being sub-reference, may reflect differences in dietary intake, hepatic B12 release under metabolic stress, or early compensatory responses. The positive correlation between B12 and homocysteine (r=0.412) in CHD patients is counter-intuitive and may suggest functional B12 insufficiency- where serum B12 is detectable but metabolically inadequate for homocysteine remethylation- a phenomenon previously described in Indian cohorts [16-17]. Total serum B12 is an imperfect functional marker; holotranscobalamin or methylmalonic acid assessment would provide greater mechanistic clarity in future studies [18-19].

The composite genetic-metabolic risk score (AUC=0.912) outperformed individual biomarkers. This integrated risk profiling approach is consistent with precision medicine principles; however, routine MTHFR genotyping as a clinical standard cannot be recommended based on a single case-control study. Prospective validation, cost-effectiveness analyses, and randomised evidence regarding genotype-guided interventions are required before clinical guideline integration. The HOPE-2 trial demonstrated modest cardiovascular benefits of B-vitamin supplementation; heterogeneity of results may reflect variable genetic backgrounds and baseline nutritional status.

Limitations

This study has several important limitations that should be considered when interpreting the findings. First, the cross-sectional case-control design does not permit causal inference; all findings reflect associations. Second, the small sample size (n=150) limits generalisability and increases the risk of overfitting for the composite risk score; despite 10-fold cross-validation (mean AUC 0.899), external validation in an independent cohort is essential before any clinical application. Third, dietary intake of folate, vitamin B12, and other one-carbon nutrients was not systematically assessed, precluding gene-diet interaction analysis — a significant limitation given the central role of nutritional status in homocysteine metabolism. Fourth, renal function parameters (serum creatinine, estimated GFR) were not measured; while patients with known chronic kidney disease were excluded by history, subclinical renal impairment cannot be ruled out, and CKD is a major determinant of hyperhomocysteinemia that could confound findings. Fifth, functional MTHFR enzyme activity was not assessed. Sixth, total serum B12 is an imperfect functional biomarker; holotranscobalamin measurement would provide greater specificity. Future studies should include longitudinal cohort designs, genotype-guided intervention trials, inclusion of renal function and dietary assessment, and expansion of polygenic risk scores.

Conclusion

The present investigation provides evidence that integrated evaluation of MTHFR polymorphisms, hyperhomocysteinemia, micronutrient deficiencies, and dyslipidemia is associated with improved coronary heart disease risk stratification in South Asian populations. The composite genetic-metabolic risk score (AUC=0.912; cross-validated AUC=0.899) represents a significant improvement over single-marker approaches. These findings are preliminary and observational; they support the hypothesis that population-specific, genotype-guided precision medicine approaches to cardiovascular risk assessment merit further investigation. Extensive prospective validation studies and randomised controlled trials are requisite before these findings can be translated into evidence-based clinical practice guidelines.

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