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**Polimorfismos del gen MTHFR y su asociación con los niveles de homocisteína
y el riesgo cardiovascular: Revisión bibliográfica**

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DEDICATORIA

“El triunfo de haber culminado esta etapa, no es solo un logro personal, sino una victoria compartida con todas las personas que estuvieron siempre junto a mí, por ello les dedico este trabajo, como muestra de mi eterna gratitud”.

María Fung

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A mis padres, por su amor incondicional, sus sacrificios y su confianza plena en mí. Gracias por acompañarme en cada desafío, por celebrar mis logros y además brindarme fortaleza en los momentos más difíciles. Este logro también les pertenece y no los pienso defraudar.

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Resumen

Las enfermedades cardiovasculares constituyen una de las principales causas de morbimortalidad a nivel mundial. Los polimorfismos del gen MTHFR C677T y A1298C, han sido ampliamente estudiados por su influencia en el metabolismo de la homocisteína y su posible relación con el riesgo cardiovascular. Para esta revisión bibliográfica se realizó una búsqueda de artículos científicos publicados entre 2016 y 2026 en PubMed y Google Scholar. Tras aplicar los criterios de inclusión y exclusión establecidos, se seleccionaron diez estudios para su análisis. Los resultados mostraron una asociación positiva entre el polimorfismo C677T, particularmente el genotipo TT, y concentraciones elevadas de homocisteína, así como un aumento en el riesgo de enfermedades cardiovasculares, aunque estos resultados variaron según los diferentes factores modificadores como la edad, la etnia y el estado nutricional de folato. En contraste, la evidencia para A1298C fue limitada e inconclusa.

Palabras clave: *MTHFR, homocisteína, hiperhomocisteinemia, polimorfismo, riesgo cardiovascular, enfermedad cardiovascular.*

Abstract:

Cardiovascular diseases are one of the leading causes of morbidity and mortality worldwide. The MTHFR C677T and A1298C gene polymorphisms have been extensively studied for their influence on homocysteine metabolism and their possible association with cardiovascular risk. For this literature review, a search was conducted in PubMed and Google Scholar for scientific articles published between 2016 and 2026. After applying the established inclusion and exclusion criteria, ten studies were selected for analysis. The results showed a positive association between the C677T polymorphism—particularly the TT genotype—and elevated homocysteine concentrations, as well as an increased risk of cardiovascular disease, although these results varied depending on different modifying factors, such as age, ethnicity, and folate status. In contrast, the evidence regarding the A1298C polymorphism was limited and inconclusive.

Keywords: *MTHFR, homocysteine, hyperhomocysteinemia, polymorphism, cardiovascular risk, cardiovascular disease.*

Introducción

Las enfermedades cardiovasculares representan uno de los principales problemas de salud pública a nivel mundial debido a su elevada incidencia y mortalidad. A pesar de los avances en la prevención y el tratamiento, continúan siendo responsables de millones de muertes anuales. Factores como la hipertensión arterial, la diabetes mellitus, la obesidad, el tabaquismo y la dislipidemia han sido reconocidos como determinantes fundamentales del riesgo cardiovascular. Sin embargo, también se han identificado otros factores biológicos y genéticos implicados en el desarrollo y progresión de estas enfermedades.

Uno de los focos de atención actuales es la homocisteína, un aminoácido sulfurado cuyo aumento en plasma (hiperhomocisteinemia) se vincula directamente con el daño vascular.

A nivel genético, las variantes C677T y A1298C del gen MTHFR son las modificaciones más estudiadas por su capacidad para ralentizar la enzima homónima y alterar el metabolismo del folato, elevando así la homocisteína. Dado que la literatura científica muestra resultados sumamente heterogéneos sobre el impacto real de estas variantes, esta revisión bibliográfica se propone integrar y analizar críticamente la evidencia disponible para esclarecer su verdadera relevancia en la clínica cardiovascular.

1. CAPÍTULO I. PLANTEAMIENTO DEL PROBLEMA

1.1. Necesidad del estudio

Las enfermedades cardiovasculares (ECV) constituyen la principal causa de mortalidad a nivel mundial y representan un problema prioritario de salud pública debido a su elevada carga de morbilidad, mortalidad y costos socioeconómicos asociados (Denis de Armas et al., 2024). A pesar de los avances en la identificación y control de factores de riesgo tradicionales, como la hipertensión arterial, la dislipidemia, la diabetes mellitus y el tabaquismo, una proporción significativa de eventos cardiovasculares continúa ocurriendo en individuos sin un perfil de riesgo claramente definido, lo que sugiere la participación de mecanismos fisiopatológicos adicionales aún no completamente esclarecidos.

En este contexto, la hiperhomocisteinemia ha sido reconocida como un factor de riesgo cardiovascular independiente, asociado a procesos aterogénicos y trombogénicos, incluyendo disfunción endotelial, estrés oxidativo y alteraciones en la coagulación (Ghattas Hasbun et al., 2025). La homocisteína es un aminoácido sulfurado cuyo metabolismo depende de vías bioquímicas reguladas por enzimas clave, entre ellas la metilentetrahidrofolato reductasa (MTHFR), cuya función está estrechamente relacionada con el metabolismo del folato y de las vitaminas del complejo B.

Diversos polimorfismos funcionales del gen MTHFR, particularmente C677T y A1298C, han sido ampliamente estudiados debido a su capacidad para reducir la actividad enzimática y favorecer el incremento de los niveles plasmáticos de homocisteína. Estas variantes genéticas han sido asociadas con un mayor riesgo de

enfermedad cardiovascular en múltiples poblaciones; sin embargo, la evidencia científica disponible muestra resultados heterogéneos e incluso contradictorios, influenciados por diferencias en el diseño de los estudios, características genéticas de las poblaciones, factores ambientales y variables clínicas concomitantes (Chita et al., 2020; Kong et al., 2022).

La existencia de una gran cantidad de estudios primarios sobre la relación entre los polimorfismos del gen MTHFR, los niveles de homocisteína y el riesgo cardiovascular, junto con la variabilidad de sus hallazgos, evidencia la necesidad de realizar una revisión bibliográfica que permita integrar y analizar de manera crítica la información disponible. Una síntesis estructurada del conocimiento existente resulta esencial para clarificar el papel real de estas variantes genéticas en el riesgo cardiovascular, identificar patrones consistentes, reconocer discrepancias y detectar vacíos de investigación que orienten futuros estudios.

1.2. Objetivos

1.2.1. Objetivo general

Analizar la evidencia científica disponible sobre la asociación entre los polimorfismos C677T y A1298C del gen MTHFR, los niveles de homocisteína y el riesgo cardiovascular, mediante una revisión sistemática de la literatura científica.

1.2.2. Objetivos específicos

1. Describir los principales polimorfismos del gen MTHFR, con énfasis en C677T y A1298C, y su impacto funcional sobre la actividad de la enzima metilentetrahidrofolato reductasa.

2. Identificar la relación reportada en la literatura entre los polimorfismos del gen MTHFR y los niveles plasmáticos de homocisteína.
3. Analizar la evidencia científica sobre la asociación entre la hiperhomocisteinemia y el riesgo cardiovascular.
4. Comparar los resultados de los estudios incluidos respecto a la influencia de factores genéticos, bioquímicos y clínicos en la relación MTHFR–homocisteína–riesgo cardiovascular.
5. Sintetizar las principales conclusiones, limitaciones metodológicas y vacíos de conocimiento identificados en la literatura revisada.

1.3. Justificación

La identificación de factores de riesgo cardiovascular más allá de los determinantes tradicionales constituye un área de interés creciente en la investigación biomédica, particularmente en el contexto de la prevención y el abordaje temprano de la enfermedad cardiovascular. En este sentido, la hiperhomocisteinemia ha emergido como un biomarcador relevante, cuya asociación con eventos cardiovasculares ha sido documentada en diversos estudios, aunque con resultados variables y no concluyentes.

Los polimorfismos del gen MTHFR, especialmente C677T y A1298C, desempeñan un papel central en la regulación del metabolismo del folato y de la homocisteína, condicionando diferencias interindividuales en la concentración plasmática de este aminoácido y en la respuesta a intervenciones nutricionales, como la suplementación con ácido fólico y vitaminas del complejo B (Meng et al., 2021). A pesar de la amplia producción científica sobre este tema, la falta de consenso y la heterogeneidad de los

hallazgos dificultan la interpretación clínica de estas asociaciones y limitan su aplicación práctica.

La realización de una revisión bibliográfica se justifica como una herramienta metodológica fundamental para integrar y evaluar críticamente la evidencia disponible sobre la relación entre los polimorfismos del gen MTHFR, los niveles de homocisteína y el riesgo cardiovascular. Este enfoque permite organizar la información de manera estructurada, identificar tendencias consistentes, contrastar resultados divergentes y valorar la calidad metodológica de los estudios incluidos.

Desde una perspectiva académica y profesional, los resultados de esta revisión serán de utilidad para investigadores, profesionales de la salud y estudiantes del área biomédica, al proporcionar una visión actualizada y sintetizada de un tema de alta relevancia genética y cardiovascular. Asimismo, el estudio podrá servir como base para el diseño de futuras investigaciones y para el fortalecimiento de estrategias de prevención y manejo del riesgo cardiovascular basadas en la evidencia científica.

2. CAPÍTULO II. MARCO TEÓRICO

2.1. Antecedentes de la investigación

2.1.1. Estudio No.1



metabolites



Article

Biochemical Association of MTHFR C677T Polymorphism with Myocardial Infarction in the Presence of Diabetes Mellitus as a Risk Factor

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Abstract: Myocardial infarction (MI) is a cardiovascular disease that occurs due to the blockage of the coronary artery. Subsequently, cardiac muscles receive a lower oxygen supply, which leads to the death of cardiac muscles. The etiology of MI is linked to various environmental, occupational, and genetic factors. Various studies have been conducted on the polymorphism of genes involved in MI. Previous studies have shown that different variants of the methylene tetrahydrofolate reductase (MTHFR) gene are involved in causing MI by altering the metabolism of folate and homocysteine. However, the genetic polymorphism of MTHFR C677T (rs1801133) and its association with MI in the presence of diabetes mellitus (DM) as a risk factor still needs to be investigated. This study recruited 300 participants who were divided into three groups, i.e., the control, MI, and MI-DM. The blood samples collected from the study participants were subjected to various biochemical tests and their clinical parameters were monitored. MTHFR C677T (rs1801133) genotyping was performed by Tetra ARMS PCR using predetermined primers. The MTHFR C677T (rs1801133) polymorphism was associated with MI in the presence of DM as a risk factor among the participants. The MTHFR C677T (rs1801133) T/T homozygous genotype was found to be significant among MI patients in the presence of DM as a risk factor.

Keywords: MTHFR gene polymorphism; myocardial infarction; myocardial infarction with diabetes mellitus; homocysteinemia; tetra-ARMS PCR



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1. Introduction

Myocardial infarction (MI) is a cardiovascular disease and the main cause of death globally [1]. It is a significant clinical issue because of its mortality [2]. It occurs by the total blockage of the coronary artery, which stops oxygen and nutrients from reaching the heart and causes cell death. MI either happens suddenly or in a devastating way that could cause hemodynamic valve deterioration and mortality [3]. Atherosclerosis leads to coronary artery disease (CAD), which is characterized by the constriction of coronary vessels that occurs after the development of atherosclerotic plaques. Ultimately, endothelial attrition and subsequent thrombosis and plaque rupture induce MI [4]. Cytokines and enzymes released by inflammatory cells weaken the plaque, break down the plaque and lead to the production of thrombus, which causes CAD and acute coronary syndrome [5]. Immune cells such as neutrophils, monocytes, macrophages, mast cells, T cells, and dendritic cells also play a significant role in directing this pathological situation [6]. CD4 T cells, CD8

T cells, B-lymphocytes, and macrophages found within the mononuclear cell infiltrates are primarily responsible for cardiomyocyte destruction [7]. CD4 T cells and their subsets activate and follow complex patterns to coordinate tissue clearance, scar formation, and immune resolution post-MI [8]. T cells prevent the production of pro-inflammatory factors and lower the apoptosis of cardiomyocytes [9]. A series of quick early innate immune responses are activated by MI and are followed by the activation of the adaptive immune system. The activation of immune responses is protective and helps to recover, creates scars, and re-establishes homeostasis [10]. A lack of CD4 T cells worsens myocardial damage and increases the chance of death [11].

MI is a complicated, multifactorial, and polygenic cardiovascular disease that results from the interplay between inherited predisposition and ecological factors [12]. Conventional risk aspects, such as nutrition, adiposity, insulin resistance, high blood pressure, hyperlipidemia, inflammatory processes, tobacco smoking, and excessive alcohol intake, have been shown to predict about 50% of an individual patient's risk of having a cardiovascular incident, with the remainder due to genetic variables [13–15]. The genetic vulnerability may result from alteration and mutations in a number of genes, primarily those implicated in the clotting of blood, maintenance of blood pressure and metabolism of lipid, glucose, and homocysteine [13]. DM increases the incidence of MI in individuals and is a risk factor for the progression of CAD [16]. Progression of atherosclerosis in DM is due to numerous factors, such as DM, oxidative stress, advanced glycation end product, cholesterol, autonomic dysfunction, metabolic abnormalities, excessive inflammatory cytokines, and hereditary factors [17]. Neutrophils, regulatory T cells, myeloid-derived suppressor cells, activated dendritic cells, central memory CD4, and CD8 T cells lead to immune cell infiltration and development of cardiomyopathy in diabetic patients [18].

There is strong evidence that a higher level of total plasma homocysteine is a major health concern for vascular diseases, such as CAD and MI. The plasma concentrations of homocysteine can be increased due to a variety of hereditary or dietary conditions [19]. The methylenetetrahydrofolate reductase (MTHFR) gene has drawn considerable attention as it has been linked to an increased plasma level of homocysteine, which has been associated with an increased risk of MI [20]. Homocysteine, an amino acid that contains sulfur, has significance in CAD and is an absolute atherosclerotic risk of coronary and peripheral vascular lesions [5].

MTHFR is an important enzyme that plays a significant role in the metabolism of homocysteine. The transformation of homocysteine to methionine requires MTHFR, which catalyzes the reaction between 5,10-methylenetetrahydrofolate and 5-methyltetrahydrofolate [21], as shown in Figure 1. The MTHFR gene has 11 exons, a length of 2.2 kb, and is found on chromosome 1p36.3 [22]. The dbSNP database reports that the MTHFR gene has at least 247 single nucleotide polymorphisms (SNPs). The most common polymorphisms of MTHFR gene's DNA sequence variants are MTHFR C677T (rs1801133) and MTHFR A1298C (rs1801131) SNPs [22,23]. The C677T (rs1801133) polymorphism involves the C to T transformation of the MTHFR gene coding sequence at 677 nucleotides, resulting in the substitution of ala-222 with valine, and the A1298C (rs1801131) polymorphism involves the A to C transformation of the MTHFR gene at 1298 nucleotides, resulting in the substitution of Glu-429 with alanine. These are the genetic factors of increased homocysteine levels and thrombotic disposition [24]. Several studies have also demonstrated that elevated homocysteine levels increase the chances of developing cardiovascular diseases [25,26].

The association between the polymorphism of MTHFR C677T (rs1801133) and MI in the presence of DM as a risk factor has not yet been investigated among the Pakistani population. In this study, we have investigated the association of MTHFR C677T (rs1801133) genotype with MI among the Pakistani population and the association of MTHFR C677T (rs1801133) gene polymorphism with MI in the presence of DM, being a risk factor, and we have compared this with healthy individuals.

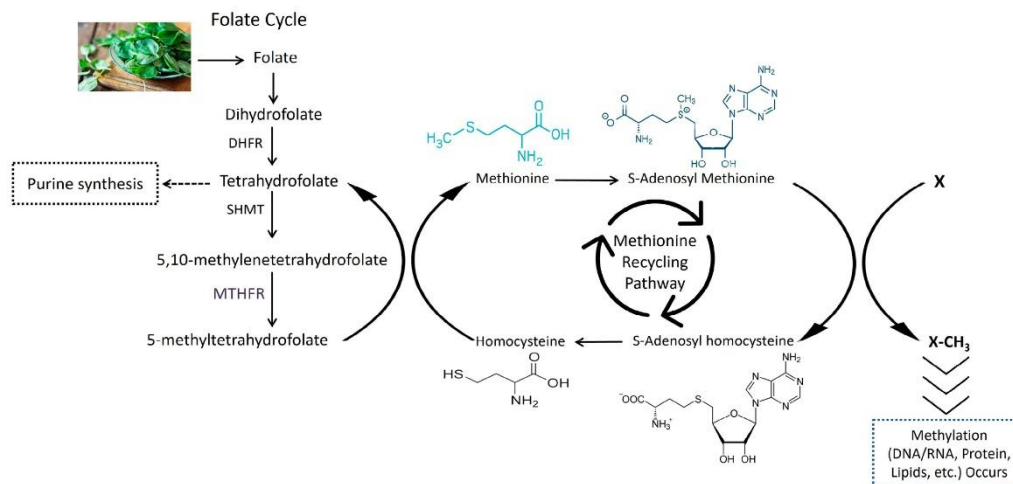


Figure 1. Mechanism of methylene tetrahydrofolate reductase (MTHFR) in folate and homocysteine metabolism.

2. Materials and Methods

2.1. Ethical Considerations

This study was approved by the Ethical Review Committee, Government College University, Faisalabad, Pakistan (Ref. No. GCUF/ERE/36). Informed written consent was obtained from all participants before collecting blood samples.

2.2. Study Design

The current study was a case-control study that was conducted to determine the relationship between the frequencies of MTHFR C677T (rs1801133) gene mutation in MI patients in the presence of DM as a risk factor by analyzing the polymorphism using Tetra-ARMS PCR. The sampling was performed in the Allied Hospital, Faisalabad and the Faisalabad institute of Cardiology, Faisalabad, Pakistan, according to the guidelines approved by the ethical committee of Government College University, Faisalabad, Pakistan. We recruited a total of 300 study participants who were divided into three groups: Group I included the control patients ($n = 100$), group II included MI patients ($n = 100$), and group III ($n = 100$) included MI with diabetic patients (MI-DM). The study participants were briefly informed of the purpose of the research and written consent was obtained from all the participants before collecting blood. The blood specimen was collected from the target population and underwent various biochemical analyses and Tetra-ARMS PCR for genotyping to observe the genetic polymorphism.

2.3. Standards for Inclusion and Exclusion of Study Participants

Study participants of age 30–70 were selected. No participants below the age of 30 or older than 70 were recruited in this study. Study participants who had any other medical history, history of alcohol intake, or any other disorder and used medication for any comorbidity for a long period were excluded from this study. Standard measurements of cardiac biomarkers and electrocardiogram (ECG) were used for the diagnosis of MI and MI-DM patients. MI-DM patients recruited for this study had an HbA1c value greater than 5.7%. Similarly, MI Patients were receiving antiplatelet (aspirin and clopidogrel) and anticoagulant therapy (heparin and streptokinase), whereas patients with MI-DM were receiving antidiabetic (insulin, glipizide, and metformin), antiplatelet (aspirin and clopidogrel), and anticoagulant (heparin and streptokinase) therapies.

2.4. Measurement of Clinical Parameters

Vital signs such as pulse, blood pressure, respiration rate, and temperature were assessed at the time of sample collection. A traditional mercury sphygmomanometer was used to measure the systolic and diastolic pressures (mmHg). A routine ECG was also performed and the patients with ECG abnormalities were noted as a diseased group. The ECG tracings of the control group were normal and there were no clinical signs of MI in the control group.

2.5. Collection of Blood Specimen

Blood was taken from the cubital vein of each subject in two different tubes (EDTA and gel clot activator vacutainer). About 3 mL of blood was taken for biochemical analysis in a gel clot activator vacutainer, while about 4 mL blood was taken for DNA extraction in an EDTA tube. The blood specimen was transported from the hospital to the laboratory in an ice box or ice pack. The gel clot activator vacutainer was then centrifuged to separate the blood serum and stored at -20°C for biochemical evaluation. However, for the extraction of DNA, blood specimens in EDTA tubes were used.

2.6. Analysis of Biochemical Parameters

The separated serum from the blood specimen of study participants was used for the assessment of various biochemical parameters, i.e., glycemic profile (random blood sugar (RBS) and glycated hemoglobin A1c (HbA1c)), cardiac biomarkers (troponin i, creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), and creatinine phosphokinase (CPK)), liver enzymes (alanine transaminase (ALT) and aspartate aminotransferase (AST)), renal profile (urea and creatinine), lipid profile (triglyceride), homocysteine levels, and folate levels, which are involved in MI and DM, by the use of their respective assay kits with the help of a biochemical analyzer (MicroLab-300 BIOBASE, China).

2.7. Extraction of DNA

Genomic DNA was extracted manually from the obtained blood specimens. Blood was collected in Eppendorf tubes and RBC lysis buffer was added to them. The sample was shaken and then centrifuged for 2 min at 7000 rpm. The pellet was broken with the help of a vortex mixer and rinsed well with RBC lysis buffer. Then, nucleic acid lysis buffer was added, followed by saturated NaCl (5 M) and chloroform into the Eppendorf tube. Then, the sample was again mixed and centrifuged for 2 min at 7000 rpm and the supernatant was transferred to a new Eppendorf tube and cold ethanol was added. The sample was shaken and centrifuged for 1 min at 12,000 rpm and then the supernatant was discarded. At the end, TE buffer was added and the mixture was vortexed. Finally, the sample was kept in the Eppendorf tube at -20°C until further analysis for genotyping.

2.8. Purification and Estimation of DNA

The DNA concentration was determined after DNA extraction. For quantitative analysis, the nano-drop method was used, while for the qualitative analysis, the gel electrophoresis technique was used. The purity of the extracted DNA was examined by placing the samples on 2% agarose gel and an absorbance ratio of 260/280 was used to calculate the number of nucleic acids.

2.9. Genotyping of MTHFR Gene (rs1801133)

To genotype the SNP of the MTHFR C677T (rs1801133) gene, Tetra-ARMS PCR was used. Three types of genotypes (C/C, C/T, and T/T) were found for the MTHFR C677T (rs1801133) gene. The base pair size used for Tetra ARMS PCR was 86 bp and 146 bp. For the amplification of the MTHFR C677T (rs1801133) gene, two forward primers and two reverse primers were used (Table 1). At the start of the reaction, two non-allele specific primers were amplified by the outer primers which produced outer fragments that served as a template for the attachment and elongation of inner primers.

Table 1. The Primer Sequence of MTHFR Variant C677T (rs1801133).

Gene Primers	Primer Sequence
Forward inner primer	5'/GAAGGAGAAGGTGTCTGCGGAAT3'
Forward outer primer	5'/CCGAAGCAGGAGCTTTGAGG3'
Reverse inner primer	5'/CCCTCACCTGGATGGGAAAGAT3'
Reverse outer primer	5'/AGCAAAGCTGCGTGATGATGAAATAGG3'

A Thermocycler Master Cycler Gradient was used to perform PCR. PCR was carried out in a 20 μ L container with 1 μ L of DNA sample, 10 μ L Master Mix, 0.1–0.5 mM of each primer, and 8 μ L of DD-H₂O. The initial denaturation temperature was 95 °C for 5 min, further followed by 40 cycles of denaturation at 94 °C for 30 s. Annealing was performed at 52 °C for 75 s, followed by extension at 72 °C for 40 s. The final extension temperature was again 72 °C for about 7 min. After the completion of the PCR reaction, 20 μ L of PCR resultant product was placed in the well with 2% agarose gel, dyed with ethidium bromide, soaked in TAE buffer, and let to run in an electric field. The gel was then examined under UV light using a gel documentation system (InGenius3, Syngene, UK).

2.10. Statistical Analysis

The data were represented as means $\pm$ SD and statistical analysis was performed using GraphPad Prism 5 (version 5.01). Statistically significant differences among the study groups were set at $p < 0.05$ and analyzed by using one way ANOVA and Tukey's Multiple Comparison test. The significant difference in genotype frequency of MTHFR C677T (rs1801133) among the study groups was calculated by using Fisher's exact test.

3. Results

3.1. Clinical Profile and Anthropomorphic Characteristics

The mean systolic blood pressure was 114.2 ± 12.41 mmHg for the controls. The mean systolic blood pressure was 146.4 ± 22.72 mmHg for the MI study group and 174.6 ± 48.46 mmHg for the MI-DM study group. The mean diastolic blood pressure was 74.74 ± 9.88 mmHg for the controls. The mean diastolic blood pressure was 94.6 ± 10.86 mmHg for the MI study group and 108.2 ± 16.29 mmHg for the MI-DM study group. The data were calculated using one way ANOVA and Tukey's Multiple Comparison test as shown in Table 2. After statistical analysis, it was found that the mean systolic blood pressure was high in the MI-DM study group when compared to the MI study group and the controls. The mean diastolic blood pressure was also high in the MI-DM study group when compared to the MI study group and the controls. The gender distribution shows that the number of females recruited in this study was high in MI and MI-DM study groups when compared to males in all groups. Females are at a high risk of developing MI and MI-DM compared to males. While on the other hand, the smoking status of the study participants showed that there were more smokers in the MI and MI-DM study groups when compared to the non-smokers in these groups. Smokers were at a higher risk of developing MI and MI-DM when compared to non-smokers.

Table 2. Comparison of clinical parameters with anthropomorphic parameters among the control, myocardial infarction (MI), and myocardial infarction with diabetes mellitus (MI-DM) study groups.

Characteristics	Control	MI	MI-DM	<i>p</i> -Value
No. of participants	100	100	100	
Age (Years)				
Age	46.94 ± 8.71	52.2 ± 9.484	52.26 ± 9.76	<0.0001

Table 2. Cont.

Characteristics	Control	MI	MI-DM	p-Value
Blood pressure (mmHg)				
Systolic Blood Pressure	114.2 ± 12.41	146.4 ± 22.72	174.6 ± 48.46	<0.0001
Diastolic Blood Pressure	74.74 ± 9.88	94.6 ± 10.86	108.2 ± 16.29	<0.0001
Gender wise distribution (sex)				
Males	30	33	49	<0.0001
Female	70	67	51	<0.0001
Smoking status				
Smoker	12	67	75	<0.0001
Non smoker	88	33	25	<0.0001
Physical activity				
Bad	14	56	51	<0.0001
Good	86	44	49	<0.0001

The p-value was calculated by GraphPad prism (version 5.01) using one-way ANOVA and Tukey's Multiple Comparison test.

3.2. Biochemical Analysis

The glycemic profile (RBS and HbA1c) was significant in MI and MI-DM study groups when compared with that of the controls. The cardiac biomarkers (Troponin i, CK-MB, LDH, and CPK) were found to be significantly high in MI and MI-DM study groups when compared with the controls. The liver enzymes (ALT and AST) were also found to be significantly high in both the MI and MI-DM study groups when compared with the controls. The renal profile (urea and creatinine) and lipid profile (TGs) were found to be significantly high in both the MI and MI-DM study groups when compared with those of the controls. The homocysteine and folate levels were also found to be high in both the MI and MI-DM study groups when compared with the controls. The values of these biochemical parameters are shown in Table 3.

Table 3. Biochemical analysis of the control, myocardial infarction (MI), and myocardial infarction with diabetes mellitus (MI-DM) study groups.

Biochemical Test	Control (n = 100)	MI (n = 100)	MI-DM (n = 100)	p-Value
Random blood sugar	94.24 ± 11.09	245.2 ± 119.5	304.1 ± 108.7	<0.05
Hemoglobin A1C	4.724 ± 0.4901	5.464 ± 1.151	7.892 ± 0.6675	<0.05
Troponin i	0.202 ± 0.770	2.610 ± 1.371	3.637 ± 1.323	<0.05
Creatinine kinase MB	21.96 ± 2.238	42.10 ± 14.75	44.82 ± 18.55	<0.05
Lactate dehydrogenase	372.0 ± 50.69	482.6 ± 100.9	518.4 ± 53.48	<0.05
Creatinine phosphokinase	128.1 ± 35.77	261.6 ± 91.96	287.6 ± 87.81	<0.05
Alanine transaminase	18.78 ± 6.137	36.12 ± 7.299	45.58 ± 8.924	<0.05
Aspartate aminotransferase	26.10 ± 9.745	38.42 ± 13.99	51.76 ± 11.05	<0.05
Urea	30.72 ± 6.523	40.42 ± 11.65	49.28 ± 25.28	<0.05
Creatinine	0.8400 ± 0.147	1.846 ± 0.6193	2.208 ± 0.954	<0.05
Triglycerides	90.76 ± 21.98	157.5 ± 4.945	164.6 ± 10.80	<0.05
Homocysteine	8.200 ± 2.636	49.80 ± 14.18	81.80 ± 13.99	<0.05
Folate	29.64 ± 6.928	8.240 ± 2.760	4.240 ± 1.615	<0.05

The p-value was calculated by GraphPad prism (version 5.01) by using one-way ANOVA and Tukey's Multiple Comparison test.

3.3. Results of Gel Electrophoresis

The MTHFR C677T (rs1801133) gene polymorphism was amplified and genotyped using Tetra-ARMS PCR method. Three different kinds of MTHFR C677T (rs1801133) genotypes (TT, CT, and CC) were confirmed via PCR results after the confirmation of the gel documentation results. In a single PCR reaction, each sample's genomic DNA was amplified using the set of two forward (inner and outer) and two reverse (inner and outer) primers. The band size of 87 bp was for the C allele, for the outer primer and the inner primer. Meanwhile, the 146 bp band was for the other inner and outer primer for the T allele. The PCR product was viewed under UV light after gel electrophoresis as shown in Figure 2.

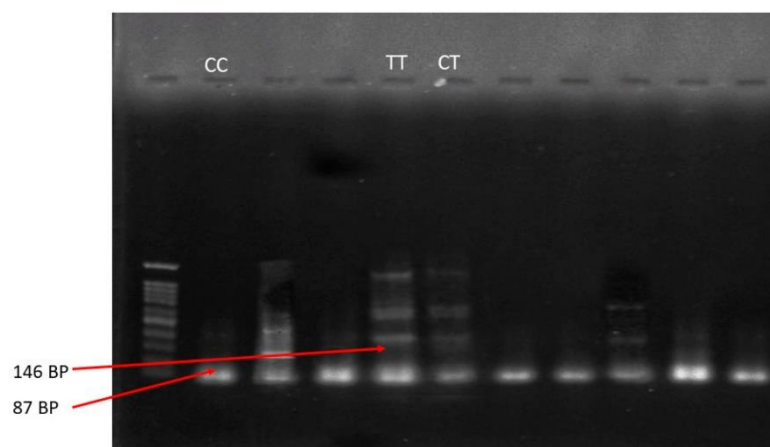


Figure 2. Agarose gel electropherogram of MTHFR (rs1801133), or genotype (CC, TT, and CT) performed by Tetra-ARMS PCR.

3.4. Genotype and Allelic Frequency for the Control, Myocardial Infarction, and Myocardial Infarction with Diabetes Study Group

The genotype (C/C, C/T, and T/T) frequency of MTHFR in the control, MI, and MI-DM study groups showed that the T/T genotype was high among MI and MI-DM study groups when compared to the controls. However, the prevalence of C/C and C/T genotypes was lower among the MI and MI-DM study groups compared to the controls. The allelic distribution of C and T alleles among the study groups showed that the T allele was more prevalent in the MI and MI-DM study groups when compared to the control (Table 4).

Table 4. Genotype and allelic frequency of MTHFR C677T (rs1801133) gene polymorphism in the control, myocardial infarction (MI), and myocardial infarction with diabetes mellitus (MI-DM) study groups.

Groups	Allele		Genotype			p-Value
	T	C	C/C	C/T	T/T	
Control	141	59	71	270	2	0.0001
MI	147	53	53	25	22	
MI-DM	146	54	59	23	18	

The *p*-value was calculated by SNP Stat software (<http://bioinfo.iconcologia.net/SNPstats>) (accessed on 1 December 2022) by using Fisher's exact test and Hardy-Weinberg equilibrium analysis.

3.5. Association of SNP among the Control, Myocardial Infarction, and Myocardial Infarction with Diabetes Mellitus among Study Groups

Association of SNP among the control, MI, and MI-DM study groups showed that there was a significant difference for homozygous genotype T/T under the codominance genetic model (OR = 1.74, CI 95% = 0.65–4.63, $p < 0.05$) and overdominance genetic model (OR = 0.50, CI 95% = 0.27–0.91, $p < 0.05$) between the control and MI study group (Table 5). A significant difference was found for homozygous genotype T/T under the codominance genetic model (OR = 0.52, CI 95% = 0.27–0.97, $p < 0.05$) and overdominance genetic model (OR = 0.45, CI 95% = 0.24–0.83, $p < 0.05$) between the control and MI-DM study groups (Table 6). There was also a significant difference for homozygous genotype T/T under the codominance genetic model between the MI and MI-DM study groups (OR = 0.65, CI 95% = 0.46–2.90, $p < 0.05$) (Table 7). SNP association between the control and diseased (MI and MI-DM) groups showed that for the homozygous genotype (T/T), there was a significant difference under the codominance genetic model (OR = 0.53, CI 95% = 0.31–0.90, $p < 0.05$) and overdominance genetic model (OR = 0.47, CI 95% = 0.28–0.79, $p < 0.05$). Hence, this result indicates that the codominant and overdominant T/T genotype might be a risk for the pathogenesis of MI (Table 8).

Table 5. Association of MTHFR C677T (rs1801133) single nucleotide polymorphism in the control and myocardial infarction (MI) study groups ($n = 200$).

Genetic Model	Genotype	Control ($n = 100$)	MI ($n = 100$)	OR (95% CI)	p -Value
Codominance	C/C	53(53%)	61 (61%)	1.00	0.04
	C/T	40(40%)	25 (25%)	0.54 (0.29–1.01)	
	T/T	7 (7%)	14 (14%)	1.74 (0.65–4.63)	
Dominance	C/C	53(53%)	61 (61%)	1.00	0.25
	C/T-T/T	47(47%)	39 (39%)	0.72 (0.41–1.26)	
Recessive	C/T-C/T	93(93%)	86 (86%)	1.00	0.1
	T/T	7 (7%)	14 (14%)	2.16 (0.83–5.61)	
Overdominance	C/C-C/T	60(60%)	75 (75%)	1.0	0.023
	T/T	40(40%)	25 (25%)	0.50 (0.27–0.91)	

The p -value was calculated by SNP Stat software by using Fisher's exact test and Hardy–Weinberg equilibrium analysis.

Table 6. Association of MTHFR C677T (rs1801133) single nucleotide polymorphism in the control and myocardial infarction with diabetes mellitus (MI-DM) study groups ($n = 200$).

Genetic Model	Genotype	Control ($n = 100$)	MI-DM ($n = 100$)	OR (95% CI)	p -Value
Codominance	C/C	53 (53%)	59 (59%)	1.00	0.006
	C/T	40 (40%)	23 (23%)	2.31 (0.89–5.96)	
	T/T	7 (7%)	18 (18%)	0.52 (0.27–0.97)	
Dominance	C/C	53 (53%)	59 (59%)	1.00	0.39
	C/T-T/T	47 (47%)	41 (41%)	0.78 (0.45–1.37)	
Recessive	C/C-C/T	93 (93%)	82 (82%)	1.00	0.17
	T/T	7(7%)	18 (18%)	2.92 (1.16–7.33)	
Overdominance	C/C-C/T	60(60%)	77 (77%)	1.00	0.009
	T/T	40(40%)	23 (23%)	0.45 (0.24–0.83)	

The p -value was calculated by SNP Stat software (<http://bioinfo.iconcologia.net/SNPstats>) (accessed on 1 December 2022) by using Fisher's exact test and Hardy–Weinberg equilibrium analysis.

Table 7. Association of MTHFR C677T (rs1801133) single nucleotide polymorphism in myocardial infarction (MI) and MI with diabetes mellitus (MI-DM) study groups ($n = 200$).

Genetic Model	Genotype	MI ($n = 100$)	MI-DM ($n = 100$)	OR (95% CI)	p -Value
Codominance	C/C	61 (61%)	59 (5%)	1.00	0.04
	C/T	38 (38%)	34 (34%)	0.86 (0.471–1.57)	
	T/T	10 (10%)	12 (12%)	0.65 (0.46–2.90)	
Dominance	C/C	61 (61%)	59 (59%)	1.00	0.77
	C/T-T/T	39 (39%)	41 (41%)	1.92 (0.62–1.91)	
Recessive	C/C-C/T	86 (86%)	82 (82%)	1.00	0.44
	T/T	14 (14%)	18 (18%)	1.35 (0.63–2.89)	
Overdominance	C/C-C/T	75(75%)	77 (77%)	1.00	0.74
	T/T	25 (25%)	23 (23%)	0.90 (0.47–1.50)	

The p -value was calculated by SNP Stat software (<http://bioinfo.iconcologia.net/SNPstats>) (accessed on 1 December 2022) by using Fisher's exact test and Hardy–Weinberg equilibrium analysis.

Table 8. Association of MTHFR C677T (rs1801133) single nucleotide polymorphism in control and myocardial infarction (MI) with and without diabetes mellitus (DM) study groups ($n = 300$).

Genetic Model	Genotype	Control ($n = 100$)	MI with and without DM ($n = 200$)	OR (95% CI)	p -Value
Codominance	C/C	53 (53%)	120 (60%)	1.00	0.004
	C/T	40 (40%)	48 (24%)	2.02 (0.84–4.86)	
	T/T	7 (7%)	32 (16%)	0.53 (0.31–0.90)	
Dominance	C/C	53 (53%)	120 (60%)	1.00	0.25
	C/T-T/T	47 (47%)	80 (40%)	0.89 (0.55–1.43)	
Recessive	C/C-C/T	93 (93%)	168 (84%)	1.00	0.096
	T/T	7 (7%)	32 (16%)	2.53 (1.07–5.96)	
Overdominance	C/C-C/T	60(54%)	152 (76%)	1.00	0.0046
	T/T	40 (46%)	48 (24%)	0.47 (0.28–0.79)	

The p -value was calculated by SNP Stat software (<http://bioinfo.iconcologia.net/SNPstats>) (accessed on 1 December 2022) by using Fisher's exact test and Hardy–Weinberg equilibrium analysis.

3.6. Clinical and Biochemical Association of MTHFR C677T (rs1801133) SNP

The data for clinical and biochemical parameters are shown in Table 9. The level of systolic blood pressure was high in MI and MI-DM study groups when compared with the control. However, the mean of homozygous genotype T/T was higher in the MI and MI-DM study groups than the C/C and C/T genotypes when compared with control. It was found that blood pressure was elevated in MI and MI-DM study groups when compared with the control, and the mean of homozygous genotype T/T was more than the C/C and C/T genotype. The overall p -value was also significant, indicating that the T/T genotype has an association with the pathogenesis of MI.

The level of glycemic profile (RBS and HbA1c) was found to be high in MI and MI-DM study groups when compared with the control, and the mean of homozygous genotype T/T was high in the MI-DM study group compared with the control. The level of cardiac biomarkers (troponin i, CK-MB, LDH, and CPK) was found to be high in MI and MI-DM study groups when compared to the control and the mean of homozygous genotype T/T was high in the MI and MI-DM study groups. The level of liver enzymes (ALT and AST) was found to be significantly increased in MI and MI-DM study groups when compared with the control, and the mean of the homozygous genotype T/T was high in MI and MI-DM study groups. The level of kidney profile (urea and creatinine) was found to be

significantly increased in MI and MI-DM study groups when compared to the controls, and the mean of homozygous genotype T/T was high in MI and MI-DM study groups. The level of lipid profile (TGs) was found to be high in MI and MI-DM study groups compared to the control, and the mean of the homozygous genotype T/T was higher in the MI and MI-DM study groups than in the control. The level of homocysteine was found to be high in MI and MI-DM study groups compared to the control, and the mean of the homozygous genotype T/T was high in MI and MI-DM study groups compared to the control. The level of folate was significantly decreased in MI and MI-DM study groups compared to the control. However, the mean of the homozygous genotype T/T was higher in MI and MI-DM study groups when compared to the control (Table 9).

Table 9. Clinical and biochemical parameters for control, myocardial infarction (MI), and myocardial infarction with diabetes mellitus (MI-DM) according to genotype (C/C, C/T, and T/T) of MTHFR C677T (rs1801133). The data are presented as means $\pm$ SD.

Parameters	Control (n = 100)			MI (n = 100)			MI-DM (n = 100)			p-Value
	C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T	
Clinical										
Systolic BP	114.2 ± 12.47	113.5 ± 12.33	122.5 ± 12.58	144.0 ± 22.51	147.6 ± 25.41	146.0 ± 11.74	176.3 ± 49.80	166.5 ± 44.71	190.0 ± 52.05	<0.05
Diastolic BP	74.40 ± 9.930	73.48 ± 9.711	85.00 ± 5.774	94.23 ± 10.91	94.74 ± 11.56	96.00 ± 8.433	110.0 ± 17.80	104.7 ± 14.82	110.0 ± 12.06	<0.05
Biochemical										
RBS	94.06 ± 11.11	95.04 ± 11.41	87.50 ± 4.509	241.0 ± 119.6	238.1 ± 117.7	294.1 ± 126.4	303.4 ± 115.8	292.4 ± 103.0	340.7 ± 89.43	<0.05
HbA1c	4.724 ± 0.492	4.743 ± 0.5027	4.500 ± 0.316	5.431 ± 1.152	5.687 ± 1.228	4.790 ± 0.354	7.863 ± 0.682	7.838 ± 0.655	8.175 ± 0.613	<0.05
Troponin i	0.0224 ± 0.0118	0.0230 ± 0.011	0.0125 ± 0.005	2.570 ± 1.367	2.728 ± 1.435	2.373 ± 1.221	3.598 ± 1.332	3.526 ± 1.329	4.128 ± 1.258	<0.05
CK-MB	21.96 ± 2.249	22.17 ± 2.122	19.50 ± 2.517	41.85 ± 14.71	41.26 ± 14.19	46.60 ± 17.69	44.02 ± 18.40	44.82 ± 18.47	48.42 ± 20.60	<0.05
LDH	372.0 ± 50.95	375.2 ± 51.54	335.0 ± 23.80	487.1 ± 103.1	477.6 ± 100.6	478.5 ± 99.15	516.4 ± 52.39	515.4 ± 48.48	535.8 ± 71.28	<0.05
CPK	128.5 ± 36.23	130.4 ± 34.16	96.25 ± 43.47	259.8 ± 91.66	264.9 ± 99.86	257.9 ± 65.86	293.7 ± 90.14	281.6 ± 89.79	276.7 ± 75.12	<0.05
ALT	18.78 ± 6.169	18.72 ± 5.935	19.50 ± 9.609	35.48 ± 7.932	36.74 ± 7.028	37.10 ± 4.630	44.26 ± 9.961	47.09 ± 8.083	47.23 ± 5.246	<0.05
AST	26.31 ± 9.786	25.72 ± 9.960	28.00 ± 8.524	38.13 ± 13.86	37.97 ± 13.72	41.60 ± 16.65	51.46 ± 10.75	50.68 ± 12.28	56.17 ± 8.032	<0.05
Urea	30.72 ± 6.556	30.74 ± 6.794	30.50 ± 3.109	40.23 ± 11.52	41.13 ± 12.56	38.70 ± 9.238	49.33 ± 26.19	52.65 ± 27.20	39.50 ± 9.463	<0.05
Creatinine	0.840 ± 0.1485	0.991 ± 0.055	1.000 ± 0.01	1.817 ± 0.627	1.832 ± 0.620	2.050 ± 0.994	2.193 ± 0.953	2.286 ± 1.058	2.058 ± 0.644	<0.05
Triglycerides	90.76 ± 22.09	91.17 ± 22.88	86.00 ± 9.201	157.4 ± 4.956	157.2 ± 4.600	159.4 ± 6.204	164.6 ± 10.64	164.6 ± 11.65	164.6 ± 9.867	<0.05
Homocysteine	8.200 ± 2.650	8.065 ± 2.703	9.750 ± 1.258	49.33 ± 14.18	49.47 ± 14.37	53.50 ± 14.35	81.76 ± 13.57	80.59 ± 14.76	85.42 ± 14.22	<0.05
Folate	29.64 ± 9.963	29.17 ± 7.012	35.00 ± 3.559	8.327 ± 2.756	8.342 ± 2.989	7.400 ± 1.776	4.241 ± 1.577	4.441 ± 1.691	3.667 ± 1.557	<0.05

The p-value was calculated by GraphPad prism (version 5.01) by using one-way ANOVA and Tukey's Multiple Comparison test.

4. Discussion

CVD has been identified as a primary cause of death over the years [27]. Numerous findings have suggested that environmental and genetic factors play a critical role in the emergence of CAD [28,29]. MTHFR is considered as an important enzyme for the metabolism of homocysteine and its impairment leads to the develop of CVD. Numerous clinical findings have shown that plasma homocysteine levels lead to CVD and predict the death in patients with CAD independent of other risk factors [30,31]. Various nutritional and genetic factors are known to affect the plasma homocysteine levels [32]. Frosst and co-workers found that the SNP of the MTHFR C677T gene lowers the enzyme's activity and thermolability. Homozygous mutant TT alleles result in noticeably higher plasma homocysteine levels [33].

Numerous researchers have conducted case-control studies to establish a link between MTHFR mutation and the occurrence of CAD or MI; however, the findings were conflicting due to small sample sizes. A key feature of meta-analysis is that it increases the sample size and statistical power to combine the comparable research to provide a more competitive conclusion [34]. The prevalence of the MTHFR C677T mutation is linked to a reduction in MTHFR activity by 50%, a rise in homocysteine levels, and a fall in folic acid concentration, which worsens endothelial dysfunction and raises the risk of CVD [35].

A schematic representation of this study is shown in Figure 3. Our data showed that in the MI and MI with diabetes (MI-DM) study groups, there was an increase in homocysteine

levels when compared with the controls. Furthermore, the folate level was decreased in MI and MI with diabetes (MI-DM) study groups.

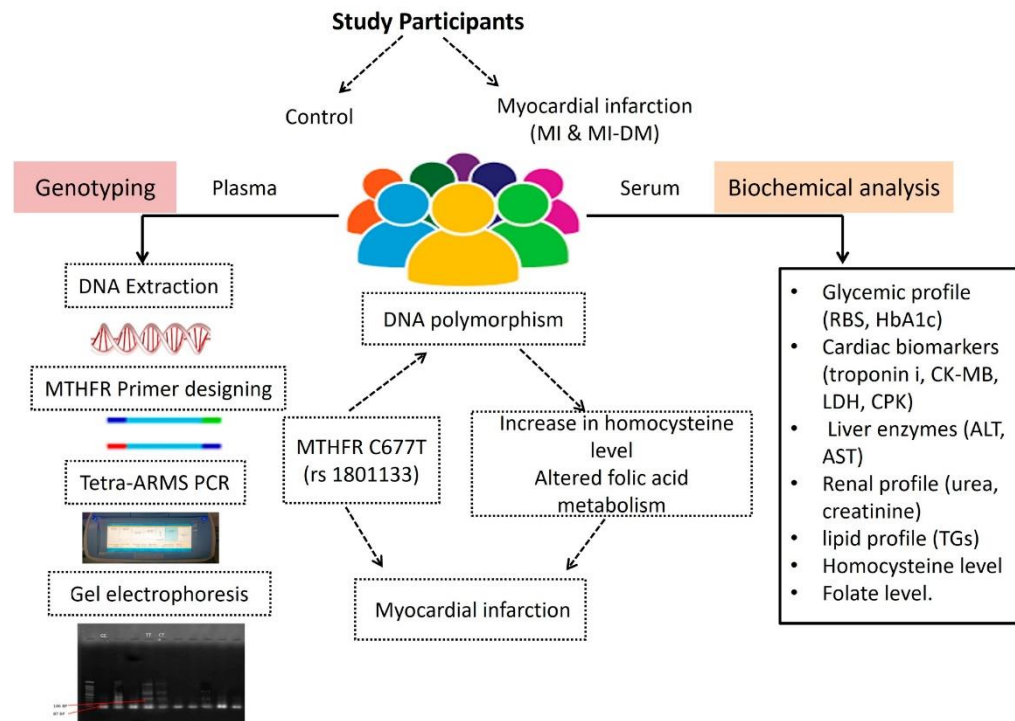


Figure 3. Genotype and biochemical analysis of MTHFR C677T (rs1801133).

In our study, we analyzed all the diagnostic markers such as cardiac biomarkers (troponin i, CK-MB, LDH, and CPK), liver function test (ALT and AST), renal profile (urea and creatinine), triglycerides, HbA1c, and random blood sugar. There was significant increase in the level of cardiac biomarkers in the MI and MI with diabetes (MI-DM) study groups when compared with the control. Liver enzymes were found to be significantly increased in the MI and MI with diabetes (MI-DM) study groups when compared with the control. The renal profile was significantly increased in MI and MI with diabetic (MI-DM) study groups when compared with the control. There was an increase in triglycerides in the MI and MI with diabetes (MI-DM) study groups when compared with the control. There was significant increase in HbA1c and random blood sugar in the MI and MI with diabetes (MI-DM) study groups when compared with the control.

Furthermore, the genotypic study of MTHFR C677T (rs1801133) showed that the codominant and overdominant homozygous T/T genotype had a high homocysteine level in MI and MI-DM study groups compared to the control and could be the risk factor for the pathogenesis of MI. In harmony with our results, Huh et al. showed that people with the homozygous T/T genotype had a high level of homocysteine, and individuals with the T/T genotype had significantly higher genotype-specific folate threshold levels than those with C/C or C/T genotypes. Interestingly, patients with low folate levels and the TT genotype had 2.2-fold higher probability of coronary artery disease (CAD), while those with high folate levels and the T/T genotype had 1.5-fold decreased probability of CAD [36].

Klerk M. et al. found a relationship between the MTHFR C677T genotype and an increased prevalence of coronary heart disease (CHD) in the presence of low plasma folate levels. These findings showed that elevated homocysteine levels are caused by poor folate metabolism and lead to CHD [37]. Contrary to the above data, Kang et al. discovered that those with the variation of MTHFR have a high chance of developing CAD compared to normal. In that study, only 5% of the controls and 17% of the cardiac patients had thermolabile MTHFR [38]. According to another report, the population of Mexico has a high occurrence of the MTHFR C677T mutation with a high frequency of the T allele. The authors of the same study also noted a significant distribution of the T allele in Korea and Italy. However, a comparatively low incidence of the T allele was discovered in Canada, as well as in North and South Africa [39].

According to Benrahma et al., there is an association between Moroccan patients and the control in terms of the allelic frequency of C677T and the occurrence of cardiovascular disease among the Moroccan population [40]. Movva et al. discovered that the MTHFR C677T variant increased the risk of developing myocardial infarction by four times in the Indian population [41].

Eftychiou et al. found no association between MTHFR polymorphism (C677T and A1298C) in patients and the control. The mutant homozygous 677T/T was present more in patients than in the control, while mutant homozygous 1298CC was also present more in patients than in the control [42]. Allawi et al. found that the MTHFR C677T C/T and T/T alleles were present in the Iraqi population and that the MTHFR C677T (T/T) genotype was a risk for developing myocardial infarction [43].

According to Alizadeh et al., the MTHFR C677T mutation was linked to an increased risk of MI in Africa and North America [2]. According to Chen et al., there was a significant interaction between the MTHFR C677T polymorphism and IHD risk. The MTHFR C677T homozygous (T/T) genotypes were highest in the Hispanic, East Asian, African, Caucasian, Middle Eastern, and South Asian populations [44].

Isordia-Salas et al. showed that the mutation of the MTHFR C677T T/T genotype did not increase the incidence of myocardial infarction in the Mexican population [15]. Patti et al. found that the prevalence of myocardial infarction with MTHFR C677T was high with T/T genotype when compared to those with C/T and C/C genotypes, and the T/T genotype independently was a risk factor for myocardial infarction with a specificity of 90%. The greatest homocysteine levels were found in myocardial infarction and the T/T genotype. The T/T homozygosity is independently linked to myocardial infarction [21].

Isordia-Salas et al. found that the T allele of the MTHFR C677T polymorphism is a risk for myocardial infarction in the adults of Mestizos in Mexico [45]. Some of the important studies are mentioned in Table 10.

In conclusion to our results, this study is the first report on the association between the MTHFR C677T (rs1801133) polymorphism and MI patients and MI-DM patients among the Pakistani population. Our data showed a significant difference in the occurrence of the homozygous genotype T/T mutation of the MTHFR C677T (rs1801133) gene among MI patients and MI-DM patients and the control, which suggests an increased risk of MI among the Pakistani population. This study also aimed to individualize the treatments by promoting the rational and direct use of medication, hence removing clinical trial and error. It will reduce the morbidity and mortality rate and will enhance the patient benefits as well as reduce the cost of treatment. This pharmacogenomics study will also help to discover new drug targets and also reduce adverse effects. For future health, it enables the prediction of individual's health risk, the development of personalized medical treatment, and leads to the prevention of disease through drug design and development. Finally, we also state some and/or certain limitations of this study, including the small sample size, limited population, classification of participants into groups, and short time duration.

Table 10. Studies showing the association between MTHFR C677T polymorphism and myocardial infarction.

Country	Control	MI	Genotype Frequency Control			Genotype Frequency in MI			p Value	Ref.
			CC	CT	TT	CC	CT	TT		
Turkey	242	231	123	111	8	119	89	23	0.004	[46]
Italy	1210	1210	363	317	230	371	547	292	0.026	[47]
Japan	2291	1192	804	1134	353	375	579	247	0.152	[48]
Italy	909	1626	311	436	162	574	754	298	0.84	[49]
Sweden	971	514	489	392	90	265	205	44	0.37	[50]
India	120	100	41	10	1	17	3	0	0.451	[51]
Egypt	50	50	22	22	6	22	24	4	0.466	[25]
Turkey	129	107	72	47	10	66	34	7	0.369	[52]
Sweden	514	971	574	754	298	311	436	162	0.66	[53]
Egypt	31	20	14	16	1	3	15	0	0.07	[54]
Mexican	167	167	42	78	47	38	75	54	0.69	[15]
Turkey	70	70	46	16	8	50	12	8	0.58	[55]
China	231	406	88	95	48	80	172	154	0.007	[56]
Tunisia	207	310	123	78	6	131	121	58	0.039	[57]
Iran	150	150	88	55	7	69	74	7	0.66	[58]

5. Conclusions

Our study indicated a high association of MI with the MTHFR polymorphism in the presence of diabetes as a risk factor. However, the predisposition to MI might be due to genetic mutation. We suggest that numerous genome-wide association studies (GWAS) and epidemiological studies must be performed to further illuminate the pathogenesis of MI. An analysis of the relationship between the disease and genetic variation can help in rationalizing the course of treatment and lowering the medication errors. Along with better health outcomes, it can also lower the treatment costs and improve quality of life. The pathophysiology of the disease can be better understood with the use of a pharmacogenomics approach, which may ultimately result in the development of novel and target-specific medications.

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Data Availability Statement: All data generated and/or analyzed during this study are included in this published article.

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Research article



Homocysteine levels, H-Hypertension, and the MTHFR C677T genotypes: A complex interaction

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ABSTRACT

Background and objectives: High homocysteine levels are associated with increased risk of hypertension and stroke. Homocysteine is metabolized by the methylenetetrahydrofolate reductase (*MTHFR*). We aimed to investigate the levels of homocysteine and their association with hypertension, stroke, and antihypertensive medication usage in patients with different *MTHFR* C677T genotypes.

Methods and results: Genotype frequency of *MTHFR* polymorphism was performed, and plasma homocysteine levels were measured in 2,640 adult Lebanese patients. Hypertension, history of stroke, and list of medications were documented, among other clinical and demographic parameters. The TT mutant genotype and the T mutant allele of *MTHFR* were more prevalent in hyperhomocysteinemia (HHcy) and H-hypertensive (H-HTN, defined as hypertension with hyperhomocysteinemia) patients when compared to non-HHcy subjects and non H-HTN patients respectively. Homocysteine levels were significantly higher in hypertensive patients specifically

Abbreviations: HTN, Hypertension; H-HTN, H-type hypertension; MTHFR, methyltetrahydrofolate reductase; HHcy, Hyperhomocysteinemia; NHANES III, the Third National Health and Nutrition Examination Survey; SHEP, Systolic Hypertension in the Elderly Program; CSPT, Chinese stroke primary prevention trial; MEIA, Microparticle enzyme immunoassay; HWE, Hardy-Weinberg equilibrium; ACE, Angiotensin-converting enzyme.

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among those on diuretics. A higher level of homocysteine was found in hypertensive patients with the *MTHFR* T allele compared to patients carrying the C allele. Among the T allele carriers, the average plasma homocysteine level was 13.3 ± 0.193 $\mu\text{mol/L}$ for hypertensive subjects compared to 11.9 ± 0.173 $\mu\text{mol/L}$ (non-hypertensives). Furthermore, homocysteine levels significantly correlated with stroke risk in patients with the T alleles.

Conclusions: We found an association of homocysteine with hypertension, hypertensive medication, and stroke risk among patients with the *MTHFR* T allele and the TT genotype. The association of diuretics therapy with higher homocysteine levels calls for routine measurements and therapeutic control of homocysteine in patients on diuretic, to improve health-related outcomes.

1. Introduction

H-type hypertension (H-HTN) is characterized by increased blood pressure associated with Hyperhomocysteinemia (HHcy), which is defined as plasma homocysteine level ≥ 10 $\mu\text{mol/L}$, due to defective metabolism of plasma homocysteine [1].

H-HTN has been under extensive investigation in recent years. Despite some controversy, elevated levels of plasma homocysteine are an emerging factor associated with several conditions such as congenital heart defect, cardiovascular disease, and neurodegenerative disorders (i.e., Alzheimer's) [2,3]. The relationship between HHcy and HTN has come to contrasting conclusions across several studies. Multiple investigations, such as the Framingham study, failed to establish HHcy as a culprit for HTN [4,5]. Other reports convincingly linked HHcy to HTN and crystallized it as a new risk factor in multiple other findings [6,7]. A large observational trial, conducted as part of the NHANES III (The Third National Health and Nutrition Examination Survey), established an association between HHcy and HTN [8]. Similar findings were provided by the SHEP (Systolic Hypertension in the Elderly Program) study, which showed a direct correlation between HHcy and elevated systolic blood pressure [7]. Of interest, the prevalence of HTN in China is 29.6%, and approximately 75% of hypertensive patients have elevated homocysteine levels [9]. Similarly, the Chinese stroke primary prevention trial (CSPPT) showed that lowering homocysteine levels in patients with H-HTN, through folate intake, had a significant impact on primary prevention of stroke in these patients [10]. H-HTN was associated with an increased risk of all-cause mortality (ACM) in Chinese and in older American patients [11–13]. Therefore, for every 1 $\mu\text{mol/L}$ increment of homocysteine, hypertensive patients had a 7% higher risk of cardiovascular disease mortality and an 8% higher risk of ACM.

High homocysteine levels are linked to an increased risk of ischemic stroke, mainly involving large but also small arteries. However, high homocysteine levels are not believed to be associated with the cardioembolic and other stroke subtypes [14,15].

There are contradictory reports in the literature regarding the association of genetically determined elevations in plasma homocysteine with risk for cerebrovascular and cardiovascular disease [16–19]. Zhao et al. investigated the impact of the *MTHFR* C677T polymorphism on the association of homocysteine with stroke. *MTHFR* codes for an enzyme involved in the breakdown of methionine, using folate and vitamin B12 [20,21]. They reported that in hypertensive patients with the CC/CT genotype, homocysteine levels were associated with an increased risk of stroke. However, this association was not seen in patients with the TT genotype, highlighting a significant role for the genetic background of patients in such association [22]. A meta-analysis involving more than 11,000 *MTHFR* homozygous patients and nearly 13,000 matched controls from 40 observational studies found that the *MTHFR* TT genotype is associated with a higher risk for cardiovascular disease [16]. On the contrary, another meta-analysis of genome-wide association studies reported no association between genetic variants that influence plasma total homocysteine concentrations and cardiovascular disease [18]. The perceived associations between mild hyperhomocysteinemia and cardiovascular diseases may be due to an overlook of confounding risk factors [23–25].

Although the prevalence of H-HTN is reaching an epidemic proportion, its management is still poorly explored in many populations, including the current study population. In fact, even observational studies on the prevalence and prognosis of H-HTN in Lebanon are inexistent. Previous studies targeted Lebanese adults and concluded a prevalence of HTN of 35.9%, with only 27% having controlled blood pressure, while another study yielded similar results with the following values 31.2% and 28.7%, respectively [26, 27]. Nonetheless, there is a paucity of information on the types of HTN and their associated treatment within the Lebanese population, particularly H-HTN. The plasma levels of homocysteine and consequently H-HTN are under the control of exogenous and endogenous factors [28]. We previously described the frequency of the *MTHFR* 677C > T polymorphism in the Lebanese population [20,21,28]. However, the correlation between the 677C > T polymorphism, HHcy, HTN, and its management was not evaluated. This study investigates the levels of homocysteine and their association with HTN, stroke, and antihypertensive medication in patients with different *MTHFR* C677T genotypes. The prevalence of HHcy and H-HTN is also determined.

2. Methods

2.1. Study subjects

A total of 2,640 Lebanese subjects were recruited for this study: out of which 1,589 were hypertensive and 1,051 were not hypertensive and served as control group. The subjects are a subset of a larger group (8,000) enrolled as part of a multicenter cross-sectional study for the FGENTCARD Consortium [29], now part of the CARDIoGRAMplusC4D consortium (<http://www.cardiogramplusc4d.org>). These patients were recruited at two major University Hospitals in Lebanon, one located in Beirut and one in

the Northern part of the country. Only patients with documented homocysteine levels and other biochemical and clinical data listed in Table 1, were included in this study. The genotyping of the samples was available for a subset of 1,337 subjects. The study was approved by the Institutional Review Board committee at the Lebanese American University. Potential participants were briefed on the purpose and the details of what is expected from them in this study; it was also highlighted that their participation is voluntary; those participants who agreed to enroll in this project signed a written informed consent prior to their participation. All protocols were performed according to the Helsinki Declaration of 1975.

Data collection forms were completed on site to record information about the patients' risk factors (hypertension, lifestyle, and family history). Data on clinical conditions, laboratory tests, and current use of medications were annotated following a medical chart/codes as previously described [28,29].

2.2. Description of variants

The description of variants was done in line with our previous report [28]. Homocysteine concentration $>10 \mu\text{mol/L}$ was defined as hyperhomocysteinemia. Clinical conditions (i.e. diabetes, hypertension, hyperlipidemia and stroke) of the participants were recorded according to the patient's treatment regimen or when an ascertained physician documented the condition in the medical chart. The family history (Fx) of hypertension was included when a close family member to the participant (parent, sibling, or second degree relative) was hypertensive. Medications intake, lipid and other metabolic profiles were also recoded from the patient's records. We calculated the body mass index (BMI) following standard measurement methods. Participants with previous and/or current history of smoking cigarettes were considered Smokers.

2.3. Genotyping and immunoassay

Sampling blood was performed by collecting 20 ml of blood (EDTA and gel separating tubes) from each participant. Blood in EDTA tubes was used for plasma collection and DNA extraction. Blood in gel separator tubes was used to collect serum. For the measurement of homocysteine and the evaluation of lipid profile, samples were centrifuged (15 min at RT), and both the plasma and serum aliquots were stored at -20°C for further handling, as previously described [28]. Using a microparticle enzyme immunoassay (MEIA) method, we determined the plasma level of homocysteine for 2,640 participants and this according to the manufacturer protocol. Cholesterol levels were measured on COBAS INTEGRA 400 Plus using fasting blood samples collected from the participants.

The genomic DNA from our group of patients was extracted using the phenol extraction method as described previously [28]. The genotyping of the extracted DNA was performed using the Illumina Human610-Quad BeadChip (Illumina, San Diego, CA, USA) and the Illumina Human660W-Quad BeadChip (Illumina, San Diego, CA, USA). Genotyping was performed according to manufacturer's

Table 1
Descriptive statistics of the 2640 subjects.

	Not Hypertensive (%)	Hypertensive (%)	Total	P-value
	1,051 (39.8%)	1,589 (60.2%)	2,640	
Gender				
Male	788 (75.0%)	986 (62.1%)	1,774 (67.2%)	$<0.0001^*$
Female	263 (25.0%)	603 (37.9%)	866 (32.8%)	
Age	57.037 (11.876)	62.556 (10.688)	60.358 (11.496)	$<0.0001^*$
Diabetes				
No	845 (80.4%)	988 (62.2%)	1,833 (69.4%)	$<0.0001^*$
Yes	206 (19.6%)	601 (37.8%)	807 (30.6%)	
Hyperlipidemia				
No	648 (61.8%)	740 (46.6%)	1,388 (52.7%)	$<0.0001^*$
Yes	401 (38.2%)	847 (53.4%)	1,248 (47.3%)	
Total Cholesterol (mg/dL)	188.512 (48.072)	182.147 (46.243)	184.665 (47.069)	$<0.0001^*$
Hyperhomocysteinemia				
No	260 (24.7%)	331 (20.8%)	591 (22.4%)	0.021*
Yes	791 (75.3%)	1,258 (79.2%)	2,049 (77.6%)	
Smoking				
No	346 (33.0%)	605 (38.1%)	951 (36.0%)	0.008*
Yes	704 (67.0%)	984 (61.9%)	1,688 (64.0%)	
Family history of Hypertension				
No	551 (52.5%)	579 (36.6%)	1,130 (42.9%)	$<0.0001^*$
Yes	498 (47.5%)	1,003 (63.4%)	1,501 (57.1%)	
Weight (kg)	79.448 (15.395)	80.263 (15.217)	79.939 (15.290)	0.186
BMI (kg/m²)	28.310 (4.704)	29.810 (5.073)	29.215 (4.983)	$<0.0001^*$
Stroke				
No	1,042 (99.2%)	1,550 (97.7%)	2,592 (98.3%)	0.004*
Yes	8 (0.8%)	37 (2.3%)	45 (1.7%)	

For categorical variables, the number of individuals and percentages are reported. For scale variables, data are mean $\pm$ standard deviation (SD). The P is generated using a chi-square test (categorical variables) and an independent *t*-test (scale variables). P is significant (*) if P-value <0.05 . Hyperhomocysteinemia: plasma homocysteine is $\geq 10 \mu\text{mol/L}$.

instructions for the Illumina 610 and 660W-Quad arrays.

2.4. Statistical analysis

We processed our data by applying chi-square tests for ordered and unordered categorical measures, and independent *t*-test for continuous variables. We applied generalized linear model (GLM) binomial logistic regression to evaluate the association of HTN and the demographic and clinical data such as gender, age, diabetes, hyperlipidemia, Fx HTN, stroke, total cholesterol and BMI. We also applied binomial regression to evaluate the association between homocysteine and the different medications used for HTN such as beta blockers, calcium channel blockers, Angiotensin-Converting Enzyme (ACE), Angiotensin II antagonists, diuretics, nitrates, and vasodilators. Adjusted odds ratios were obtained using the same regression, after controlling for age and/or sex. *P* value of <0.05 indicated statistical significance. R package was used to perform the above-mentioned statistical analyses. We performed allele and genotype frequency of *MTHFR* polymorphism (rs1801133) to relate the presence of 677C > T mutation of *MTHFR* with HHcy. Grouped Barplots by the percentage of *MTHFR* alleles and genotypes in HHcy and non-HHcy groups were plotted. The Hardy-Weinberg equilibrium (HWE) was tested with Chi-square (χ^2) goodness of Fit test. The distribution of genotypes agrees with HWE if the *p* value > 0.05. In addition, Fisher's exact test using R package was applied to test the differences between frequencies. Differences were considered significant at *p* value < 0.05. We evaluated homocysteine levels among T or C allele carriers according to HTN, diuretics intake, and stroke. Two-way ANOVA with Tukey posthoc test was used to determine whether *MTHFR* variants and genotypes, HTN, diuretics or stroke impact homocysteine levels and to determine whether there is an interaction between *MTHFR* alleles/genotypes and HTN, diuretics or stroke, with homocysteine levels.

3. Results

Our study targeted a group of Lebanese adults with a mean age of 60.358 (± 11.496) years, a mean body mass weight of 79.939 (± 15.290) Kg, a mean BMI of 29.215 (± 4.983) Kg/m², and a mean total cholesterol 184.665 (± 47.069) mg/dL (Table 1). In our groups of patients, 1,774 of the subjects were males (67.2%) and 866 were females (32.8%). A total of 1,589 (60.2%) of the subjects were hypertensive, 807 subjects were diabetic (30.6%), 1,248 were hyperlipidemic (47.3%), 2,049 had hyperhomocysteinemia (77.6%) (HHcy: homocysteine > 10 μ mol/L), 1,501 had family history (Fx) of hypertension (57.1%), 45 had history of stroke (1.7%), and 1,688 subjects were smokers (64%). Significant differences between hypertension patients and control individuals for age, gender, stroke, diabetes, hyperlipidemia, HHcy, smoking, Fx HTN, total cholesterol, BMI and homocysteine levels were detected. About 37.9% of the hypertensive subjects were females and 62.1% were males, while 25% of the non-hypertensive subjects were females and 75% were males. This gender-linked component was statistically significant (*P* < 0.001). Diabetes and hyperlipidemia were both significantly elevated in people with HTN when compared with non-hypertensive subjects, from 19.6% to 37.8% and from 38.2% to 53.4%, respectively. HHcy were significantly higher in hypertensive patients compared to non-hypertensive patients (79.2% vs 75.3%). It is worth noting that 48.8% of our participants suffer from H-hypertension. Stroke history was also significantly higher in hypertensive patients compared to non-hypertensives (2.3% vs 0.8%). Family history of hypertension was significantly increased from 47.5% to 63.4% in hypertensive patients compared to non-hypertensives. BMI levels were significantly different between HTN and non-HTN groups (*P* < 0.0001).

Following adjustment for age and sex, we identified significant association between HTN and the clinical and biochemical features (Table 2). Most notably diabetes, hyperlipidemia, and Fx HTN were shown to be significantly associated with HTN, with adjusted odds ratio (OR) of 2.32 [95% C.I. 1.92–2.80], 1.89 [1.60–2.23], and 2.21 [1.86–2.61], respectively. The risk of HTN was significantly correlated with HHcy with OR of 1.28 [1.06–1.56]. BMI was also positively correlated with HTN with OR of 1.06 [1.04–1.08]. Stroke was negatively associated with HTN with OR of 0.41 [0.17–0.85].

We explored the relationship between antihypertensive medications intake and plasma homocysteine levels. The intake of beta-blockers (*P* = 0.014) or ACE (Angiotensin-Converting Enzyme) inhibitors (*P* = 0.03) was significantly associated with HHcy (Table 3). The intake of diuretics showed the strongest association (OR = 2.03 [1.54–2.70]; *P* < 0.0001) with plasma homocysteine

Table 2
Association analysis between hypertension and different sample metadata.

	P-value	OR	95%C.I.	
Gender	<0.0001*	1.69	1.42	2.02
Age	<0.0001*	1.04	1.04	1.05
Diabetes	<0.0001*	2.32	1.92	2.80
Hyperlipidemia	<0.0001*	1.89	1.60	2.23
Total Cholesterol	0.016*	1.00	1.00	1.00
Hyperhomocysteinemia	0.011*	1.29	1.06	1.57
Fx Hypertension	<0.0001*	2.21	1.86	2.61
BMI	<0.0001*	1.07	1.05	1.09
Stroke	0.025*	0.41	0.17	0.85

After adjusting for age and/or sex, logistic regression was used to assess the association between hypertension and different clinical and biochemical features. OR > 1 indicates increased occurrence of HTN. Association with HTN is significant (*) if *P*-value < 0.05. OR: odd ratio; Fx: family history; 95% C.I.: 95% confidence interval. A patient is considered Hyperhomocysteinemic if plasma homocysteine is ≥ 10 μ mol/L.

levels. In contrast, the intake of calcium channel blockers, angiotensin II receptor antagonists, nitrates, or vasodilators did not significantly correlate with plasma homocysteine levels.

We sought to compare the genotypes and allele frequency in HHcy and non-HHcy groups. Fisher's exact test was used to examine the difference in the genotype distribution among HHcy groups ($P = 0.3757$) and the difference in the allelic distribution among HHcy groups ($P = 0.583$) (Fig. 1 A & B). The T allele frequency was 35% in the HHcy group compared to 33.1% in non-HHcy, while the C allele frequency was 65% in the HHcy group compared to 66.9% in the non-HHcy group (Fig. 1A). *MTHFR* genotype frequency distribution in the HHcy group was: CC 43.6%, CT 42.8% and TT 13.6%. In the non-HHcy group, the *MTHFR* genotypes frequency distribution was: CC 46.8%, CT 40.6%, and TT 12.9%. Moreover, The T allele frequency was 36.1% in the H-HTN group compared to 30.1% in non the H-HTN group, while the C allele frequency was 63.9% in the H-HTN group compared to 69.9% in the non H-HTN group (Supplementary Fig. 1). *MTHFR* genotypes frequency distribution in the H-HTN group was: CC 42.9%, CT 42.1%, and TT 15.1% while in the non H-HTN group was: CC 50.3%, CT 39.3%, and TT 10.4%.

Homocysteine levels varied significantly with HTN and *MTHFR* alleles ($P < 0.001$), though the interaction between HTN and *MTHFR* was not significant ($P = 0.2445$). The results showed that homocysteine levels for C allele carriers with HTN were higher than that for C allele carrier without HTN ($P < 0.0001$). In addition, homocysteine levels for T allele carriers with HTN were higher than that for T allele carriers without HTN ($P < 0.0001$), $13.3 \pm 0.193 \mu\text{mol/L}$, $11.9 \pm 0.173 \mu\text{mol/L}$, respectively (Fig. 2a). Higher levels of homocysteine were found in hypertensive patients carrying the T allele ($13.3 \pm 0.193 \mu\text{mol/L}$) compared with patients carrying the C allele ($12.8 \pm 0.127 \mu\text{mol/L}$). The Two-way ANOVA analysis showed that there was a significant difference in the levels of homocysteine between diuretics and non-diuretics groups ($P < 0.001$). In the C allele carriers, the homocysteine level was significantly higher in those who are on diuretics compared to those who are not on diuretics ($P < 0.001$). Increased levels of homocysteine in T carriers with diuretics treatment were observed when compared to the non-diuretics treatment group (14.1 ± 0.385 vs $13.1 \pm 0.230 \mu\text{mol/L}$, $P < 0.001$). In the diuretics group, the average plasma homocysteine level for the T allele carriers was $14.1 \pm 0.385 \mu\text{mol/L}$ compared to $13.6 \pm 0.262 \mu\text{mol/L}$ for the C allele carriers (Fig. 2b).

Moreover, since stroke was associated with HTN, we sought to evaluate the levels of homocysteine in the different alleles and genotypes of *MTHFR*. The levels of homocysteine correlated significantly with stroke and the different *MTHFR* alleles ($P < 0.05$). However, no significant interaction was found between stroke and the *MTHFR* alleles ($P = 0.99$). For the C allele carriers, homocysteine levels in subjects with stroke were higher than those without stroke ($P < 0.0001$). For the T allele carriers, the homocysteine levels for patients with stroke were higher than those without stroke, $15.2 \pm 1.42 \mu\text{mol/L}$ vs $12.4 \pm 0.124 \mu\text{mol/L}$ ($P < 0.0001$) (Fig. 2c). Higher levels of homocysteine were found in stroke patients carrying the T allele compared with patients carrying the C allele (15.2 ± 1.42 vs. $14.9 \pm 0.761 \mu\text{mol/L}$, respectively).

In the TT genotypes, an average plasma homocysteine level of $13.6 \pm 0.424 \mu\text{mol/L}$ was observed in hypertensives compared with $12.3 \pm 0.442 \mu\text{mol/L}$ for the non-hypertensive subjects. Among hypertensive patients, higher levels of homocysteine were found in those carrying the TT genotype compared to the CC carriers (13.6 ± 0.424 vs $12.3 \pm 0.442 \mu\text{mol/L}$). Increased levels of homocysteine in the TT carriers with diuretic treatments were observed when compared to the non-diuretic treatment group (14.5 ± 0.857 vs $13.1 \pm 0.491 \mu\text{mol/L}$). The average plasma homocysteine level for the TT genotype in the diuretics group was $14.5 \pm 0.857 \mu\text{mol/L}$ compared to $13.4 \pm 0.419 \mu\text{mol/L}$ and $13.8 \pm 0.517 \mu\text{mol/L}$ for the CC and CT genotypes, respectively. In addition, higher levels of homocysteine were found in stroke patients carrying the TT genotype compared with patients carrying the CC genotype (16.7 vs. 15.1 , respectively) (Supplementary Fig. 2).

4. Discussion

Elevated homocysteine is a predictor of cardiovascular disease, but its role in HTN remains controversial [30,31]. While HTN has been associated with HHcy, the role of HHcy in the development of HTN and its complication has been contentious for the past decade. Despite the fact that studies showed a causal link between HHcy and HTN [7,8,32], others failed to reproduce similar results and came to contrasting conclusions [33]. The potential role of HHcy in the pathogenesis of high blood pressure was supported by the reduction in both systolic and diastolic blood pressures, which correlated with homocysteine-lowering treatment [10,34,35]. Our study brings additional information to the ongoing debate, while addressing the causal link between HTN and HHcy, and shows that high serum levels of homocysteine are associated with HTN.

Table 3
Association analysis between hyperhomocysteinemia and different medications for hypertension.

	P value	OR	95% C.I.	
Beta blockers	0.014*	1.28	1.05	1.57
Calcium channel blockers	0.47	0.91	0.70	1.19
ACE inhibitors	0.036*	1.28	1.02	1.63
Angiotensin II antagonists	0.66	1.06	0.81	1.42
Diuretics	<0.0001*	2.03	1.54	2.70
Nitrates	0.12	1.42	0.93	2.27
Vasodilators	0.17	1.22	0.92	1.64

After adjusting for age and sex, logistic regression was used to assess the association between hyperhomocysteinemia and different medications used for hypertension. OR >1 indicates increased occurrence of hyperhomocysteinemia (homocysteine >10 $\mu\text{mol/L}$). Association with hyperhomocysteinemia is significant (*) if P-value <0.05. ACE: Angiotensin-Converting Enzyme.

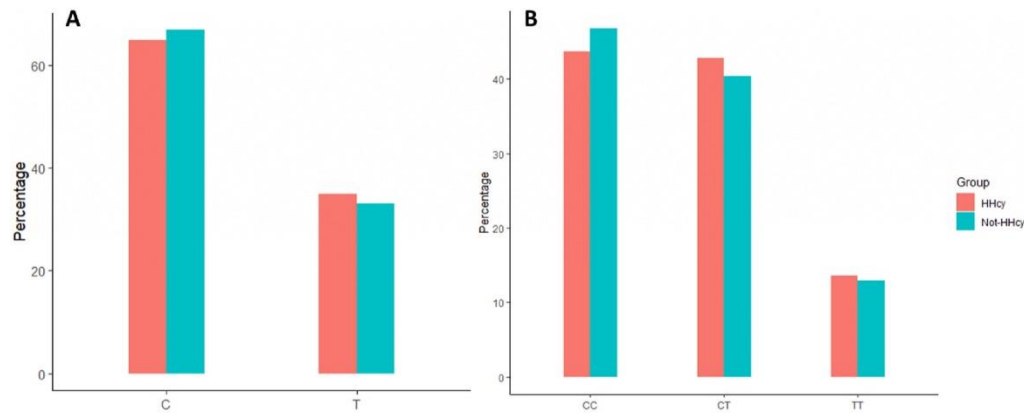


Fig. 1. Genotypes and allele frequencies (in %) of *MTHFR* C677T according to hyperhomocysteinemia (HHcy) groups.

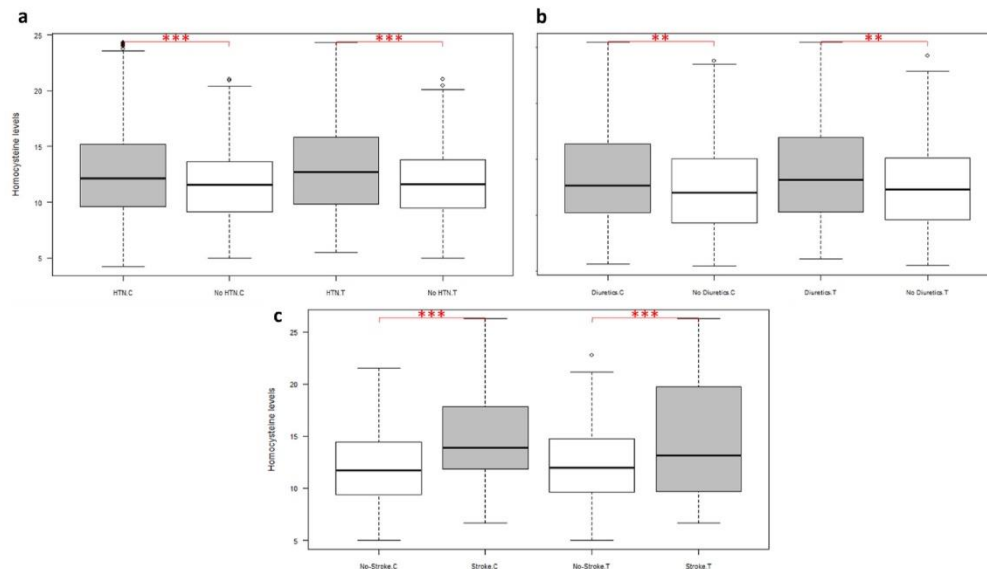


Fig. 2. Homocysteine levels and *MTHFR* alleles according to (a) hypertension status, (b) diuretics intake and (c) stroke.

Our data show an association between high homocysteine levels and HTN, HTN medication, and stroke risk among patients with the *MTHFR* T allele, particularly the TT genotype. The association strengthened further after correction for several confounding predisposing factors, thus confirming that the seen effect is not due to an indirect association between HHcy and other risk factors. The *MTHFR* 677C > T is associated with elevated plasma levels of homocysteine. The *MTHFR* enzyme plays an important role in homocysteine metabolism. Thus, the mutant allele 677C > T, which results in a reduced activity of the enzymes involved in homocysteine metabolism, is now known to be the most prevalent genetic cause of hyperhomocysteinemia [36–38]. We found an increase in the prevalence of the TT mutant genotype and the T mutant allele in the HHcy group, compared to non-HHcy group. Previous studies reported a median homocysteine value of 11.5 $\mu\text{mol/L}$ for people with HTN, and 9.9 $\mu\text{mol/L}$ for control subjects [7]. Although the *MTHFR* 677C > T polymorphism was not genotyped, these values are in line with our recordings for hypertensive patients with a CC genotype. Genotyping this polymorphism in other populations is crucial to test whether a TT genotype would correlate with higher systolic and/or diastolic blood pressure levels and with higher homocysteine levels.

Our results also indicate a positive association of HHcy with the use of beta blockers, and ACE inhibitors. Both of these medications

were shown to decrease plasma homocysteine levels in hypertensive patients [39]. Studies investigating the impact of antihypertensive medications on plasma homocysteine levels remain however scarce.

Patients on diuretics carrying the TT genotype or the T allele have higher levels of homocysteine than those carrying the CC genotype or the C allele. In fact, patients carrying the C allele who are on diuretics have almost similar homocysteine levels (13.6 $\mu\text{mol/L}$) than those carrying the T allele and are not on diuretic therapy (13.1 $\mu\text{mol/L}$). These findings demonstrate the complex and non-linear interaction between the *MTHFR* genotype, HTN, homocysteine levels and diuretic use. Previous studies have shown a significant increase in serum homocysteine concentration associated with the chronic use of diuretics [40,41], while others showed a modest association between these two factors [42]. Our data show that elevated levels of serum homocysteine are seen in correlation with the TT genotype and the T allele, regardless of the intake of diuretics. We posit that the TT genotype is directly correlated with increased levels of homocysteine with no evidence for any association between diuretics intake and HHcy, thus ruling out a causation effect of diuretics on HHcy.

The *MTHFR* 677C > T polymorphism is reported to be associated with high levels of homocysteine and stroke [43], we therefore investigated the association between stroke, homocysteine and the *MTHFR* C677T polymorphism. High levels of homocysteine were found in patients with stroke regardless of the *MTHFR* genotype. In addition, a higher level of homocysteine was found to be associated with the TT genotype and the T allele in stroke patients, compared to the CC genotype and the C allele. This suggested that the risk of stroke might increase with high homocysteine level in the presence of TT mutant genotype in our population.

Our data suggest that patients with high levels of homocysteine who are carriers of the TT genotype are at a higher risk of developing severe HTN, necessitating treatment by a combination of antihypertensive medications including diuretics. These patients are likely to be at higher risk of developing stroke and may be requiring homocysteine lowering medications. Furthermore, HHcy has been linked to deficiencies in folate, vitamins B6 and B12. In fact, B-vitamins supplementation has been shown to reduce homocysteine [44]. A recent meta-analysis of randomized trials showed that folic acid supplementation could efficiently decrease the risk of cardiovascular diseases, including stroke [45]. Folic acid and/or a vitamin B complex might be useful in the prevention of morbidity associated with H-HTN.

Our study has several limitations, the most prominent one being the low number of individuals genotyped for the *MTHFR* 677C > T polymorphism. Despite the fact that only 1.7% of our population experienced stroke, there was a strong statistical significance ($p = 0.004$) between stroke and hypertension. Unfortunately, due to this lower number we could not directly evaluate the association between H-Hypertension and stroke after adjustment for anti-hypertensive medication use. Our study demonstrated however, that H-Hypertension, a phenotype that has not been previously addressed in this population, is prevalent and is a major modulator of health risks that are associated with elevated blood pressure including stroke.

In conclusion, we have shown that high levels of plasma homocysteine are partly associated with the *MTHFR* 677C > T polymorphism. We also showed that a high risk of stroke might be associated with high levels of homocysteine and the presence of the mutant T allele of *MTHFR* in our subjects. Further, we showed that the T allele is correlated with high homocysteine levels irrespective of the intake of diuretics, thus ruling out the causation effect of diuretics on HHcy. Our findings promote our knowledge on the etiology of HHcy with direct impact on public health. Finally, we posit that early detection of HHcy may lead to the prevention of the high levels morbidity related to HTN and its complications, namely stroke.

Author contribution statement

Cynthia Al Hageh, Eman Alefishat, Michella Ghassibe-Sabbagh: Analyzed and interpreted the data; Wrote the paper.

Daniel E Platt: Analyzed and interpreted the data.

Hamdan Hamdan, Raya Tcheroyan, Siobhán O'Sullivan: Performed the experiments.

Elie Chammas, Antoine Abchee: Contributed reagents, materials, analysis tools or data.

Binyan Wang, Xiping Xu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Moni Nader, Pierre Zalloua: Conceived and designed the experiments.

Data availability statement

Data will be made available on request.

Support

Chinese National Key Research and Development Program.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2023.e16444>.

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2.1.3. Estudio No.3

Original Investigation | Cardiology

Association of Homocysteine, Methionine, and *MTHFR* 677C>T Polymorphism With Rate of Cardiovascular Multimorbidity Development in Older Adults in Sweden

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Abstract

IMPORTANCE Strong evidence links high total serum homocysteine (tHcy) and low methionine (Met) levels with higher risk of ischemic disease, but other cardiovascular (CV) diseases may also be associated with their pleiotropic effects.

OBJECTIVES To investigate the association of serum concentrations of tHcy and Met with the rate of CV multimorbidity development in older adults and to explore the role of methylenetetrahydrofolate reductase (*MTHFR*) 677C>T polymorphism in this association.

DESIGN, SETTING, AND PARTICIPANTS The Swedish National Study on Aging and Care in Kungsholmen is a cohort study of randomly selected individuals aged 60 years or older. The present study included data on 1969 individuals with complete information and without CV diseases at baseline, collected from the baseline examination (2001-2004) to the fourth follow-up (2013-2016). Data analysis was conducted from January to May 2019.

EXPOSURES Concentrations of tHcy and Met were measured from nonfasting venous blood samples. The Met:tHcy ratio was considered a possible indicator of methylation activity. *MTHFR* status was dichotomized as any T carriers vs noncarriers.

MAIN OUTCOME AND MEASURES The number of CV diseases at each wave was ascertained based on medical interviews and records, laboratory test results, and drug data. Linear mixed models were used to study the association of baseline tHcy and Met levels and the rate of CV multimorbidity development, adjusting for sociodemographic characteristics, CV risk factors, chronic disease burden, and drug use.

RESULTS Of 1969 participants, most were women (1261 [64.0%]), with a mean (SD) age of 70.9 (9.8) years; 1703 participants (86.6%) had at least a high school level of education. Baseline measurements of serum tHcy, Met, and the Met:tHcy ratio were associated with the rate of CV disease accumulation (tHcy: $\beta = 0.023$ per year; 95% CI, 0.015 to 0.030; $P < .001$; Met: $\beta = -0.007$ per year; 95% CI, -0.013 to -0.001 ; $P = .02$; Met:tHcy ratio: $\beta = -0.017$ per year; 95% CI, -0.023 to -0.011 ; $P < .001$). The association between low Met concentrations and the rate of CV multimorbidity development was restricted to the group with CT/TT alleles of *MTHFR* ($\beta = 0.023$ per year; 95% CI, 0.006 to 0.041; $P = .009$). Results remained largely significant when individual CV diseases were removed from the total count 1 at a time (eg, ischemic heart disease, tHcy: $\beta = 0.023$ per year; 95% CI, 0.013 to 0.027; $P < .001$; Met: $\beta = -0.006$ per year; 95% CI, -0.011 to -0.0003 ; $P = .04$; Met:tHcy ratio: $\beta = -0.015$ per year; 95% CI, -0.020 to -0.009 ; $P < .001$).

(continued)

Key Points

Question Are serum concentrations of homocysteine and methionine associated with the rate of cardiovascular multimorbidity development in older adults?

Findings In this cohort study of 1969 older adults in Sweden, high homocysteine levels, low methionine levels, and a low methionine to homocysteine ratio were associated with faster development of cardiovascular multimorbidity. The *MTHFR* 677C>T polymorphism was further associated with accelerated cardiovascular multimorbidity development in participants with a low methionine concentration.

Meaning The findings of this study suggest that these biomarkers may have a potentially meaningful role of in the pathogenesis of cardiovascular aging, possibly through impaired methylation activity.

+ Supplemental content

Author affiliations and article information are listed at the end of this article.

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May 20, 2020 1/14

Abstract (continued)

CONCLUSIONS AND RELEVANCE In this study, high tHcy and low Met levels were associated with faster CV multimorbidity development in older age. The interactive association of Met concentrations and *MTHFR* polymorphism, together with the association found for the Met:tHcy ratio, point toward the relevance of impaired methylation in the pathogenesis of CV aging.

JAMA Network Open. 2020;3(5):e205316. doi:10.1001/jamanetworkopen.2020.5316

Introduction

Most age-related cardiovascular (CV) diseases share common underlying biological mechanisms. Telomere attrition,¹ epigenetic modifications,² defects in autophagy or mitophagy,³ and cell senescence⁴ eventually contribute to a pro-inflammatory environment that is common in different disorders, such as atherosclerosis, cardiomyopathies, heart failure, ischemic heart disease, and stroke. The rate at which multiple CV diseases, ie, CV multimorbidity, accumulate is a marker of CV aging.⁵ Thus, the identification of cellular and molecular biomarkers of accelerated CV multimorbidity development may be helpful for secondary prevention and prognostic counselling.

Homocysteine, an amino acid generated via demethylation of dietary methionine, is associated with atherosclerosis and its complications, such as myocardial infarction and stroke.^{6,7} In fact, high concentrations of total homocysteine (tHcy) can lead to endothelial cell damage, impaired vascular compliance, and alterations of hemostasis.⁸ Besides an unhealthy lifestyle, poor diet, impaired renal function, and intake of certain drugs,⁹ moderate increases in tHcy have been associated with a 677C>T variant in the gene coding for methylenetetrahydrofolate reductase (*MTHFR*; OMIM 607093).¹⁰

Methionine (Met), obtained both from diet and from the remethylation pathway of tHcy metabolism, is an essential amino acid functioning as a precursor for cysteine and glutathione, 2 major antioxidants. Studies in mice suggest that low Met availability affects the development of atherosclerotic lesions and the regulation of inflammatory response and hepatic cholesterol metabolism,^{11,12} but its role in CV diseases is unclear. One study found an association between low blood Met levels and incident myocardial infarction¹³; another study corroborated this association prospectively but only in subjects with high low-density lipoprotein (LDL) cholesterol levels¹⁴; a third study showed no such association.¹⁵ A positive association between low Met concentration and venous thrombosis risk has also been reported.¹⁶

High tHcy and low Met blood concentrations could have antagonistic pleiotropic effects on CV aging, not only via atherosclerosis, the most studied mechanism, but also through other pathways, such as lipid metabolism, DNA synthesis and repair, oxidative damage, development of inflammation, and telomere attrition.¹⁷ Therefore, it is plausible that tHcy and Met may play a role in multiple physiologic processes underlying CV aging, including left ventricular wall thickening, increased left atrial size, and vascular intimal thickening, stiffening, and fibrosis.¹⁸ To our knowledge, no prior study has examined the association of tHcy and Met with multiple CV diseases as a group or with the rate at which these disorders accumulate.

We aimed to investigate the association of serum concentrations of tHcy and Met with the rate of CV multimorbidity development in older adults. We further intended to explore the role of *MTHFR* 677C>T polymorphism in this association.

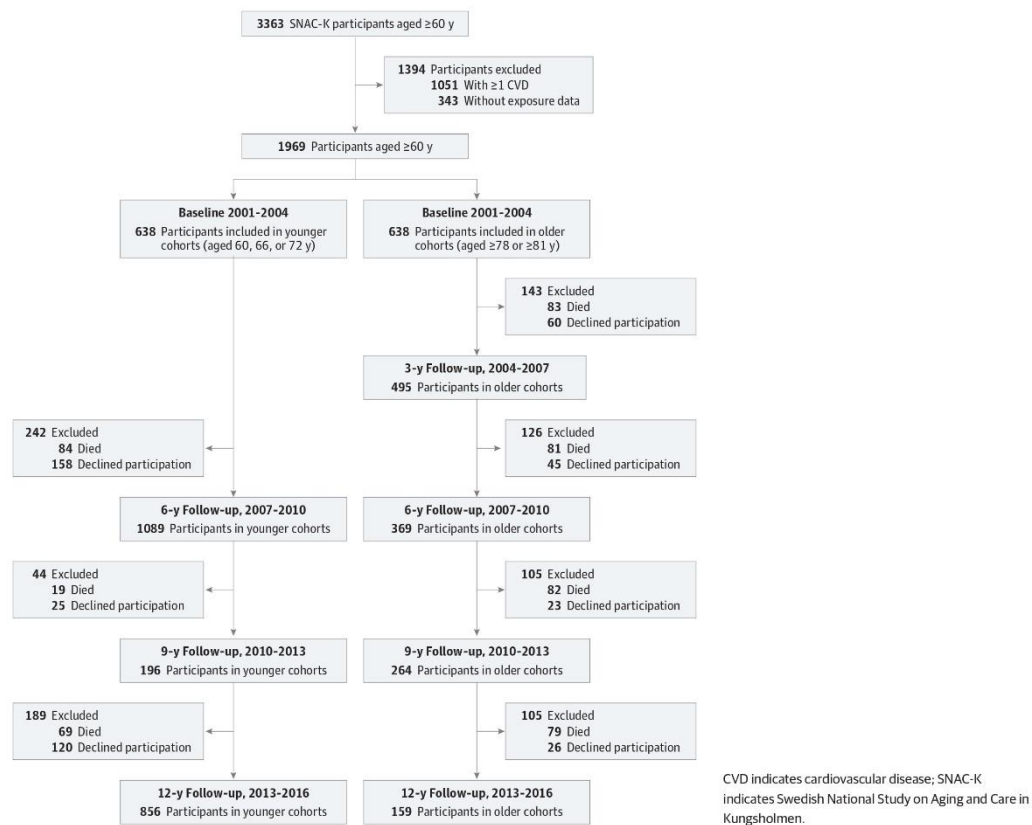
Methods

Data Collection

This research is based on data from the Swedish National Study on Aging and Care in Kungsholmen (SNAC-K). This is a community-based longitudinal study of randomly selected individuals aged 60 years or older living at home or in institutions in the Kungsholmen district of Stockholm between

2001 and 2004. Of 4590 eligible participants, 3363 (73.3%) participated in the baseline examination. Participants underwent extensive clinical examinations, interviews, and assessments by physicians, nurses, and psychologists. Data on sociodemographic characteristics, medical history, drug use, laboratory test results, and cognitive function were collected according to structured protocols. Data on medical history and vital status were also obtained by linking SNAC-K data with the National Patient Register and the Swedish Cause of Death Register. Since baseline, SNAC-K participants have been followed up regularly, every 6 years for the cohorts younger than 78 years and every 3 years for those aged 78 years or older. This study included data from baseline and the first 4 follow-ups (Figure 1). In the present study, we excluded 1051 individuals (31.3%) with at least 1 CV disease at baseline plus 343 individuals (10.2%) with missing data on the exposures, leaving 1969 participants (58.5%) in the study cohort. SNAC-K baseline and follow-up protocols have been approved by the Ethics Committee at Karolinska Institutet and the Regional Ethics Review Board in Stockholm, and written informed consent was obtained from participants or their next of kin for those with cognitive impairment.

Figure 1. Population Flow Chart for Baseline and First 4 Follow-up Assessments



Outcome Variable

Chronic diseases were coded using the *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10)* and further categorized according to a comprehensive list proposed by Calderon-Larrañaga et al.¹⁹ For this study, the following CV diseases were considered: ischemic heart disease, stroke, heart failure, atrial fibrillation, cardiac valve diseases, bradycardias and conduction disorders, peripheral artery disease, and other CV diseases (eTable 1A in the Supplement). In addition to the clinical diagnoses made by SNAC-K physicians (based on participant self-report, medical records, inpatient and outpatient registers, anamnestic details, and information gathered from participants' proxies), complementary clinical and drug-related parameters were used for certain conditions (eTable 1B in the Supplement). Considering the primarily chronic nature of the cardiovascular diseases under study, once detected in any data source, the disease remained present in all following waves. The sum of these diseases at each wave composed the CV multimorbidity.

Biomarkers

Nonfasting venous blood samples were taken from SNAC-K participants at baseline, and the serum was stored at -80°C for 10 to 12 years. The concentrations of tHcy and Met were measured using tandem mass spectrometry at the Department of Pharmacology, University of Oxford.²⁰ Interassay coefficients of variation were 4.4% for tHcy and 4.0% for Met. The Met:tHcy ratio was considered a possible indicator of methylation activity, with higher ratios indicating higher methylation activity.²¹

Genotyping was performed using matrix-assisted laser desorption/ionization time-of-flight analysis on the MassARRAY platform (Agena Bioscience) at the Mutation Analysis Facility, Karolinska Institutet. Successful genotyping call rate for rs1801133 (ie, MTHFR variation 677C>T) was 94%.

Covariates

Several covariates were measured at baseline, as follows: age, sex, highest level of formal education (ie, elementary school, high school, or university), use of any type of antihypertensive drug, use of any type of B vitamin supplement, hypertension (based on self-report, medical records, or a SNAC-K blood pressure measurement $\geq 140/90$ mm Hg), diabetes (based on self-report, medical records, SNAC-K glycated hemoglobin measurement $\geq 6.5\%$ [to convert to proportion of total hemoglobin, multiply by 0.01], or use of antidiabetic drugs), hypercholesterolemia (based on self-report, medical records, or a SNAC-K serum total cholesterol measurement ≥ 240.2 mg/dL [to convert to millimoles per liter, multiply by 0.0259]), chronic kidney disease (based on self-report, medical records, SNAC-K glomerular filtration rate measurement <60 mL/min/1.73m² according to the Chronic Kidney Disease Epidemiology Collaboration equation), total number of drugs, physical activity (ie, inadequate, health-enhancing, and fitness-enhancing), body mass index (BMI, calculated as weight in kilograms divided by height in meters squared; in 4 groups, as follows: <18.5 , 18.5 – 24.9 , 25.0 – 29.9 , and ≥ 30.0), smoking (ie, never, former, or currently), alcohol consumption (ie, never or occasional, light or moderate, or heavy), serum C-reactive protein (CRP) concentration (ie, ≤ 0.5 mg/dL and >0.5 mg/dL [to convert to milligrams per liter, multiply by 10]), and time to death or dropout.

Statistical Analysis

The main outcome variable was the rate of CV multimorbidity development during the 12-year follow-up period. Linear mixed models were used to estimate β coefficients and 95% CIs for the association between baseline concentrations of the biomarkers and the rate of CV multimorbidity development. To measure the association between the exposures of interest and the mean annual increase in the number of CV diseases, interactions between follow-up time and biomarker concentrations were included as fixed effects.^{22,23} Random effects were defined for the intercept and time of follow-up; unstructured covariance was assumed. The exposures (ie, tHcy and Met concentrations) were operationalized both as a standardized continuous variables (z scores) and according to tertiles. We created an indicator variable with 4 mutually exclusive categories by cross-classifying individuals' concentrations of tHcy or Met (ie, high vs low according to medians) and their

MTHFR categories (ie, CC vs CT/TT) to further explore the combined association of both exposures with the outcome. We dichotomized MTHFR status (ie, any T carriers vs noncarriers) to increase group size and thus maintain statistical power. Models were adjusted for sex, age, and education in the minimally adjusted model and for other potential confounders, such as smoking, alcohol consumption, physical activity, BMI, CRP level, number of drugs, dyslipidemia, diabetes, hypertension, chronic kidney disease, use of antihypertensive drugs, use of B vitamin supplements, and time of death or dropout in the fully adjusted model. Confounder selection was evidence informed. In humans, serum concentrations of tHcy are mainly determined by B vitamin levels (ie, folate and B₁₂) and renal function, and lifestyle behaviors are among the major associated factors.⁹ Dyslipidemia and CRP level are 2 biomarkers that strongly and independently predict systemic atherosclerosis.²⁴ Cardiovascular risk factors have also been included as potential confounding or mediating factors in previous studies.¹⁴

In a sensitivity analysis, to evaluate whether the associations between baseline concentrations of the biomarkers and the rate of CV multimorbidity development were driven by specific chronic conditions either at baseline or at follow-ups, we reran the models after removing the 8 CV diseases, 1 at a time, from the original count of CV diseases. Analyses were performed using Stata version 15 (StataCorp). Statistical significance was set at $P < .05$, and all tests were 2-tailed.

Results

The study population consisted of 1969 individuals without any CV disease at baseline, of whom 1261 (64.0%) were women. The mean (SD) age of the study population was 70.9 (9.8) years, and 1703 participants (86.6%) had at least a high school level of education. Most had normal weight (851 [44.5%]) and performed health-enhancing physical activity (995 [50.5%]) (eTable 2 in the Supplement). More than half had hypertension (1342 [68.2%]) and dyslipidemia (994 [50.5%]), but proportions were lower for diabetes (123 [6.3%]) and chronic kidney disease (529 [26.9%]). Nearly one-fifth of the study population used antihypertensive drugs (367 [18.7%]), and 202 (10.3%) used B vitamin supplements. The mean (SD) number of drugs taken at baseline was 2.9 (2.8). Differences between the study population and the entire SNAC-K population can be found in eTable 2 in the Supplement.

The median (interquartile range [IQR]) baseline concentrations were 1.68 (1.38-2.08) mg/dL for tHcy (to convert to micromoles per liter, multiply by 7.397) and 0.35 (0.30-0.40) mg/dL for Met (to convert to micromoles per liter, multiply by 67.02). A significantly higher median (IQR) concentration of tHcy and a lower median (IQR) Met:tHcy ratio were found in the higher age group vs the lower age group (tHcy: 1.596 [1.325-1.920] mg/dL vs 1.893 [1.542-2.420] mg/dL; $P < .001$; Met:tHcy ratio: 2.0 [1.6-2.6] vs 1.6 [1.2-2.0]; $P < .001$), in men vs women (tHcy: 1.785 [1.515-2.164] mg/dL vs 1.623 [1.312-2.042] mg/dL; $P < .001$; Met:tHcy ratio: 1.8 [1.4-2.3] vs 1.9 [1.5-2.5]; $P < .001$), in those with only elementary education vs with a high school or university education (tHcy: 1.893 [1.555-2.393] mg/dL vs 1.704 [1.393-2.137] mg/dL vs 1.596 [1.339-1.920] mg/dL; $P < .001$; Met:tHcy ratio: 1.6 [1.2-2.1] vs 1.8 [1.4-2.3] vs 2.0 [1.6-2.6]; $P < .001$), in those who currently smoke vs formerly or never smoked (tHcy: 1.677 [1.366-2.055] mg/dL vs 1.650 [1.379-2.042] mg/dL vs 1.758 [1.420-2.204] mg/dL; $P = .003$; Met:tHcy ratio: 1.9 [1.4-2.4] vs 1.9 [1.5-2.4] vs 1.8 [1.3-2.3]; $P = .003$), in those with inadequate levels of physical activity vs health-enhancing or fitness-enhancing levels of physical activity (tHcy: 1.839 [1.461-2.366] mg/dL vs 1.677 [1.379-2.069] mg/dL vs 1.582 [1.352-1.880] mg/dL; $P < .001$; Met:tHcy ratio: 1.7 [1.2-2.2] vs 1.8 [1.4-2.4] vs 2.0 [1.6-2.5]; $P = .001$), those with higher vs lower concentrations of CRP (tHcy: 1.650 [1.366-2.055] mg/dL vs 1.785 [1.461-2.177] mg/dL; $P = .04$; Met:tHcy ratio: 1.9 [1.5-2.4] vs 1.7 [1.3-2.2]; $P = .02$), those taking less than 4 drugs vs 4 or more drugs (tHcy: 1.690 [1.420-2.069] mg/dL vs 1.650 [1.312-2.096] mg/dL; $P = .006$; Met:tHcy ratio: 1.9 [1.5-2.4] vs 1.9 [1.4-2.5]; $P = .004$), and those not taking vs taking B vitamin supplements (tHcy: 1.542 [1.258-2.880] mg/dL vs 1.690 [1.393-2.109] mg/dL; $P < .001$; Met:tHcy ratio: 2.0 [1.5-2.6] vs 1.9 [1.5-2.4]; $P < .001$) (Table 1). We found a significantly higher concentration of tHcy and a lower

Table 1. Serum Concentrations of tHcy and Met and Met:tHcy Ratio According to Baseline Sociodemographic, Lifestyle, Clinical, and Genetic Characteristics

Characteristic	tHcy, median (IQR), mg/dL	P value ^a	Met, median (IQR), mg/dL	P value ^a	Met:tHcy, median (IQR)	P value ^a
Age, y						
<78	1.596 (1.325-1.920)	<.001	0.357 (0.305-0.417)	<.001	2.0 (1.6-2.6)	<.001
≥78	1.893 (1.542-2.420)		0.329 (0.284-0.381)		1.6 (1.2-2.0)	
Sex						
Men	1.785 (1.515-2.164)	<.001	0.354 (0.306-0.414)	.02	1.8 (1.4-2.3)	<.001
Women	1.623 (1.312-2.042)		0.341 (0.290-0.400)		1.9 (1.5-2.5)	
Education						
Elementary	1.893 (1.555-2.393)	<.001	0.333 (0.288-0.387)	.01	1.6 (1.2-2.1)	<.001
High school	1.704 (1.393-2.137)		0.339 (0.288-0.396)		1.8 (1.4-2.3)	
University	1.596 (1.339-1.920)		0.359 (0.312-0.421)		2.0 (1.6-2.6)	
Smoking						
Never	1.677 (1.366-2.055)	.003	0.348 (0.293-0.406)	.15	1.9 (1.4-2.4)	.009
Former	1.650 (1.379-2.042)		0.350 (0.299-0.406)		1.9 (1.5-2.4)	
Current	1.758 (1.420-2.204)		0.341 (0.294-0.394)		1.8 (1.3-2.3)	
Alcohol consumption						
Never or occasional	1.785 (1.474-2.272)	.88	0.333 (0.288-0.391)	.55	1.7 (1.2-2.2)	.87
Light or moderate	1.650 (1.366-2.015)		0.354 (0.302-0.411)		1.9 (1.5-2.5)	
Heavy	1.623 (1.339-2.042)		0.348 (0.291-0.411)		1.9 (1.5-2.5)	
Physical activity						
Inadequate	1.839 (1.461-2.366)	<.001	0.338 (0.293-0.402)	.42	1.7 (1.2-2.2)	.001
Health-enhancing	1.677 (1.379-2.069)		0.348 (0.294-0.400)		1.8 (1.4-2.4)	
Fitness-enhancing	1.582 (1.352-1.880)		0.357 (0.302-0.411)		2.0 (1.6-2.5)	
BMI						
<18.5	1.609 (1.393-2.407)	.38	0.332 (0.280-0.424)	.77	1.8 (1.2-2.2)	.05
18.5-24.9	1.636 (1.339-2.028)		0.345 (0.294-0.405)		1.9 (1.5-2.5)	
25.0-29.9	1.690 (1.420-2.069)		0.351 (0.297-0.406)		1.9 (1.5-2.4)	
≥30	1.717 (1.447-2.137)		0.353 (0.299-0.405)		1.9 (1.4-2.3)	
CRP level, mg/L						
≤5	1.650 (1.366-2.055)	.04	0.350 (0.297-0.408)	.24	1.9 (1.5-2.4)	.02
>5	1.785 (1.461-2.177)		0.335 (0.291-0.394)		1.7 (1.3-2.2)	
Drugs, No.						
<4	1.690 (1.420-2.069)	.006	0.351 (0.299-0.408)	.76	1.9 (1.5-2.4)	.004
≥4	1.650 (1.312-2.096)		0.336 (0.290-0.397)		1.9 (1.4-2.5)	
Dyslipidemia						
Yes	1.677 (1.379-2.096)	.32	0.342 (0.296-0.402)	.32	1.9 (1.4-2.4)	.13
No	1.663 (1.366-2.069)		0.350 (0.296-0.406)		1.9 (1.4-2.5)	
Diabetes						
Yes	1.650 (1.393-2.123)	.68	0.353 (0.309-0.418)	.27	1.9 (1.5-2.5)	.02
No	1.677 (1.379-2.082)		0.347 (0.294-0.403)		1.9 (1.4-2.4)	
Hypertension						
Yes	1.704 (1.406-2.137)	.008	0.342 (0.294-0.399)	.08	1.8 (1.4-2.3)	.003
No	1.623 (1.339-1.974)		0.356 (0.299-0.418)		2.0 (1.5-2.6)	
Chronic kidney disease						
Yes	1.947 (1.596-2.515)	<.001	0.345 (0.291-0.393)	.27	1.6 (1.1-2.0)	<.001
No	1.596 (1.325-1.947)		0.348 (0.299-0.409)		2.0 (1.5-2.5)	
Use of antihypertensive drugs						
Yes	1.690 (1.352-2.096)	.45	0.344 (0.299-0.406)	.08	1.9 (1.4-2.4)	.11
No	1.677 (1.379-2.069)		0.348 (0.296-0.403)		1.9 (1.4-2.4)	
Use of B vitamin supplements						
Yes	1.542 (1.258-1.880)	<.001	0.333 (0.284-0.385)	.93	2.0 (1.5-2.6)	<.001
No	1.690 (1.393-2.109)		0.348 (0.296-0.406)		1.9 (1.4-2.4)	

(continued)

Table 1. Serum Concentrations of tHcy and Met and Met:tHcy Ratio According to Baseline Sociodemographic, Lifestyle, Clinical, and Genetic Characteristics (continued)

Characteristic	tHcy, median (IQR), mg/dL	P value ^a	Met, median (IQR), mg/dL	P value ^a	Met:tHcy, median (IQR)	P value ^a
<i>MTHFR</i> 677C>T polymorphism						
CC	1.636 (1.352-2.015)		0.348 (0.294-0.402)		1.9 (1.5-2.5)	
CT	1.690 (1.393-2.069)	<.001	0.345 (0.297-0.403)	.96	1.8 (1.5-2.4)	<.001
TT	1.799 (1.488-2.474)		0.353 (0.297-0.415)		1.8 (1.2-2.3)	

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); CC, *MTHFR* 677CC polymorphism; CRP, C-reactive protein; CT, *MTHFR* 677CT polymorphism; IQR, interquartile range; Met, methionine; *MTHFR*, methylenetetrahydrofolate reductase; tHcy, homocysteine; TT, *MTHFR* 677TT polymorphism.

SI conversion factors: To convert Met to micromoles per liter, multiply by 67.02; tHcy to micromoles per liter, multiply by 7.397.

^a Linear regression with tHcy and Met as the dependent variable and with the rest of the variables in the left column of the table as independent variables.

Table 2. Association of Baseline Concentrations of tHcy, Met, and Met:tHcy Ratio With Annual Rate of Cardiovascular Disease Accumulation During the 12-Year Follow-up

Biomarker	Minimally adjusted model ^a		Fully adjusted model ^b	
	β coefficient (95% CI) ^c	P value	β coefficient (95% CI) ^c	P value
tHcy	0.017 (0.011 to 0.024)	<.001	0.023 (0.015 to 0.030)	<.001
Met	-0.006 (-0.012 to -0.0002)	.04	-0.007 (-0.013 to -0.001)	.02
Met:tHcy	-0.018 (-0.024 to -0.012)	<.001	-0.017 (-0.023 to -0.011)	<.001

Abbreviations: Met, methionine; tHcy, homocysteine.

^a Adjusted for age, sex, and education.

^b Adjusted for age, sex, education, smoking habit, alcohol consumption, physical activity, body mass index, C-reactive protein level, number of drugs, dyslipidemia, diabetes, hypertension, chronic kidney disease, use of antihypertensive drugs, use of B vitamin supplements, and time of death or dropout.

^c β coefficient for 1 standard deviation change in each biomarker.

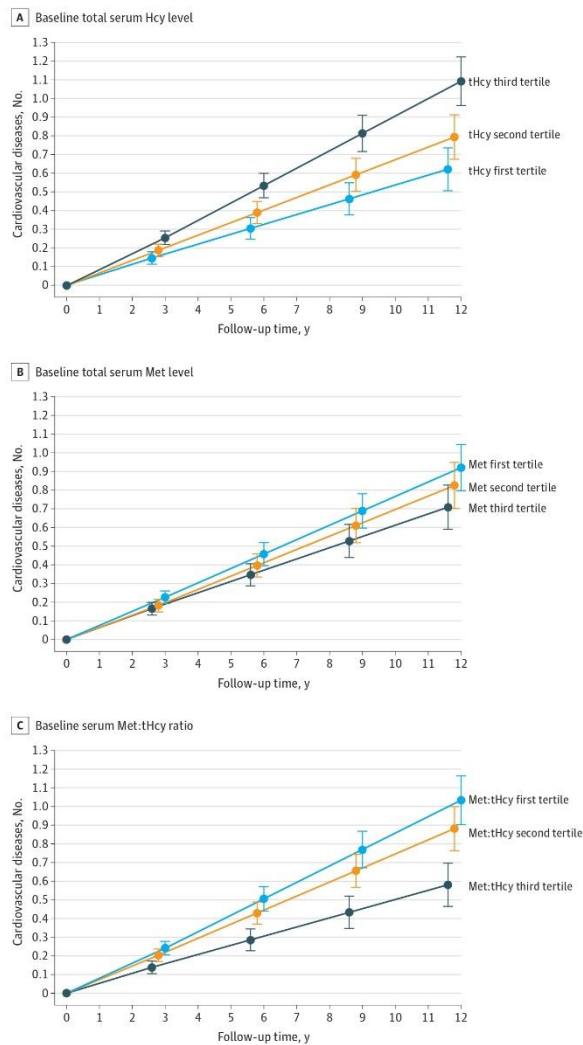
Met:tHcy ratio among participants diagnosed with hypertension vs no hypertension (tHcy: 1.704 [1.406-2.137] mg/dL vs 1.623 [1.339-1.974] mg/dL; $P = .008$; Met:tHcy ratio: 1.8 [1.4-2.3] vs 2.0 [1.5-2.6]; $P = .003$), chronic kidney disease vs no chronic kidney disease (tHcy: 1.947 [1.596-2.515] mg/dL vs 1.596 [1.325-1.947] mg/dL; $P < .001$; Met:tHcy ratio: 1.6 [1.1-2.0] vs 2.0 [1.5-2.5]; $P < .001$), and those with a *MTHFR* 677 TT allele vs a *MTHFR* 677 CT or CC allele (tHcy: 1.636 [1.352-2.015] mg/dL vs 1.690 [1.393-2.069] mg/dL vs 1.799 [1.488-2.474] mg/dL; $P < .001$; Met:tHcy ratio: 1.9 [1.5-2.5] vs 1.8 [1.5-2.4] vs 1.8 [1.2-2.3]; $P < .001$). The median (IQR) concentration of Met was significantly lower among older participants vs younger participants (0.357 [0.305-0.417] mg/dL vs 0.329 [0.284-0.381] mg/dL; $P < .001$), women vs men (0.354 [0.306-0.414] mg/dL vs 0.314 [0.290-0.400] mg/dL; $P < .001$), and those with only elementary education vs those with high school or university educations (0.333 [0.288-0.387] mg/dL vs 0.339 [0.288-0.396] mg/dL vs 0.359 [0.312-0.421] mg/dL; $P < .001$).

In the longitudinal analyses during the 12-year follow-up, tHcy showed a significant association with the rate of CV disease accumulation over time ($\beta = 0.023$ per year; 95% CI, 0.015 to 0.030; $P < .001$). In contrast, a significant inverse association was seen with Met ($\beta = -0.007$ per year; 95% CI, -0.013 to -0.001; $P = .02$) and with the Met:tHcy ratio ($\beta = -0.017$ per year; 95% CI, -0.023 to -0.011; $P < .001$) (Table 2). The analysis by tertiles showed a likely concentration-response association between the biomarkers and the rate of CV disease accumulation (highest vs lowest tertile of tHcy: $\beta = 0.040$ per year; 95% CI, 0.025 to 0.055; highest vs lowest tertile of Met: $\beta = -0.017$ per year; 95% CI, -0.032 to -0.002; highest vs lowest tertile of tHcy:Met ratio: $\beta = -0.039$ per year; 95% CI, -0.054 to -0.024) (Figure 2). *MTHFR* 677 C>T polymorphism was not significantly associated with the outcome in any of the models (eTable 3 in the Supplement). In the sensitivity analysis excluding 1 CV disease at a time from the main outcome variable, all models provided similar results (eg, ischemic heart disease, tHcy: $\beta = 0.023$ per year; 95% CI, 0.013 to 0.027;

$P < .001$; Met: $\beta = -0.006$ per year; 95% CI, -0.011 to -0.0003 ; $P = .04$; Met:tHcy ratio: $\beta = -0.015$ per year; 95% CI, -0.020 to -0.009 ; $P < .001$) (eTable 4 in the Supplement).

The associations between high concentrations of tHcy and the rate of CV disease accumulation were similar for both *MTHFR* genotypes (CC: $\beta = 0.034$ per year; 95% CI, 0.016 - 0.051 ; $P < .001$; CT/TT: $\beta = 0.035$ per year; 95% CI, 0.019 - 0.052 ; $P < .001$) (Table 3). For Met, a significant association was only seen in participants with a low concentration of Met and the CT/TT genotype

Figure 2. Estimated Rate of Cardiovascular Disease Accumulation During the 12-Year Follow-up by Baseline Concentrations of Homocysteine (tHcy), Methionine (Met), and the Met:tHcy Ratio



Fully adjusted models were adjusted for age, sex, education, smoking habit, alcohol consumption, physical activity, body mass index, C-reactive protein level, number of drugs, dyslipidemia, diabetes, hypertension, chronic kidney disease, use of antihypertensive drugs, use of B vitamin supplements, and time of death or dropout. Tertiles for tHcy were defined as follows: first tertile, less than 1.49 mg/dL; second tertile, 1.50-1.91 mg/dL; third tertile, greater than 1.91 mg/dL. Tertiles for Met were defined as follows: first tertile, less than 0.31 mg/dL; second tertile, 0.31-0.38 mg/dL; third tertile, greater than 0.38 mg/dL. Tertiles for Met:tHcy ratio were defined as follows: first tertile, less than 1.6; second tertile, 1.6-2.2; third tertile, greater than 2.2. To convert Met to micromoles per liter, multiply by 67.02; tHcy to micromoles per liter, multiply by 7.397.

($\beta = 0.023$ per year; 95% CI, 0.006-0.041; $P = .009$) (Table 3; eFigure in the Supplement). These results were further corroborated by the positive association with the rate of CV multimorbidity development in participants with low Met:tHcy ratios and both the CC group ($\beta = 0.039$ per year; 95% CI, 0.021-0.057; $P < .001$) and the CT/TT group ($\beta = 0.037$ per year; 95% CI, 0.021-0.054; $P < .001$) (Table 3).

Discussion

In this longitudinal population-based study of older adults, a higher concentration of tHcy, a lower concentration of Met, and a lower Met:tHcy ratio (suggesting impaired methylation activity) were all associated with an increased rate of CV multimorbidity development during a 12-year period. Genetic predisposition (ie, the *MTHFR* 677T allele) further contributed to accelerated CV multimorbidity development in participants with low Met concentrations. Results were independent of sociodemographic, lifestyle, and clinical factors and did not seem to be driven by the incidental diagnosis of a specific CV disease but rather with the global burden of CV multimorbidity. Even if the effect sizes were relatively small, the biological coherence, the concentration-response associations, and the statistical robustness of the findings point to a potentially meaningful role of these biomarkers in the detection of CV multimorbidity and possibly also its etiology.

The prevalence of CV multimorbidity is increasing rapidly in the older population,²⁵ and the combination of any of the conditions included in CV multimorbidity is associated with a multiplicative mortality risk.²⁶ Moreover, CV multimorbidity is highly disabling in older adults and is associated with faster cognitive decline,^{27,28} thus affecting quality of life as well as the ability to live independently. Therefore, knowledge of the rate of CV multimorbidity development could help to target individuals who would benefit most from primary and secondary prevention of CV disease. In addition, if specific biological markers can be identified to capture CV aging, measuring these could track the efficacy of interventions aimed precisely at slowing CV dysfunction and decline. This will undoubtedly be the next phase of improvement in human longevity, for which CV diseases are still the leading threat.²⁹

The pathophysiologic mechanisms underlying the increased risk of CV diseases in individuals with hyperhomocysteinemia have been comprehensively studied.^{30,31} Increased tHcy concentrations have consistently been shown to be associated with increased risk of new CV events,³² in line with our findings. Thus, in patients with CV diseases, tHcy concentrations could be used as a prognostic factor and for stricter surveillance of lifestyle and treatment.⁹ Other nonatherogenic mechanisms may explain the nonselective association between tHcy and CV disease accumulation described in our study. Indeed, hyperhomocysteinemia, which has been described to

Table 3. Association of Baseline Concentrations of tHcy, Met, and Met:tHcy Ratio With the Rate of Cardiovascular Disease Accumulation During the 12-Year Follow-up Stratified by *MTHFR* 677C>T Polymorphism^a

<i>MTHFR</i> polymorphism	tHcy				Met				Met:tHcy			
	Levels ^b	Participants, No.	β coefficient (95% CI)	P value	Levels ^b	Participants, No.	β coefficient (95% CI)	P value	Levels ^b	Participants, No.	β coefficient (95% CI)	P value
CC, wild type	Low	494	1 [Reference]	NA	High	443	1 [Reference]	NA	High	486	1 [Reference]	NA
	High	410	0.034 (0.016 to 0.051)	<.001	Low	461	0.012 (−0.010 to 0.024)	.20	Low	418	0.039 (0.021 to 0.057)	<.001
CT and TT	Low	432	0.012 (−0.005 to 0.029)	.17	High	449	0.007 (−0.008 to 0.025)	.43	High	428	0.014 (−0.003 to 0.030)	.11
	High	478	0.035 (0.019 to 0.052)	<.001	Low	461	0.023 (0.006 to 0.041)	.009	Low	482	0.037 (0.021 to 0.054)	<.001

Abbreviations: CC, *MTHFR* 677CC polymorphism; CT, *MTHFR* 677CT polymorphism; Met, methionine; *MTHFR*, methylenetetrahydrofolate reductase; tHcy, homocysteine; TT, *MTHFR* 677TT polymorphism.

^a Models adjusted for age, sex, education, smoking habit, alcohol consumption, physical activity, body mass index, C-reactive protein level, number of drugs, dyslipidemia, diabetes, hypertension, chronic kidney disease, use of antihypertensive drugs, use of B vitamin supplements, and time of death or dropout.

^b Levels for tHcy, Met, and Met:tHcy ratio established according to the median of the distribution (tHcy, 1.68 mg/dL [to convert to micromoles per liter, multiply by 7.397]; Met, 0.35 mg/dL [to convert to micromoles per liter, multiply by 67.02]; Met:tHcy ratio, 1.9).

induce both ventricular systolic and diastolic dysfunction, has been associated with heart failure even in the absence of ischemic heart disease.^{33,34} The increased deposition of interstitial and perivascular collagen triggered by high tHcy concentrations may explain its effect on heart failure via nonatherosclerotic mechanisms. Moreover, telomere attrition in cardiomyocytes, potentially accelerated by higher tHcy blood concentrations, can promote faster aging of myocardium, inducing pump insufficiency and chambers dilation.^{1,35,36} Increased tHcy concentrations have been also associated with impaired autophagy of both cardiomyocytes and smooth vascular cells, leading to the accumulation of senescent cells and consequently organ dysfunction.^{37,38}

Much less is known regarding the role and pathophysiology of Met. A possible pathogenic pathway may involve glycine N-methyltransferase (GNMT), the most abundant liver methyltransferase regulating the availability of the biologic methyl donor, S-adenosylmethionine. Met deficiency leads to reduced GNMT flux,³⁹ which is linked to hepatic lipid accumulation, hyperlipidemia, and the deposition of oxidized LDL in vascular walls, all of which have been associated with increased risk of atherosclerosis.^{11,12,40} Another possible pathway is through the role of Met in methylation reactions. Met deficiency can induce site-specific hypomethylation,⁴¹ leading to dysfunctional epigenetic modifications described recently in the expression of proprotein convertase subtilisin/kexin type 9, a serine protease involved in the degradation of both hepatic and extrahepatic LDL receptors, thereby increasing circulating LDL cholesterol concentrations.⁴² Indeed, higher concentrations of Met and an improved methylation activity, as indicated by the Met:tHcy ratio, were both associated with a decreased rate of CV disease accumulation in our study, which is consistent with findings from animal experimental studies^{11,12} and a 2019 study examining the risk of dementia development and structural brain changes.²¹ Finally, Met deficiency may lead to deficiency of glutathione, a major intracellular antioxidant, which has been linked to metabolic and CV diseases.⁴³

The *MTHFR* 677C>T polymorphism leads to a modest increase in tHcy. Individuals with the homozygous TT genotype have approximately 0.34 mg/dL higher tHcy levels than those with the wild-type CC variant.^{44,45} The difference depends mainly on folate status.⁴⁶ Lack of power may therefore be the reason why most studies, including ours, have failed to show any difference in the risk of CV diseases in people with this polymorphism,³² but meta-analyses have revealed such an effect consistently, as first shown in 2002 by Klerk et al.⁴⁵ It has recently been hypothesized that the *MTHFR* 677C>T polymorphism could influence CV pathology through mechanisms that are independent of tHcy, such as via blood pressure disorders.⁴⁷ Interestingly, in our study, the association of low Met with the rate of CV multimorbidity development was restricted to the group with the CT/TT alleles of *MTHFR*, suggesting an interactive effect of both Met and genotype. Such a finding, together with the significant protective association of the Met:tHcy ratio in our models, suggests that we need to further examine methylation-derived modifications of gene expression in relation to CV multimorbidity.

Strengths and Limitations

The main strength of our study is the use of a longitudinal population-based study in older adults with detailed clinical characterization and available data on potential confounders. The identification of CV diseases was done through a clinically driven algorithm, integrating different sources of data. Thus, the risk of misclassification and underdetection of the outcome was limited, as previously shown.¹⁹ Individuals with CV diseases at baseline were excluded from our analyses, which minimized the risk of reverse causality.

Several limitations should be considered. Time-varying measurements for the biomarkers (ie, the marker may not be constant over time) could lead to biased associations due to regression dilution. It has been shown that failure to correct for regression dilution may underestimate the relative risks of disease by 50% after 10 years.⁴⁸ Moreover, blood measurements were performed under nonfasting conditions, which could have direct consequences on serum Met levels following a meal.⁴⁹ However, in previous studies, adjustment for fasting status did not alter the association

between plasma Met concentrations and acute myocardial infarction¹⁴ or other outcomes.²¹ The low number of participants with the TT genotype forced us to explore its association in combination with the CT genotype, even if the latter had tHcy concentrations that were closer to the CC rather than the TT genotype. Moreover, we could not control for confounding from ancestry or technical bias in genotyping. Even after adjustment for potential confounders, the possibility of residual confounding cannot be discarded. Furthermore, although this is a prospective study, reverse causality should not be ruled out given that elevated tHcy is associated with risk factors often targeted for treatment in relation to CV health.

Conclusions

This study adds further epidemiologic evidence to the hypothesis that serum tHcy and Met concentrations and Met:tHcy ratio are important independent risk factors not only for the incidence of multiple CV diseases of diverse nature, but also for the rate at which these different CV diseases accumulate in older age. The interactive associations of Met concentrations and the MTHFR 677C>T polymorphism, together with the association found for the Met:tHcy ratio, point to the relevance of impaired methylation in the pathogenesis of CV aging but also call for further studies investigating alternative pathways.

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SUPPLEMENT.

eTable 1. ICD-10 Codes and Additional Clinical and Drug-Related Parameters

eTable 2. Descriptive Analysis of the Baseline SNAC-K Population and the Cardiovascular Disease-Free Study Sample, Stratified by Sex

eTable 3. Association Between Baseline Concentrations of tHcy, Met, Met:tHcy Ratio (Continuous), MTHFR 677C>T Polymorphism and the Annual Rate of Cardiovascular Disease Accumulation During the 12-Year Follow-up

eTable 4. Sensitivity Analysis: Association Between Baseline Concentrations of tHcy, Met and Met:tHcy Ratio (Continuous) and the Rate of Cardiovascular Disease Accumulation During the 12-Year Follow-up Removing 8 Cardiovascular Diseases Individually From Original Total

eFigure. Estimated Rate of Cardiovascular Disease Accumulation During the 12-Year Follow-up by Baseline Concentrations of tHcy, Met, and Met:tHcy Ratio and MTHFR 677C>T Polymorphism

2.1.4. Estudio No.4

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ORIGINAL RESEARCH ARTICLE

Association of *MTHFR* C677T (*rs1801133*) and A1298C (*rs1801131*) Polymorphisms with Serum Homocysteine, Folate and Vitamin B12 in Patients with Young Coronary Artery Disease

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Abstract C677T (*rs1801133*) and A1298C (*rs1801131*) *MTHFR* gene polymorphisms and/or nutritional deficiency of folate/vitamin B12 leading to hyperhomocysteinemia is an established risk factor for CAD. The objective of this study was to evaluate the clinical usefulness of association between *MTHFR* C677T (*rs1801133*) and A1298C (*rs1801131*) polymorphisms with serum homocysteine, folate and vitamin B12 in addition to conventional cardiovascular risk factors in patients with young CAD. Genomic DNA was isolated from the whole blood.

Genotyping of *MTHFR* C677T (*rs1801133*) and *MTHFR* A1298C (*rs1801131*) polymorphisms in young CAD patients and healthy controls was performed by ARMS-PCR method. Serum homocysteine, vitamin B12 and folate were estimated by CMIA and lipid profile parameters were measured by automated chemistry analyzers. Serum homocysteine levels were significantly higher but serum folate and vitamin B12 levels were not significantly different among young CAD group as compared to control group. Statistically significant hyperhomocysteinemia was observed in carriers of T allele for *MTHFR* 677C/T (*rs1801133*) genotype in young CAD group but this association was not significant for *MTHFR* 1298A/C (*rs1801131*) polymorphism. The association between hyperhomocysteinemia and CAD in young group was not independent of conventional cardiovascular risk factors. Risk of hyperhomocysteinemia and young CAD could be monitored by *MTHFR* polymorphism detection followed by serum homocysteine, folate and vitamin B12 measurements. The findings could help to prevent or delay the occurrence of young CAD through appropriate measures.

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Keywords Young CAD · *MTHFR* C677T · *MTHFR* A1298C · Homocysteine · Folate · Vitamin B12

Introduction

Atherosclerosis leading to coronary artery disease (CAD) in young Indian population less than 45 years of age is looming large as a cause of morbidity and mortality in recent years. In young CAD patients, established risk factors like hypertension, diabetes mellitus, smoking, central obesity, family history of heart disease, dyslipidemia are as important as in old CAD patients [1]. But many variations

are observed in the prevalence of these risk factors in young CAD leaving the blank arena for the research. An elevated level of serum homocysteine ($> 15 \mu\text{mol/L}$) is an independent established risk factor for CAD due to its role in the initiation of endothelial dysfunction [2]. Mild to moderate elevation in serum homocysteine levels could be due to nutritional factors like deficiency of vitamin B12 (cobalamin) and folate. This is because of their involvement as cofactor and methyl group donor respectively in the methylation of homocysteine to methionine. Methylene tetrahydrofolate reductase (MTHFR) enzyme is responsible for reducing serum homocysteine levels by converting it into methionine. Methyltetrahydrofolate is methyl group donor in homocysteine re-methylation metabolism. Genetic factors like mutation in the gene coding for the MTHFR, the regulatory enzyme in the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, is responsible for hyperhomocysteinemia [2, 3].

MTHFR gene is located on the short arm of chromosome 1 at 1p36.3. A common single nucleotide polymorphism (SNP) *C677T (rs1801133)*, where cytosine is replaced by thymine at 677 position (exon 4) of *MTHFR* gene which results in substitution of an alanine to a valine at codon 222 in N-terminal catalytic domain of protein. The encoded protein has reduced activity at temperature 37°C and higher hence called thermolabile variant [4]. *MTHFR TT* genotype has been associated with increased serum homocysteine level which is an established risk factor for CAD. Another polymorphism in *MTHFR* gene is seen in exon 7 at 1298 position where adenine (A) is replaced by cytosine (C) with subsequent substitution of a glutamate codon at position 429 to an alanine in amino acid sequence within C-terminal regulatory domain of protein. *A1298C (rs1801131)* mutation in *MTHFR* has also been described as a cause of reduced enzyme activity though it is considered less than conferred by *C677T* [4, 5]. Several studies have reported that *C677T (rs1801133)* and *A1298C (rs1801131)* *MTHFR* gene polymorphisms are crucial in determining the serum homocysteine levels, which if elevated, can lead to CAD [3–5]. However, there is paucity of data on the serum homocysteine, folate and vitamin B12 levels and their association with *MTHFR C677T (rs1801133)* and *A1298C (rs1801131)* gene polymorphism in young CAD patients. Hence, the present study was carried out to evaluate the association between serum homocysteine levels with *MTHFR C677T (rs1801133)* and *A1298C (rs1801131)* polymorphism in young CAD patients. Association between *C677T (rs1801133)* polymorphism and serum homocysteine levels are also affected by dietary folate intake [4, 5]. Hence, additionally, we also evaluated the correlation between serum homocysteine and serum folate as well as vitamin B12 and studied association between serum folate and vitamin B12 levels and *MTHFR*

C677T (rs1801133) and *A1298C (rs1801131)* polymorphism in young CAD patients.

Materials and Methods

In the present case—control study, we included total ninety participants further divided into two groups with forty five patients with young CAD as cases and forty five healthy volunteers as controls. The young adult CAD patients included in this study were diagnosed first time for CAD at the Cardiovascular Department of our University affiliated Medical College and Tertiary Care Hospital, Pune, India, between June 1, 2018 and September 30, 2019. The study protocol was approved by institutional ethical committee and informed written consent was obtained from all patients and healthy volunteers prior to their enrollment. Inclusion criteria for young CAD group was confirmed diagnosis of CAD by diagnostic percutaneous coronary angiography with maximum luminal narrowing estimated by visual analysis of 15 coronary segments by the cardiologist. Clinically relevant CAD was defined as the occurrence of ≥ 1 stenosis of $\geq 40\%$ in ≥ 1 of 15 coronary segments. Patients enrolled in young CAD group were 34 males and 11 females in between age group of 18–45 years. Control group was formed by healthy volunteers (males = 34, females = 11) of age group ranging 18–45 years who attended the institute for routine yearly health check-up. Patients with diabetes mellitus, hepatic and kidney diseases, family history of heart disease or on vitamin supplementation which could affect serum homocysteine levels were excluded from the study. 2.5 ml of blood sample was collected from ante-cubital vein in EDTA vacutainer and 2 ml in plain vacutainer under all aseptic precautions. Serum was separated after centrifugation at 2000 g for 10 min from plain vacutainer.

Estimation of Serum TC, TG, HDL-C, Vitamin B12, Folate and Homocysteine

Serum total cholesterol (TC) levels were measured by cholesterol oxidase-peroxidaseaminoantipyrine (CHOD-PAP) enzymatic colorimetric method [6]. Serum high-density lipoprotein cholesterol (HDL-C) levels were measured by direct enzymatic method [7]. The glycerol phosphate oxidase-peroxidaseaminoantipyrine (GPO-PAP) enzymatic colorimetric test was used to estimate serum triglyceride levels [8]. Friedewald formula was used to calculate serum low-density lipoprotein cholesterol (LDL-C) levels. Serum homocysteine, vitamin B12 and folate were measured by Chemiluminiscent Microparticle Immuno Assay (CMIA) according to instructions of kit manufacturers [9]. The required kits were purchased from

Abbott ARCHITECT. All the parameters were analysed on automated biochemistry analyzers in NABL (National Accreditation Board for Testing and Calibration Laboratories) accredited Biochemistry department of central clinical laboratory of our hospital.

Isolation of Genomic DNA and *MTHFR* Polymorphism Analysis by ARMS PCR

Genomic DNA was isolated from the whole blood collected in EDTA vacutainer using commercially available DNA isolation kit (GeNei™ Whole Blood DNA Extraction Kit, Genei Laboratories Private Laboratories) according to protocols of the manufacturer [10]. Genotyping of *MTHFR* C677T (*rs1801133*) and *MTHFR* A1298C (*rs1801131*) polymorphisms was performed by amplification-refractory mutation system- polymerase chain reaction (ARMS-PCR) method using a commercially available kit (GeNei™ PCR Master Mix, Genei Laboratories Private Laboratories). The primer sequences were:

For *MTHFR*- *rs1801133* (C/T) Forward inner primer-A (T allele): TTGAAGGAGAAGGTGCTGCGGGCGT,

Reverse inner primer -B (C allele): CAAAGAAAA GCTGCGTGATGATGAAATAGG,

Forward outer primer- C (5'—3'):CCCAGCCA CTCACTGTTTATGTTTCAGGC,

Reverse outer primer- D (5'—3'): GTGAGAGTGGG GTGGAGGGAGCTTATG.

For *MTHFR*- *rs1801131* (A/C) Forward inner primer- A (A allele): GTGGGGGAGGAGCTGACCAGTGAGGA Reverse inner primer-B (C allele): GGTAAGAACG AAGACTTCAAAGACACCTG,

Forward outer primer- C (5'—3'): GGCCTGCAGA CCTTCCTTGCAAATACAT,

Reverse outer primer- D (5'—3'): ACTTACCCTTC TCCCTTGCCATGTCCA.

The PCR protocol followed was: Initial denaturation at 94 °C for 5 min, then 40 cycles (Denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s), and final extension at 72 °C for 7 min. In post-PCR process, 5 µl of PCR product was loaded on 3% agarose gel along with DNA ladder to confirm size of PCR product and results were interpreted. In C677T (*rs1801133*) genotyping, TT mutant genotype amplicon size was 189 bp while for CC Wild it was 273 bp. In case of A1298C (*rs1801131*) genotyping, AA wild genotype amplicon size was 232 bp while for CC mutant it was 334 bp.

Statistical Analysis

Data collected by using a structured form was entered in MS excel sheet. Using SPSS 24.0 version IBM USA data was analyzed. Normality test was carried out by using

kolmogorov Smirnov test where the p value was 0.2 which was statistically not significant and hence data was normally distributed. Large variance can exist depending upon the observed values and wide normal range of few parameters in this study like for serum folate and serum vitamin B12. Qualitative data was expressed in terms of percentages and proportions. Quantitative data was expressed in terms of Mean $\pm$ standard deviation. Chi square test was used to determine association between two qualitative variables. Comparison of mean and SD between two groups was done by using unpaired 't' test to assess significance of the mean difference between groups. Descriptive statistics of each variable was presented in terms of mean, standard deviation and standard error of mean. Correlation between two quantitative variables was assessed by using Pearson's correlation coefficient test (r). $P \leq 0.05$ was considered as statistically significant. P value of < 0.001 was considered as statistically highly significant.

Results

In the present study, we observed statistically significantly higher levels of systolic blood pressure, diastolic blood pressure and waist to hip ratio in the cases as compared to controls. However, there was no statistically significant difference in BMI between cases and controls (Table 1). Mean age of control group was 32.49 ± 7.11 years and of cases was 36.46 ± 6.98 years. P value of comparison was 0.008 making the difference statistically significant.

Further, significantly higher serum levels of total cholesterol (Sr. TC), triglycerides (Sr. TG) and VLDL cholesterol were observed in the cases as compared to levels in controls. However, serum HDL cholesterol (Sr. HDL-C) and serum LDL cholesterol (Sr. LDL-C) between patients and control group were not statistically significantly different. However, young CAD group had significantly higher TC/HDL-C ratio and LDL-C/HDL-C ratio as compared to ratio in control group (Table 2).

Significantly higher serum homocysteine levels were found in the young CAD group as compared to levels in the control group. However, serum folate and serum vitamin B12 were not statistically significantly different in cases as compare to levels in controls (Table 3).

For *MTHFR* 677 C/T (*rs1801133*) polymorphism the frequencies of genotype distribution CC, CT and TT were 75.6% versus 62.2%, 20% versus 37.8% and 4.4% versus 0.0% in cases versus controls respectively. However, no statistically significant difference was observed between young CAD group and control group for C677T (*rs1801133*) polymorphism ($P = 0.23$, chi square = 2.87). For *MTHFR* 1298 A/C (*rs1801133*) variant, the frequencies

Table 1 Clinicopathological characteristics of study groups

Group		N	Mean	SD	P	Inference
Systolic BP	Cases	45	130.00	15.83	< 0.0001	Highly significant
	Controls	45	120.18	3.44		
Diastolic BP	Cases	45	90.73	11.23	< 0.0001	Highly significant
	Controls	45	81.33	3.19		
BMI	Cases	45	25.81	4.58	0.364	Not significant
	Controls	45	24.81	2.85		
W/H ratio	Cases	45	1.11	0.13	< 0.0001	Highly significant
	Controls	45	0.88	0.06		

Table 2 Depicts the results of serum lipid profile

Group		N	Mean	SD	P	Inference
Sr. TC (mg/dL)	Cases	45	181.93	51.80	0.009	Significant
	Controls	45	158.20	28.85		
Sr. TG (mg/dL)	Cases	45	171.98	106.83	0.002	Significant
	Controls	45	113.13	59.86		
Sr.HDL-C (mg/dL)	Cases	45	32.89	9.84	0.141	Not significant
	Controls	45	35.89	6.07		
Sr.VLDL-C (mg/dL)	Cases	45	50.01	66.35	0.019	Significant
	Controls	45	24.72	19.76		
Sr.LDL (mg/dL)	Cases	45	112.44	50.80	0.139	Not significant
	Controls	45	99.85	24.77		
TC/HDL	Cases	45	5.85	1.99	< 0.0001	Highly significant
	Controls	45	4.49	0.92		
LDL/HDL	Cases	45	3.58	1.41	0.003	Significant
	Controls	45	2.84	0.74		

Table 3 Depicts serum levels of folate, homocysteine and vitamin B12

Parameter		N	Mean	SD	P	Inference
Sr. Folate (ng/ml)	Cases	45	5.70	4.39	0.898	Not significant
	Controls	44	5.90	4.60		
Sr. Homocysteine (μmol/L)	Cases	45	26.48	15.69	< 0.0001	Highly significant
	Controls	45	16.49	6.10		
Sr. vitamin B12 (pg/ml)	Cases	44	302.02	421.83	0.438	Not significant
	Controls	45	265.80	197.24		

of AA, AC and CC were 8.9% versus 13.3%, 62.2% versus 77.8% and 28.9% versus 8.9% in young CAD patient group versus control group respectively. The most frequent genotype in both cases (62.2%) and controls (77.8%) was AC. Whereas CC mutant phenotype was found in much higher frequency in cases (28.9%) as compared to controls (8.9%). When we did overall comparison for *A1298C* (*rs1801131*) polymorphism in both groups we got *P* value of 0.051 (chi square = 5.94) which is slightly greater than the level of significance 0.05 making this association statistically not significant but this may be due to the small sample size used in our study.

When we compared results of serum homocysteine, folate and vitamin B12 with *MTHFR* genotyping, we found that for *MTHFR 677 C/T* (*rs1801133*) genotyping, carrier of T allele has higher mean concentration of serum homocysteine in both cases and controls. However, this association was statistically significant in only cases of young CAD patients (*P* = 0.019). While for *MTHFR1298 A/C* genotyping, significant association between serum homocysteine and three genotypes was noted in control group (*P* = 0.003), while in cases, this association was not statistically significant (*P* = 0.82), though serum homocysteine was higher in concentration in cases as compared to controls (Table 4). For *MTHFR 677 C/T* (*rs1801133*)

Table 4 Depicts comparison of serum homocysteine according to the *MTHFR* genotype

Group		Parameter	Genotype	N	Mean	SD	P	Inference
Cases	Sr. Homocysteine	<i>MTHFR</i> 677 <i>C/T</i>	<i>CC</i> WILD	34	23.29	14.27	0.019	Significant
			<i>CT</i> Hetero	9	33.29	16.72		
			<i>TT</i> Mutant	2	50.00	0.00		
		<i>MTHFR</i> 1298 <i>A/C</i>	<i>AA</i> WILD	4	28.48	18.81	0.95	Not significant
			<i>AC</i> Hetero	28	26.05	16.15		
			<i>CC</i> Mutant	13	26.78	15.02		
Controls		<i>MTHFR</i> 677 <i>C/T</i>	<i>CC</i> WILD	28	15.80	5.56	0.82	Not significant
			<i>CT</i> Hetero	17	17.61	6.92		
			<i>AA</i> WILD	6	12.33	3.48		
		<i>MTHFR</i> 1298 <i>A/C</i>	<i>AC</i> Hetero	35	16.24	5.05		
			<i>CC</i> Mutant	4	24.88	10.30		

genotyping, carrier of T allele has lower serum folate (Table 5) and serum vitamin B12 levels (Table 6) in both cases and controls which go with the finding of higher serum homocysteine observed with T allele, however, this association was not statistically significant. For *MTHFR*1298 A/C (*rs1801131*), carrier of C allele has lower serum vitamin B12 values in both cases and controls. However, no genotypic association was statistically significant in both cases and controls.

Using Pearson's correlation coefficient test, we observed that serum homocysteine levels were negatively correlated with both serum folate ($r = -0.333$, $P = 0.025$) and serum vitamin B12 ($r = -0.202$, $P = 0.188$) in cases but this negative correlation was statistically significant with only serum folate. In the control, statistically significant negative correlation was found between serum homocysteine and serum folate as well as serum vitamin B12 ($r = -0.411$, $p = 0.006$ and $r = -0.492$, $P < 0.001$ respectively). Negative correlation of serum homocysteine

with serum folate and vitamin B12 marks the importance of role of both folate and vitamin B12 in homocysteine metabolism.

Discussion

Hyperhomocysteinemia is a strong and independent risk factor for coronary artery disease. Serum homocysteine levels are affected by various genetic and/or nutrient related factors playing important role in trans-sulfuration or remethylation reactions of homocysteine [2, 3].

In the present study, statistically significantly higher levels of serum homocysteine were observed in young CAD patients (cases) as compared to controls which were supported by many studies [2, 3, 11, 12]. Previous studies have reported positive association between *C677T* (*rs1801133*) polymorphism with cardiovascular diseases [13, 14] while others found no association between *C677T*

Table 5 Depicts comparison of serum folate according to the *MTHFR* genotype

Group		Parameter	Genotype	N	Mean	SD	P	Inference
Cases	Sr. Folate	<i>MTHFR</i> 677 C/T	CC WILD	34	6.32	4.71	0.21	Not Significant
			CT Hetero	9	3.41	2.04		
			TT Mutant	2	5.55	4.45		
		<i>MTHFR</i> 1298 A/C	AA WILD	4	4.83	0.96	0.85	Not Significant
			AC Hetero	28	5.60	4.45		
			CC Mutant	13	6.18	5.02		
Controls		<i>MTHFR</i> 677 C/T	CC WILD	28	6.71	5.45	0.12	Not Significant
			CT Hetero	17	4.49	1.94		
			TT Mutant	NIL				
		<i>MTHFR</i> 1298 A/C	AA WILD	6	9.43	7.58	0.12	Not Significant
			AC Hetero	35	5.43	4.00		
			CC Mutant	4	4.60	1.02		

Table 6 Depicts comparison of vitamin B12 according to the *MTHFR* genotype

Group		Parameter	Genotype	N	Mean	SD	P	Inference
Cases	Sr. B12	MTHFR 677 C/T	CC WILD	34	350.18	469.29	0.38	Not Significant
			CT Hetero	9	142.33	66.36		
			TT Mutant	2	102.00	0.00		
		MTHFR 1298 A/C	AA WILD	4	378.00	239.81	0.79	Not Significant
			AC Hetero	28	320.21	507.64		
			CC Mutant	13	234.25	198.52		
Controls		MTHFR 677 C/T	CC WILD	28	310.54	236.35	0.051	Not Significant
			CT Hetero	17	192.12	58.69		
		MTHFR 1298 A/C	AA WILD	6	380.50	262.82	0.26	Not Significant
			AC Hetero	35	254.77	189.56		
			CC Mutant	4	190.25	113.45		

(*rs1801133*) polymorphism and risk of CAD [15, 16] and the available data on *MTHFR* C677T (*rs1801133*) polymorphisms and risk of cardiovascular disease is conflicting. In this study, we did not find any association between C677T (*rs1801133*) polymorphism and serum homocysteine levels which is consistent with some earlier studies [3, 15–17]. However, in our study, we found higher serum homocysteine levels among the carrier of T allele in cases and controls (Table 4). Ilhan N et al. [18] concluded in their study that homocysteine level was higher in TT genotypes in CAD patients compared with CC and CT genotypes and *MTHFR* gene polymorphism is an independent risk factor for essential hypertension but not for CAD. In the present study also homozygous mutant TT polymorphism in group I (n = 2), were having serum homocysteine levels of 50 µmol/L.

Vitamin folate and B12 plays important role in homocysteine metabolism. In this study, lower levels of serum folate and vitamin B12 were found in carrier of T allele in both cases and control groups. When we compared average serum folate and B12 levels, no significant difference was found between cases and controls (Tables 5 and 6) but when we correlated serum homocysteine levels with serum folate and B12, negative association was found between them in cases as well as controls. This negative association was statistically significant for serum folate in cases as well as for serum folate and B12 in controls. John C. Chambers has shown elevated serum homocysteine levels associated with low serum folate and B12 levels in Asian population which could be due to genetic or environmental factors [17–19]. Moreover, they reported that *MTHFR* C677T (*rs1801133*) mutation does not contribute to elevated plasma homocysteine concentrations or increased CHD risk in Indian Asians compared with European whites [17]. Kadziela J et al. [20] reported that serum folic acid and vitamin B12 levels were not related to the *MTHFR* genotype but homozygotes TT had significantly higher homocysteine concentration than heterozygotes (CT) and

homozygotes (CC). Plasma homocysteine concentration was significantly higher in patients with CAD compare to controls and correlated significantly with folic acid and vitamin B12. The findings of our study were consistent with their reports. Some non-Indian population studies have shown that hyperhomocysteinemia and the T/T genotype was a risk factor for early onset of CAD [21, 22]. However, some studies reported no association between C677T (*rs1801133*) mutation and risk of CAD [23, 24].

The genotype polymorphism comparison for *MTHFR* A1298C (*rs1801131*) among young CAD patients and controls were very near to significant level but could not attain it which could be due to small sample size of our study. When *MTHFR* A1298C (*rs1801131*) polymorphism was compared with serum homocysteine, folate and vitamin B12 levels, no significant association was observed between them (Tables 4, 5, 6). However, serum homocysteine concentration was higher in young CAD patients as compared to control group (Table 4). Contradictory to C677T (*rs1801133*) polymorphism findings, serum folate and vitamin B12 levels were lower in control group in C allele carrier for A1298C (*rs1801131*) polymorphism (Table 6). Kolling K et al. [25] demonstrated that *MTHFR* gene C677T (*rs1801133*) or A1298C (*rs1801131*) polymorphisms were not associated with the presence of angiographic CAD. Although, an apparent association was noted between elevated level of homocysteine and CAD, it was not independent of conventional cardiovascular risk factors. Spiroski I et al. [26] also could not confirm a significant association of *MTHFR* C677T (*rs1801133*) and *MTHFR* A1289C (*rs1801131*) polymorphisms with occlusive artery disease or deep venous thrombosis in Macedonians, except for the safeguarding effect of *MTHFR*/CA:CC diplotype against occlusive artery disease. Domagala TB et al. [27] reported that the polymorphisms C677T(*rs1801133*) and A1298C(*rs1801131*) of *MTHFR* and fasting plasma homocysteine levels do not seem to be significant risk factors for venous thromboembolic disease.

Hanson NQ et al. [28] have shown that the prevalence of the *C677T* (*rs1801133*) and *A1298C* (*rs1801131*) polymorphisms did not differ among individuals with CAD, DVT, or those without documented vascular disease. In contrast to the *C677T* (*rs1801133*) polymorphism, the *A1298C* (*rs1801131*) polymorphism was not associated with increased fasting total serum homocysteine. Our findings were in similar line with their reports. The prevalence of *MTHFR C677T* (*rs1801133*) and *A1298C* (*rs1801131*) polymorphism was subjected to variation in different geographical regions, ethnicity, races, environmental influences, dietary folate intake and other life-style factors giving conflicting results. Friso S et al. [5] reported no additional effect of *A1298C* (*rs1801131*) on total plasma homocysteine in 148 combined heterozygotes compared with 98 heterozygotes for the *C677T* alone. These findings emphasize the hypothesis that *MTHFR* genotypes may interfere with coronary artery disease risk only when an unbalanced nutritional status leads to raised total plasma homocysteine levels. We found significantly elevated serum homocysteine levels in young CAD group as compare with control group which was significantly associated with *C677T* (*rs1801133*) polymorphism in young CAD patients but not in *A1298C* (*rs1801131*) polymorphism. This supports the hypothesis that *C677T* (*rs1801133*) mutation affects the thermolability and decreases activity of *MTHFR*, the regulatory enzyme in remethylation of homocysteine and thus leading to hyperhomocysteinemia which is independent risk factor for CAD. We did not find significant difference between serum folate and vitamin B12 levels among young CAD patients and control group but negative association was found between serum homocysteine and folate as well as vitamin B12 levels in young CAD group and control group, earmarking the role of vitamin folate and B12 in homocysteine metabolism.

In addition to this, in the present study, we found significantly higher levels of serum total cholesterol, triglycerides, TC/HDL-C and LDL-C/HDL-C ratio. Serum LDL-C was higher and HDL-C was lower in young CAD group as compare to controls but it was not statistically significant (Table 2). These findings were much similar to the reports of Gupta S et al. [6]. Moreover, significantly higher systolic BP, diastolic BP and w/h ratio was observed in young CAD group as compared to control group (Table 1). These conventional risk factors might also be contributing to the pathogenesis of CAD among young age patients.

We were among very few researchers who could successfully show that *MTHFR C677T* (*rs1801133*) and *A1298C* (*rs1801131*) polymorphisms in young CAD patients could lead to hyperhomocysteinemia which fills the role of cardiac biomarker in prediction of young CAD risk. The novelty of this study was that we have studied

association between serum folate, vitamin B12 status along with traditional risk factors with *MTHFR* polymorphisms as well as hyperhomocysteinemia in young CAD for the first time. This would help to shift focus of preventive cardiology test panel on single nucleotide gene polymorphisms especially in young CAD which has got polygenic pathophysiology in different ethnic population. The limitation of our study was that we have conducted preliminary study with a small sample size to establish link between *MTHFR C677T* (*rs1801133*) and *A1298C* (*rs1801131*) polymorphism with serum homocysteine, folate and B12 levels in young CAD group. Additional studies with larger sample size are needed to define the influence of *MTHFR C677T* (*rs1801133*) and *A1298C* (*rs1801131*) genotyping in pathogenesis of young CAD patients.

In conclusion, the findings of our study suggests that elevated serum homocysteine levels were associated with increased risk of CAD in young age group (< 45 years) independent of *MTHFR C677T* (*rs1801133*) and *A1298C* (*rs1801131*) polymorphisms. The significant hyperhomocysteinemia (> 15 $\mu\text{mol/L}$) was associated with *C677T* (*rs1801133*) polymorphism in young CAD group. This association between hyperhomocysteinemia and CAD in young group was not independent of conventional cardiovascular risk factors. Young age population being important part in population pyramid of any country, risk of hyperhomocysteinemia and CAD could be monitored by *MTHFR* polymorphism detection followed by serum homocysteine, folate and vitamin B12 measurement and appropriate measures could be taken to prevent or delay the occurrence of CAD. In further scope, longitudinal follow up of group I participants could be done for predicting development of future cardiac event.

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Declarations

Conflict of interests Authors state no conflict of interest.

Informed consent Informed consent was obtained from all individuals included in this study.

Ethical approval Research involving human subjects complied with all relevant national regulations, institutional policies and has been approved by the institutional ethical committee of Bharati Vidyapeeth

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Cardiovascular Disease

MTHFR and risk of stroke and heart disease in a low-folate population: a prospective study of 156 000 Chinese adults

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Abstract

Background: The relevance of folic acid for stroke prevention in low-folate populations such as in China is uncertain. Genetic studies of the *methylenetetrahydrofolate reductase* (*MTHFR*) C677T polymorphism, which increases plasma homocysteine (tHcy) levels, could clarify the causal relevance of elevated tHcy levels for stroke, ischaemic heart disease (IHD) and other diseases in populations without folic acid fortification.

Methods: In the prospective China Kadoorie Biobank, 156 253 participants were genotyped for *MTHFR* and 12 240 developed a stroke during the 12-year follow-up. Logistic regression was used to estimate region-specific odds ratios (ORs) for total stroke and stroke types, IHD and other diseases comparing TT genotype for *MTHFR* C677T (two thymine alleles at position 677 of *MTHFR* C677T polymorphism) vs CC (two cytosine alleles) after adjustment for age and sex, and these were combined using inverse-variance weighting.

Results: Overall, 21% of participants had TT genotypes, but this varied from 5% to 41% across the 10 study regions. Individuals with TT genotypes had 13% (adjusted OR 1.13, 95% CI 1.09–1.17) higher risks of any stroke [with a 2-fold stronger association with intracerebral haemorrhage (1.24, 1.17–1.32) than for ischaemic stroke (1.11, 1.07–1.15)] than the reference CC genotype. In contrast, *MTHFR* C677T was unrelated to risk of IHD or any other non-vascular diseases, including cancer, diabetes and chronic obstructive lung disease.

Conclusions: In Chinese adults, the *MTHFR* C677T polymorphism was associated with higher risks of stroke. The findings warrant corroboration by further trials of folic acid and implementation of mandatory folic acid fortification programmes for stroke prevention in low-folate populations.

Keywords: *MTHFR*, folate, homocysteine, stroke, Mendelian randomization, China.

Introduction

Homocysteine (tHcy) is a sulphur-containing amino acid that plays a key role in one-carbon metabolism ([Supplementary Figure S1](#), available as [Supplementary data at IJE online](#)) and genetic defects or deficiency of folic acid or vitamin B12 cause elevated plasma tHcy levels.¹ Elevated plasma tHcy levels have been associated with higher risks of cardiovascular disease (CVD)^{2–3} and some non-CVD outcomes,⁴ but the causal relevance of these associations is uncertain and may differ importantly between populations with or without folic acid fortification.

Methylenetetrahydrofolate reductase (*MTHFR*) is an enzyme that controls a key branch point in folate metabolism

and regulates the supply of one-carbon units required for protein synthesis. *MTHFR* uses folate to metabolize and thereby remove tHcy ([Supplementary Figure S1](#), available as [Supplementary data at IJE online](#)). A common genetic variant *MTHFR* C677T (rs1801133) (T allele frequency ~10% in European ancestry populations) greatly reduces enzyme efficiency. TT genotypes for this polymorphism (i.e. two thymine alleles at position 677 of *MTHFR* C677T) have ~25% (~3 µmol/L) higher plasma tHcy concentrations than the reference genotype (denoted as CC as they have two cytosine alleles).^{5,6} However, the tHcy-elevating effects of TT homozygotes for *MTHFR* C677T on plasma tHcy concentrations are substantially attenuated by folic acid fortification.

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Key Messages

- Individuals with the TT genotype for *MTHFR* C677T (two thymine alleles at position 677 of *MTHFR* C677T polymorphism), which occurs in ~10% in Western populations, have ~25% (~3 µmol/L) higher plasma homocysteine (tHcy) than those with the CC genotype (two cytosine alleles) in populations without folic acid fortification, but the effects are almost fully attenuated by effective folic acid fortification.
- Variable implementation of folic acid fortification worldwide has resulted in 2- to 4-fold differences in mean plasma folate concentrations between countries.
- In a prospective study of 156 253 participants in the China Kadoorie Biobank, ~21% had TT homozygotes for *MTHFR* C677T. TT homozygotes had 13% higher risks of all strokes (24% higher risks of intracerebral haemorrhage and 11% higher risks of ischaemic stroke) than CC homozygotes.
- *MTHFR* C677T was unrelated to ischaemic heart disease or to any of the other non-vascular diseases studied.
- The findings should prompt further trials of folic acid supplementation for stroke prevention in China and implementation of effective mandatory folic acid fortification programmes for stroke prevention.

Folic acid fortification has been widely implemented worldwide over the last three decades and it has reduced the incidence of neural tube defects,^{7–9} but variable implementation of different types of folic acid fortification has resulted in substantial differences in mean plasma folate levels in populations with mandatory, voluntary or no folic acid fortification (30–40 vs 20–25 vs 10–15 nmol/L, respectively).^{10–12} The UK government has recently signalled its intention to change from voluntary to mandatory folic acid fortification but many other countries, including China, have not yet implemented any effective food fortification with folic acid.¹⁰

Mendelian randomization (MR) studies have reported positive associations of TT vs CC homozygotes for *MTHFR* C677T with total stroke in non-fortified populations, but not in populations with folic acid fortification.⁶ The *MTHFR* TT frequency also varies substantially between populations (5–40%) and hence many studies of *MTHFR* C677T have been constrained by incomplete control for population stratification. Randomized trials have reported no beneficial effects of folic acid supplementation on stroke in Western populations but the Chinese Stroke Primary Prevention Trial (CSPPT) reported that folic acid supplementation lowered risk of total stroke by 21%.¹⁴ The discrepant results of the folic acid trials^{13,14} and MR studies^{15–17} between Chinese and Western populations may reflect the differences in folic acid fortification policies.

The aims of this study were to: (i) assess the associations of the *MTHFR* C677T polymorphism with total stroke, including ischaemic stroke (IS) and intracerebral haemorrhage (ICH) types, and with ischaemic heart disease (IHD), including myocardial infarction (MI), and other IHD types after stratifying for the 10 study regions and adjusting for principal components; and (ii) examine the associations of the *MTHFR* C677T polymorphism with site-specific cancers and other diseases previously linked with low-folate status in a large prospective Chinese cohort study.

Methods**Study population**

Details of the design, survey methods, procedures and participant characteristics of the China Kadoorie Biobank (CKB) study have been previously reported.^{18,19} In brief, the CKB is a prospective cohort study of 512 715 adults who were recruited between 25 June 2004 and 15 July 2008 from 10

diverse rural and urban regions of China ([Supplementary Methods](#), available as [Supplementary data](#) at *IJE* online).

Follow-up for disease outcomes

Information about incident disease and cause-specific mortality was obtained by electronic linkage, via the unique national identification number, to established local mortality (cause-specific) and morbidity (for stroke, IHD, cancer and diabetes) registries and to the health insurance system that records any hospitalization episodes and procedures.^{20,21} All disease diagnoses were coded using the Tenth International Classification of Diseases (ICD-10), blinded to any baseline information.

Major disease outcomes

For the present study, the main vascular disease outcomes included first non-fatal or fatal IS, ICH, total stroke, acute MI, other acute IHD and all IHD ([Supplementary Table S1](#), available as [Supplementary data](#) at *IJE* online). Stroke types were classified into IS, ICH and other pathologic types.^{20,21} The main non-vascular disease outcomes included total incident cancer and cancers of the colon, lung, stomach and breast. Other non-vascular diseases included diseases previously reported to be associated with elevated tHcy levels ([Supplementary Table S1](#), available as [Supplementary data](#) at *IJE* online) in addition to a phenome-wide analysis of 41 major disease groups.

Genotyping procedures

Genotyping of the *MTHFR* C677T single-nucleotide polymorphism (SNP) was performed at BGI Genomics Company, Shenzhen, China by genotyping 384 candidate SNPs using Illumina Golden Gate technology (Illumina, San Diego) in 92 987 randomly selected participants. In addition, genome-wide genotyping using a custom-designed Affymetrix 800K SNP arrays (including *MTHFR* C677T) was performed in 75 982 participants who were randomly selected from the CKB population and an additional 24 658 CKB participants were selected for nested case-control studies of incident cardiovascular or respiratory diseases.

After excluding a small overlap, the present analyses involved 162 366 participants with genotyping data for *MTHFR* C677T (rs1801133), of whom 151 393 were randomly selected participants and 10 973 were selected because they had a stroke or IHD event during follow-up.

(Supplementary Figure S2, available as Supplementary data at *IJE* online). After exclusion of 6133 participants with non-local ancestry or missing data for regional principal components (PCs), a total of 156 253 participants were included in the final analyses (Supplementary Figure S2, available as Supplementary data at *IJE* online).

For all vascular disease outcomes, a set of common controls were used that excluded individuals with a prior history of IHD, stroke or transient ischaemic attack, incident IHD or incident major vascular events (fatal or non-fatal MI, fatal or non-fatal stroke or death from cardiovascular disease: Supplementary Figure S2, available as Supplementary data at *IJE* online). For non-vascular disease outcomes, we used controls that separately excluded individuals with any of the specific non-vascular diseases for each comparison.

Statistical methods

Baseline characteristics were analysed for the overall CKB cohort and the genotyped subset. The *MTHFR* C677T genotype was assessed for departure from Hardy-Weinberg Equilibrium (HWE) by region and overall using a HWE chi-square test. Principal component analysis identified 11 PCs informative for CKB population structure (Supplementary Methods, available as Supplementary data at *IJE* online).²² The associations of *MTHFR* C677T genotypes with continuous traits were assessed by using linear regression after stratification by region and adjustment for age, sex and regional PCs. Binary traits were stratified by region; adjusted for age, sex and regional PCs; and presented as adjusted prevalence.

Logistic regression was used to estimate the odds ratios (ORs) comparing TT vs CC genotypes of the *MTHFR* C677T for each disease outcome after stratification by region and adjusting for age, sex and regional PCs. The region-specific risk estimates were meta-analysed using inverse-variance weighting to yield overall ORs for disease outcomes,

with the statistical importance of the effect sizes assessed using a likelihood ratio (LRT) chi-square statistic.

Additional analyses undertaken included: (i) using tHcy weighted analyses to obtain the effect of a 25% *MTHFR*-derived higher plasma tHcy concentration; and (ii) estimating effects per T allele for *MTHFR* C677T. Further analyses included stepwise comparisons of incremental adjustments in the logistic regression models by: (i) age, sex, region and regional PCs;²² (ii) additional adjustments for potential pleiotropic factors and (iii) other CVD risk factors to account for confounding (Supplementary Methods, available as Supplementary data at *IJE* online). Subgroup analyses were performed to assess potential effect modification for the main stroke outcomes by CVD risk factors and by genetic variants influencing alcohol consumption.^{23,24} Sensitivity analyses assessed the robustness of the main results after excluding prior disease in both cases and controls, or in addition after restricting analyses to population subsets with genome-wide association study data. All statistical analyses were conducted in SAS 9.4.

Results

The baseline characteristics of the genotyped subset were similar to those of the overall CKB population (Supplementary Table S2, available as Supplementary data at *IJE* online). Among control participants, the prevalence of *MTHFR* C677T genotypes did not differ by age, sex, education, lifestyle factors, systolic blood pressure (SBP) or by measures of adiposity (Table 1). However, there were associations of *MTHFR* C677T with mean levels of blood pressure indices (diastolic blood pressure (DBP), pulse pressure and mean arterial pressure), height, weight and random blood glucose concentrations, but the absolute differences were modest. The associations of *MTHFR* C677T with blood pressure indices provide evidence for some pleiotropic effects on blood

Table 1. Associations of *MTHFR* C677T genotypes with control participant characteristics in China Kadoorie Biobank^a

Participant characteristics	<i>MTHFR</i> genotypes in controls			P for trend
	CC (n = 38 908)	CT (n = 51 479)	TT (n = 23 185)	
Age, years ^b	50.0 (10.3)	50.1 (9.9)	50.0 (10.3)	0.52
Female ^c	60.3%	60.4%	59.7%	0.26
High school education or above ^c	19.7	19.7	20.3	0.22
Current smoker (men) ^c	74.5	75.1	74.2	0.76
Current drinker (men) ^c	35.7	35.5	34.9	0.32
Fresh fruit >3 days per week ^c	27.7	27.4	27.7	0.87
Meat >3 days per week ^c	47.3	47.2	47.0	0.58
Dairy >3 days per week ^c	10.6	10.5	10.6	0.97
Systolic blood pressure (mmHg) ^b	128.9 (19.0)	129.0 (18.4)	128.6 (19.0)	0.21
Diastolic blood pressure (mmHg) ^b	77.0 (10.8)	77.1 (10.4)	77.6 (10.8)	<0.0001
Pulse pressure (mmHg) ^b	51.9 (13.2)	51.8 (12.8)	51.0 (13.2)	<0.0001
Mean arterial pressure (mmHg) ^b	94.3 (12.6)	94.4 (12.2)	94.6 (12.6)	<0.0001
Height (cm) ^b	158.6 (5.6)	158.8 (5.5)	158.9 (5.6)	<0.0001
Weight (kg) ^b	59.3 (9.4)	59.5 (9.1)	59.6 (9.4)	<0.0001
Body mass index (kg/m ²) ^b	23.5 (3.3)	23.5 (3.2)	23.5 (3.3)	0.15
Body fat (%) ^b	27.9 (6.7)	27.9 (6.5)	27.8 (6.7)	0.78
Physical activity (MET-h/day) ^b	22.7 (12.8)	22.8 (12.4)	22.8 (12.8)	0.15
Random blood glucose (mmol/L) ^{b,d}	5.9 (2.1)	5.9 (2.0)	5.9 (2.1)	0.01

^a All analyses are stratified by region and adjusted for age, age-squared, sex and regional principal components. CC, CT and TT refer to two cytosine, one cytosine and one thymine, and two thymine alleles at position 677 of the *MTHFR* polymorphism, respectively. Physical activity was quantified as metabolic equivalents of task hours per day (MET-h/d) based on the type, frequency and duration of specific activities.

^b Estimates are adjusted means (standard error).

^c Estimates are adjusted prevalences.

^d There were missing values for random blood glucose by *MTHFR* genotypes. There were 38 406, 50 883 and 22 982 participants with random plasma glucose data for CC, CT and TT, respectively.

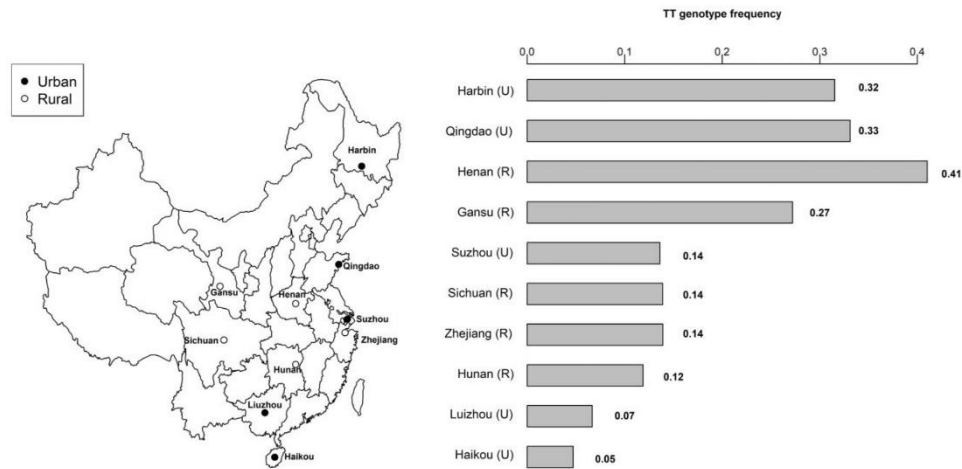


Figure 1. Locations of sites and frequency of the *MTHFR* C677T TT genotype by study area. TT refers to two thymine alleles at position 677 of the *MTHFR* polymorphism

pressure (Table 1). Consistently with the findings in controls only, similar associations of *MTHFR* C677T with blood pressure types were observed for all participants (Supplementary Table S3, available as Supplementary data at *IJE* online).

Overall, 21% of participants were homozygotes for the TT genotype, with an 8-fold difference in TT prevalence between the Northern and Southern regions in China (Figure 1). Across the 10 study regions, the prevalence of TT genotype varied from 41% in Henan to 5% in Haikou (Figure 1). Across 10 study regions, there was no evidence of departure from HWE in seven regions (Supplementary Table S4, available as Supplementary data at *IJE* online) but there was evidence of departure from HWE in Liuzhou and minimal departure in Sichuan and Zhejiang.

Associations of *MTHFR* C677T with vascular disease outcomes

Figure 2 shows the region stratified and adjusted results for all primary vascular disease outcomes. Individuals with TT genotypes for *MTHFR* C677T had 13% (adjusted OR = 1.13, 95% CI 1.09–1.17) higher risks of any stroke, with a 2-fold stronger association with intracerebral haemorrhage ($n = 3189$ cases; 1.24, 1.17–1.32) than for IS ($n = 8762$; 1.11, 1.07–1.15). In contrast, the *MTHFR* C677T polymorphism was unrelated to MI (1.03; 0.96–1.12), other IHD (1.05; 1.01–1.09) or all IHD (1.04; 1.00–1.08) outcomes after accounting for multiple testing (Figure 2).

Supplementary Figure S3 (available as Supplementary data at *IJE* online) assesses the impact of additional adjustment for different blood pressure indices over and above adjustment for age, sex, region and regional PCs. Adjustment for DBP attenuated the associations of *MTHFR* with total stroke (1.13 vs 1.09 for TT vs CC) and likewise for mean arterial pressure (1.13 vs 1.11) whereas adjustment for pulse pressure inflated the associations with total stroke (1.13 vs 1.16, respectively). The patterns were similar for both IS and ICH,

whereby adjustment for DBP attenuated ORs but pulse pressure increased the ORs and similarly for IHD.

Supplementary Figure S4 (available as Supplementary data at *IJE* online) demonstrates the importance of appropriate adjustment for region and regional PCs because, in the absence of such adjustments, the ORs for stroke were much more extreme than those with such adjustments. For example, the ORs for total stroke declined after full adjustment from 1.55 (95% CI: 1.50–1.60) to 1.13 (1.09–1.17). Likewise, the ORs for MI without such adjustments was 1.51 (1.40–1.63), which was attenuated to 1.04 (0.96–1.13) after adjustment (Supplementary Figure S4, available as Supplementary data at *IJE* online), illustrating the substantial confounding by failure to take account of regional differences in TT prevalence. There was no evidence of heterogeneity in the strength of the associations of *MTHFR* C677T TT vs CC with ICH, IS or total stroke (Supplementary Figure S5, available as Supplementary data at *IJE* online) across the 10 study regions.

Table 2 shows the associations of all three *MTHFR* C677T genotypes (TT, CT and CC) with risks of incident vascular disease outcomes. Compared with CC, the heterozygote CT genotype (one cytosine and one thymine allele at position 677 for the *MTHFR* C677T polymorphism) had less than half the effect of the TT genotype on stroke risk, with ORs per T allele of 1.09 (1.04–1.13), 1.05 (1.02–1.07) and 1.05 (1.03–1.08) for ICH, IS and total stroke, respectively. In the analyses corresponding to a 25% higher tHcy, the adjusted ORs were 1.19 (1.10–1.28) for ICH, 1.10 (1.04–1.16) for IS and 1.12 (1.07–1.17) for total stroke, respectively (Table 2), consistently with the results for the TT vs CC genotypes in Figure 2. Likewise, there was no evidence of an association per T allele or per 25% higher tHcy with MI or total IHD after taking account of multiple testing. The effects of *MTHFR* C677T on stroke and IHD outcomes were largely unaltered in sensitivity analyses that excluded prior vascular disease in cases and controls, or when analyses were restricted to a

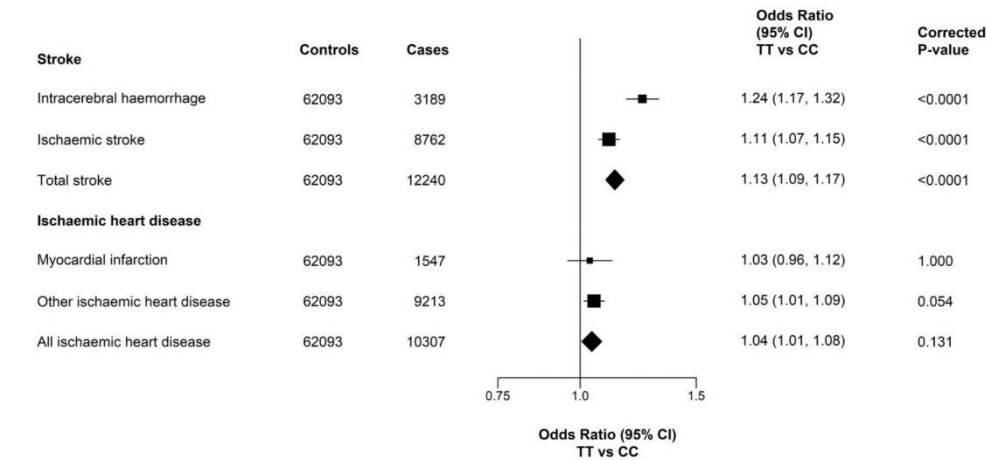


Figure 2. Association of *MTHFR* C677T with incident cases of stroke and ischaemic heart disease. Values shown are the odds ratios (95% CIs). The sizes of the squares are proportional to the inverse variance of each effect size. All analyses are region stratified and adjusted for age, age-squared, sex and regional principal components. TT and CC refer to two thymine alleles and two cytosine alleles, respectively, at position 677 of the *MTHFR* polymorphism

Table 2. Effects of *MTHFR* C677T genotype on incident vascular outcomes

Outcome	Number of cases ^a			Odds ratio (95% CI) ^b				
	CC	CT	TT	CC	CT	TT	Per T allele	Per 25% higher tHcy ^c
Intracerebral haemorrhage	1805	2684	1384	1.00 (0.95–1.05)	1.09 (1.04–1.13)	1.24 (1.17–1.32)	1.09 (1.05–1.13)	1.19 (1.10–1.28)
Ischaemic stroke	4670	7220	4092	1.00 (0.97–1.03)	1.05 (1.02–1.07)	1.11 (1.07–1.15)	1.05 (1.02–1.08)	1.10 (1.04–1.16)
Total stroke	6658	10 142	5582	1.00 (0.97–1.03)	1.05 (1.03–1.08)	1.13 (1.09–1.17)	1.06 (1.03–1.08)	1.12 (1.07–1.17)
Myocardial infarction	846	1335	701	1.00 (0.93–1.07)	1.05 (0.99–1.11)	1.03 (0.96–1.12)	1.02 (0.96–1.08)	1.03 (0.92–1.14)
Other ischaemic heart disease	5103	7956	4110	1.00 (0.97–1.03)	1.03 (1.01–1.06)	1.05 (1.01–1.09)	1.00 (0.98–1.03)	1.00 (0.95–1.05)
All ischaemic heart disease	5671	8945	4636	1.00 (0.97–1.03)	1.03 (1.01–1.06)	1.04 (1.01–1.08)	1.01 (0.98–1.03)	1.01 (0.96–1.05)

^a The cases are compared with a common set of controls for each outcome. The number of controls for each *MTHFR* C677T genotype was 38 908 for CC, 51 479 for CT and 23 185 for TT. CC, CT and TT refer to two cytosine, one cytosine and one thymine, and two thymine alleles at position 677 of the *MTHFR* polymorphism, respectively. tHcy refers to plasma total homocysteine levels.

^b All analyses were region stratified and adjusted for age, age-squared and regional principal components.

^c OR per 25 higher tHcy is the odds ratio weighted by the expected effects of *MTHFR* C677T genotypes on homocysteine levels: TT = 1, CT = 0.25, CC = 0.

random population subset with genome-wide genotyping data with additional exclusions of those recruited for nested case-control studies (Supplementary Table S5, available as Supplementary data at *IJE* online).

The effects of *MTHFR* C677T on risk of total stroke were more extreme in younger than in older people and in individuals living in rural than in urban areas, but did not differ by sex, education, smoking, alcohol or diagnosed hypertension (Supplementary Figure S6, available as Supplementary data at *IJE* online). Moreover, there was no evidence of effect modification for stroke by the two alcohol genetic variants rs1229984 and rs671 (Supplementary Figure S7, available as Supplementary data at *IJE* online).

Associations with non-vascular disease outcomes

There was no evidence of any association of *MTHFR* C677T genotypes with any of the pre-specified non-vascular disease outcomes in the adjusted models (Figure 3). In sensitivity analyses without stratification for 10 study regions or adjustment for PCs, there was substantial confounding that resulted

in much more extreme ORs for non-vascular disease outcomes [e.g. ~30% lower risks for lower respiratory tract infections, chronic obstructive pulmonary disease (COPD) and fracture, respectively: Supplementary Figure S8, available as Supplementary data at *IJE* online]. In a phenome-wide analysis of *MTHFR* C677T and 41 disease outcomes (Supplementary Figure S9, available as Supplementary data at *IJE* online), there were no associations of *MTHFR* C677T with any other diseases apart from stroke after correction for multiple testing.

Discussion

This genetic study of 156 253 Chinese adults reported that the overall TT homozygote frequency for the *MTHFR* C677T polymorphism was 2-fold greater in Chinese than in Western populations but the TT frequency also differed by 8-fold between the 10 study regions (41% in North vs 5% in South) in China. Individuals with TT compared with CC genotypes for *MTHFR* C677T had a 13% higher risk of stroke

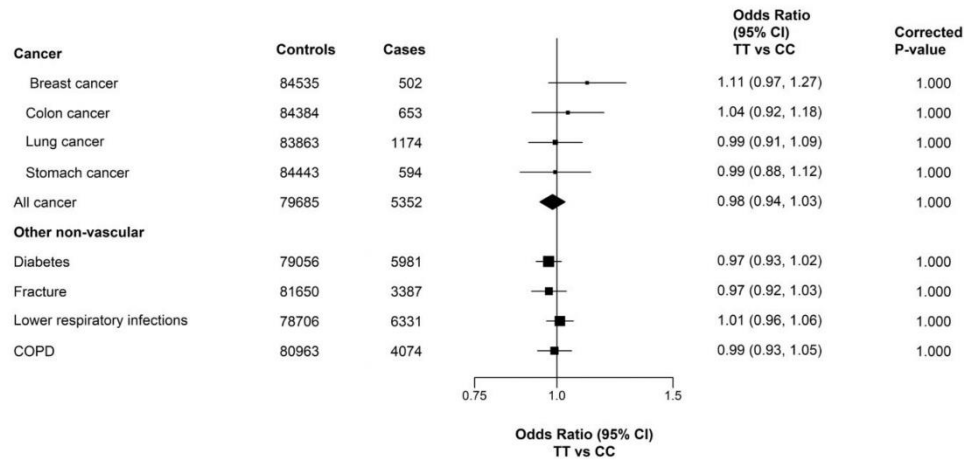


Figure 3. Association of *MTHFR* C677T with incident cases of cancer and other non-vascular diseases. Values shown are the odds ratios (95% CIs). The sizes of the squares are proportional to the inverse variance of each effect size. All analyses are region stratified and adjusted for age, age-squared, sex and regional principal components. TT and CC refer to two thymine alleles and two cytosine alleles, respectively, at position 677 of the *MTHFR* polymorphism. COPD, chronic obstructive pulmonary disease

(24% higher risk of ICH and 11% higher risk of IS) but had no excess risk of IHD or any of the other diseases studied.

In contrast with previous studies,^{15–17} this genetic study in a Chinese population that stratified the analyses by the 10 study regions and adjusted for regional PCs should have minimized any risk of population stratification. This study provides important new evidence for the population relevance of elevated tHcy levels for risk of stroke prevention (particularly for ICH, but also for IS). However, the present study provided no additional support for the relevance of elevated tHcy levels for risk of IHD or for cancer, diabetes or respiratory diseases.

Consistently with the discrepant findings of *MTHFR* C677T levels with stroke incidence in Chinese vs European populations, a meta-analysis of trials of folic acid reported conflicting results in populations with or without folic acid fortification.^{13,14} Supplementary Figure S10 (and Supplementary Methods, available as Supplementary data at *IJE* online) includes results of a meta-analysis of randomized trials of folic acid and shows that folic acid supplements had no protective effects on total stroke in populations with mandatory or voluntary folic acid fortification but had a protective effect in populations without folic acid fortification. The findings also reflect the effects of the CSPPT trial, which reported that folic acid supplements (~0.8 mg daily in 20 702 Chinese adults with hypertension but no prior history of CVD) reduced the risk of total stroke by 21%. In contrast with the present genetic study, the CSPPT trial reported that folic acid lowered IS [$n=515$ by 24% (95% CI: 9 to 36%), $n=515$ cases] but had no power to detect an effect on ICH ($n=120$ outcomes) or on IHD ($n=49$ outcomes).¹⁴ The discrepant results of folic acid trials for stroke prevention between Chinese and Western populations should prompt further trials of folic acid for stroke prevention in the Chinese population.²⁵

The *MTHFR* C677T polymorphism results in elevated tHcy concentrations (Supplementary Figure S1, available as Supplementary data at *IJE* online) but the tHcy-elevating

effects of this polymorphism are attenuated by folic acid fortification.³ The effects of the *MTHFR* C677T polymorphism are also highly sensitive to population stratification but the present report involved meta-analysis of region-specific analyses with additional adjustment for PCs to minimize such biases. Moreover, the concordant results of the sensitivity analyses that excluded prior disease in cases and controls or restricted analyses to random population subsets should mitigate other potential biases.

High alcohol intake and related alcoholic liver disease are associated with folate deficiency,^{23,24} which could possibly modify the associations of *MTHFR* C677T with risks of stroke and other diseases. However, we observed no effects of adjustment for reported alcohol consumption on risks of stroke whereas *MTHFR* C677T only had a modest effect on blood pressure measures (albeit with opposing effects on SBP and DBP), indicating that this variant may have minor pleiotropic effects on blood pressure. However, stepwise adjustment for SBP, DBP, pulse pressure and mean arterial pressure had only a modest effect on the excess risks of stroke associated with TT vs CC genotypes for *MTHFR* C677T.

Previous studies had reported conflicting results about the associations of elevated plasma tHcy levels with risk of diabetes, fracture or cancer of the stomach or colon.^{26–29} The present study involving a phenome-wide analysis of 41 disease outcomes found no evidence that *MTHFR* C677T was related to any other major non-CVD outcomes in Chinese adults.

Mandatory food fortification with folic acid has been widely implemented in the Americas and Australia, and voluntary fortification has been implemented in Europe but many low- and middle-income countries, including China, have not yet implemented any effective folic acid fortification.^{30,31} A randomized trial of two types of flour—one fortified with vs one without folic acid—conducted in 16 648 Chinese women reported that folic acid fortification reduced the incidence of neural tube defects by 69%.^{32,33} The present

study suggests that mandatory folic acid fortification may be beneficial in China and the absolute benefits may be greater in the north of China, where stroke incidence rates are 10-fold higher and prevalence rates of *MTHFR* TT genotypes are 8-fold greater than in the south of China.

The chief strengths of the present study include a large number of well-characterized stroke and other disease outcomes, control of population stratification in a population without folic acid fortification and high prevalence of the *MTHFR* TT genotypes. However, the present study also had some limitations. First, no measurements of plasma folate or tHcy concentrations were available but previous surveys in China have reported population mean plasma folate concentrations that are less than one-third of those in populations with mandatory folic acid fortification.³⁴ Second, the present study had limited power to detect the effects of the *MTHFR* C677T polymorphism on site-specific cancers or diabetes.

Consistently with the well-established beneficial effects of folic acid for prevention of neural tube defects,^{34,35} the present study provides important evidence for the relevance of the *MTHFR* polymorphism for stroke in Chinese adults. The findings should also prompt further trials of folic acid for stroke prevention in China and for effective mandatory folic acid fortification for stroke prevention in the Chinese population.³⁶

Ethics approval

Ethics approval was obtained from relevant local (Oxford Tropical Research Ethics Committee 025-04), national and international ethics committees, and all participants provided written informed consent.

Data availability

The observational data that support the findings of this study are available to bona fide researchers on application under the China Kadoorie Biobank Open Access Data Policy (www.ckbiobank.org). Sharing of genotyping data is constrained by the Administrative Regulations on Human Genetic Resources of the People's Republic of China. Access to these is available through collaboration with CKB researchers.

Supplementary data

Supplementary data are available at *IJE* online.

Author contributions

D.A.B., R.Cl., S.P. and Z.C. designed the study. D.A.B. conducted the statistical analyses and generated all tables and figures. D.A.B., R.Cl. and Z.C. wrote the first draft of the report. All other authors contributed to the revised report. L. L. and Z.C. jointly supervised the study. Y.G., Y.C., L.Y. and Y.W. co-ordinated the data collection. I.Y.M. and R.W. supervised the genotyping analyses. G.D.S. contributed to critical feedback on methods, results and the interpretation of the study findings.

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Conflict of interest

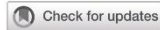
None declared.

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2.1.6. Estudio No.6



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5,10-methylenetetrahydrofolate reductase C677T gene polymorphism as a risk factor for premature coronary artery disease in patients with type 2 diabetes mellitus

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Background: Africa, like the rest of the world, is experiencing an increasing prevalence of diabetes mellitus. Diabetes increases the risk for coronary artery disease (CAD) by fourfold compared to people without diabetes. C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR) and hyperhomocysteinemia were reported by many studies as risk factors for CAD among patients with type 2 diabetes mellitus (T2DM). Early detection of modifiable risk factors for CAD is an important aspect of management of diabetes. This is the only study in Sudan which investigates the association between MTHFR genotypes and plasma homocysteine levels, and their role in premature CAD (PCAD) among patients with T2DM.

Methods: This study is a comparative study. We enrolled 226 Sudanese patients with T2DM, age range 25-60 years, recruited from Alshaab and Omdurman teaching hospitals in Khartoum State. 113 patients had CAD confirmed by angiography and electrocardiography (ECG) and 113 had no evidence of CAD. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), using Hinf1 restriction enzyme, were used to determine MTHFR genotypes. Plasma homocysteine levels were determined by enzymatic assay on the Hitachi Cobas Integra® 400 plus. Data was analyzed using statistical package for Social Sciences (SPSS) 23, using Mann-Whitney U test, general linear model, Chi-square test and logistic regression analysis.

Results: The frequencies of TT, CT, and CC genotypes were 16,40 and 44% among T2DM patients with PCAD. In T2DM patients without PCAD, the frequencies of TT, CT, and CC genotypes were 00,19 and 83%. The T allele showed strong association with PCAD among T2DM patients, $p < 0.001$, odds ratio (OR) 6.2, 95% CI (3.4-11.6). Patients with PCAD showed higher plasma homocysteine levels than patients without PCAD (13.5 $\mu\text{mol/L}$ versus 10 $\mu\text{mol/L}$, $p < 0.001$). The T allele had significant effect on homocysteine level, ($p < 0.001$). Plasma homocysteine levels were higher in individuals with TT genotype than

those with CT or CC genotypes in patients with PCAD (16.2 ± 5.3 , 14.3 ± 5.7 and 12.9 ± 5.02 $\mu\text{mol/L}$, $p=0.017$). Homocysteine levels showed a significant association with CAD, $p<0.001$, OR 3.2, 95% CI (1.9–5.5).

Conclusions: Our study suggests that C677T polymorphism of MTHFR gene and hyperhomocysteinemia are risk factors for PCAD in Sudanese population with T2DM.

KEYWORDS

diabetes mellitus, hyperhomocysteinemia, MTHFR, CAD, polymorphism

Introduction

Globally, the burden of diabetes mellitus (DM) continues to increase. The International Diabetes Federation (IDF) estimates that in 2022, 537 million adults live with diabetes worldwide (1).

Africa, like the rest of the world, is experiencing an increasing prevalence of DM, 1 in 22 adults live with DM in Africa (1, 2). In Sudan, previous studies have shown that there is high prevalence of diabetes in the adult populations (1, 3). The IDF reported, in 2021, that Sudan is one of the top five countries in the prevalence of diabetes in the Middle East and North Africa (4).

Diabetes increases the risk for coronary artery disease (CAD) by fourfold compared to people without diabetes (5, 6). CAD leads to significant morbidity and mortality. Identification and management of risk factors for CAD is an important aspect of management of DM.

Hyperhomocysteinemia has been identified as an independent risk factor for CAD (7, 8). Hyperhomocysteinemia has been associated with genetic defects in enzymes involved in its metabolism and/or with nutritional deficiencies of vitamin B₆, B₁₂ and folic acid (9, 10). Numerous studies have documented the influences of a common polymorphism (C677T) of methylenetetrahydrofolate reductase (MTHFR) on homocysteine levels. However, the relationship between this mutation and cardiovascular diseases (CVD) has remained a controversial issue (11–13).

Homocysteine and diabetes appear to have a negative synergistic effect on the cardiovascular system, and it is therefore important to investigate this effect in patients with diabetes who have higher risk for CAD than the normal population (14).

Homocysteine (Hcy) is metabolized by either remethylation to methionine or transsulphuration to cysteine. The former reaction is catalyzed by the vitamin B₁₂-dependent methionine synthase. The transsulphuration pathway is catalyzed by cystathionine- β -synthase (CBS) which is a vitamin B₆ dependent process (13, 15).

MTHFR catalyzes the reduction of 5,10-methylene tetrahydrofolate to 5-methyltetrahydrofolate, which is the methyl donor in the remethylation of Hcy to methionine. The most common mutation in the MTHFR gene is the 677T variant

which has been shown to encode a thermolabile enzyme with reduced activity (16, 17).

Several studies investigated possible association between MTHFR genotypes and plasma homocysteine levels and the incidence of different MTHFR genotypes in CAD patients (18–22). The results of these studies have been controversial. Many studies failed to show association between MTHFR genotypes and plasma homocysteine levels and their role in CAD. The variation was thought to be caused by ethnic or geographical differences, at least in some of these studies (23–25).

There is paucity in the studies which included patients with diabetes, who are already at increased risk for CAD (2, 5).

In Sudan, there is a gap in our knowledge about the most important modifiable risk factors for PCAD; therefore, identifying these factors in our population can improve prevention of these serious diabetic complications.

In this study, we investigated the association between MTHFR genotypes and plasma homocysteine (Hcy) levels and their role in PCAD in Sudanese patients with type 2 diabetes mellitus.

Materials, methods and study subjects

This is a comparative study, using a non-probability, selective sampling technique. 226 patients with diabetes were enrolled in this study according to statistical power formula.

113 patients had CAD and 113 had no evidence of CAD.

CAD was confirmed by angiography ($\geq 50\%$ stenosis of at least one of coronary arteries) and electrocardiography (ST segment depression or ST segment elevation) (26).

T2DM was diagnosed according to the WHO criteria (Glycated hemoglobin (HbA1c), $\geq 6.5\%$, 48 mmol/mol) or fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL) (27). Type 1 diabetes mellitus patients (T1DM) were excluded from the study. None of our patient was on insulin treatment. The age range of our study population was 25–60 years. The study was conducted in 2 hospitals (Alshaab and Oumdurman teaching hospitals) in Khartoum State, which treats Sudanese patients from different ethnic backgrounds.

Smoking was defined as the use of any tobacco at the time of conducting the study, while hypertension was defined with as systolic blood pressure >130 mmHg and diastolic blood pressure > 90 mmHg or using anti-hypertensive medication. The study protocol was approved by the Sudanese ministry of health ethics committee. Informed consent was obtained from all patients prior to data collection; investigation was conducted in accordance with the Declaration of Helsinki.

Biochemical analysis

Venous blood was obtained in Ethylenediamine tetraacetate (EDTA) after an overnight fast. Plasma Hcy level was determined by enzymatic assay on Hitachi Cobas Integra[®] 400 plus, the principle of the test is based on measuring the co-substrate conversion product, NAD spectrophotometrically at 340 nm. The lipid profile was estimated using Roche Cobas c311 based on enzymatic method. Glycated hemoglobin (HbA1c) was measured by a turbidimetric inhibition immunoassay (TINA) using Roche Cobas c311 analyzer. Authentic reagents were obtained from Roche diagnostics dealer in Sudan. The biochemical analysis was performed in East Model hospital in Khartoum.

DNA preparation and genotyping

The PCR procedure was performed in the centre of Sudanese association for supporting patients with kidney disease.

Buffy coat was used for DNA extraction following the manufacturer's guide of QIAquick Gel Extraction Kit (1000) From QIAGEN (Manchester, United Kingdom). The MTHFR C677T single-nucleotide polymorphism (SNP) was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using the *Hinf* I restriction endonuclease enzyme (BioLabs, Frankfurt, Germany). The primers used were forward (5'- TGA AGG AGA AGG TGT CTG CGG GA -3'), and reverse, 5'- AGG ACG GTG CGG TGA GAG TG -3'). The PCR mixture used contained 18.5 µl 5xFIREPoL[®] Master Mix from SOLIS BIODYNE company, Tartu, Estonia, 5 µl of DNA template and 0.5µl of each primer were added.

PCR was performed under the following cycling conditions: 94°C for 3 min (initial denaturation), followed by 94°C for 1 min, 61°C for 1 min (annealing), 72°C for 2 min, and 72°C for 3 min (final extension), for a total of 40 cycles using MULTI GENE OPTI MAX from Cleaver Scientific Ltd (Rugby, United Kingdom).

PCR products were electrophoresed on agarose gel (2%, 0.5 mg/mL ethidium bromide) and visualized using Gel documentation system CSL-MICRODOC System from Cleaver Scientific (Rugby, United Kingdom).

PCR-RFLP was used to determine the MTHFR C677T genotypes. PCR products were subjected to overnight restriction digestion by *Hinf*I (5Units) at 37°C. Restriction products were electrophoresed on agarose gel (2%, 0.5 mg/mL ethidium bromide) and visualized as was the PCR product.

Presence of the C-allele resulted in no cleavage of the PCR product. Heterozygote C677T yielded two fragments of 198 bp, 175 bp. Homozygote C677T yielded two fragments of 175 bp (Figure 1).

Statistical analysis

The data collected was analyzed using Statistical Package for the Social Sciences (SPSS) 23. Categorical variables were expressed as numbers and percentages. Mann-Whitney-U test was used to compare differences between quantitative variables among the two groups and expressed as median and Interquartile Range (IQR), general linear model was used to determine the significant differences in homocysteine levels according to genotypes, chi square test was used to detect the association of MTHFR polymorphisms with CAD and logistic regression analyses was used to test the association between (selected significant independent variables) and CAD among T2DM patients, $p < 0.05$ was considered statistically significant.

Results

The clinical and biochemical characteristics of patients were summarized in Table 1.

Gender had no significant effect on CAD, $p > 0.05(0.3)$. Age, smoking, duration of diabetes and hypertension were significantly

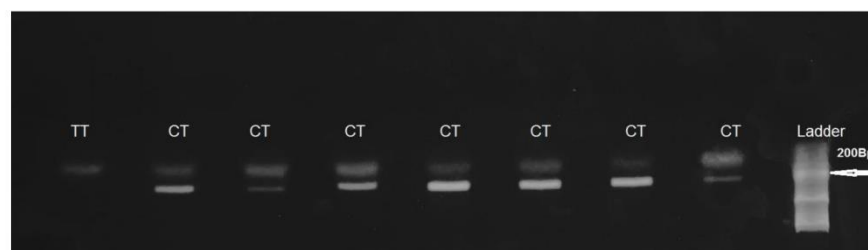


FIGURE 1
HinfI-treated MTHFR gene PCR fragments separated on 2% agarose gel. Presence of the C-allele resulted in no cleavage of the PCR product. Heterozygote C677T, (CT genotype) yielded two fragments of 198 bp and 175 bp. Homozygote C677T (TT genotype) yielded two fragments of 175 bp.

TABLE 1 Clinical and biological characteristics of diabetic patients with and without CAD.

Variables	T2DM with CAD (n=113)	T2DM without CAD (n=113)	p value
Sex (Male/female)	70/43	63/50	0.3
Smoking n (%)	37(33%)	14 (12%)	< 0.001
Age/year's	50 (42–55)	51(48–55)	0.02
Duration of Diabetes/Years	7 (3.0–10)	6 (1.0–8.0)	0.02
Duration of Hypertension/ Years	10 (6.0–12.0)	7 (5.0–10.0)	0.01
Cholesterol level	3.6 (2.9–4.9)	3.8 (3.3–4.3)	0.7
Triglyceride level	1.2 (1.01–1.5)	0.9 (0.8–1.7)	< 0.001
LDL Cholesterol	0.8 (0.7–1.1)	1.0 (1.23–1.1)	< 0.001
HDL cholesterol	0.9 (0.8–1.01)	1.1 (0.9–1.3)	< 0.001
Non-HDL cholesterol	2.5 (2.01–3.9)	2.5 (2.3–3.2)	0.5
HbA1c	75 (64–86)	75 (53–86)	0.4
Hcy level	13.5 (11.0–18.3)	10 (9.0–15)	< 0.001

All quantifiable parameters are reported as median and interquartile range (IQR). IQR is the distance between the 25th (Q1) and 75th (Q3) percentiles. Lipid levels are in millimoles per liter (mmol/L). HbA1c levels are in millimoles per mole (mmol/mol). Homocysteine levels are in micromoles per liter (μmol/L).

different between the two groups, $p < 0.02$, < 0.001 , 0.02 and 0.01 respectively, Diabetic patients with CAD have significantly higher levels of plasma triglycerides, LDL cholesterol and lower levels of HDL cholesterol, $p < 0.001$ but no difference noted in total cholesterol, and non-HDL cholesterol levels between the two groups, $p > 0.05$, 0.7 and 0.5 respectively. Plasma homocysteine levels were significantly higher in diabetic patients with P CAD, $p < 0.001$.

Association of MTHFR gene polymorphisms with PCAD in patients with T2DM

The frequencies of TT, CT and CC genotypes among patients with T2DM and CAD were 16, 40 and 44%, and were 00, 19 and 83% in T2DM patients without CAD, ($p < 0.001$). The frequency of the T allele was higher in patients with PCAD than without CAD (0.36 versus 0.08%, $p < 0.001$). The odds ratio (OR) for CAD in T2DM patients who carry the T allele was 6.2, CI 95% (3.4–11.6) (Table 2).

Correlation between MTHFR genotypes and plasma homocysteine levels

Plasma homocysteine levels were significantly different between MTHFR genotypes:

TABLE 2 Distribution of MTHFR genotypes and alleles in type 2 diabetic patients with and without CAD.

	T2DM with CAD (n=113)	T2DM without CAD (n=113)	p value
Genotypes			
CC	50 (44%)	94 (83%)	< 0.001
CT	45 (40%)	19 (17%)	
TT	18 (16%)	00(0%)	
Allele			
C	0.64	0.92	< 0.001
T	0.36	0.08	

C is the wild-type allele and T is the mutant allele.

CC genotype is homozygous for the wild-type C677T allele, CT genotype is heterozygous for the mutant 677T allele and TT genotype is homozygous for the mutant 677T allele.

16.2 ± 5.3 , 14.3 ± 5.7 and 12.9 ± 5.02 μmol/L in TT, CT and CC genotypes respectively, $p = 0.017$. *Post hoc* analysis showed significantly higher levels of homocysteine in the TT genotype than CC genotype, $p = 0.03$. Homocysteine levels showed significant association with CAD, $p < 0.001$, OR 3.2, 95% CI (1.9–5.5), (Table 3).

Logistic regression analysis

Logistic regression analysis was performed to ascertain the effects of selected significant risk factors in the study on likelihood that patients have PCAD.

It showed that age, duration of hypertension and duration of diabetes were not associated with PCAD, $p = 0.17$, 0.6 and 0.1 respectively, while other significant factors remained associated with PCAD (Table 4).

Discussion

Worldwide, the frequency of MTHFR gene mutations varies among racial and ethnic groups (28, 29). In Africa, MTHFR gene polymorphism is markedly low (below 10%) for 677 T allele (30, 31). In Europeans and North Americans, the frequency ranges from 10–18%, in contrast, the frequency of T allele is markedly high among Chinese populations at around 45% (32). In our study, the

TABLE 3 Effect of MTHFR genetic polymorphism on homocystiene levels.

Genotypes	Homocystiene Levels (μmol/L) ± SD	p. value
CC	12.9 ± 5.02	< 0.05 (0.017) *
CT	14.3 ± 5.7	
TT	16.2 ± 5.3	

*General linear model showed a significant difference in homocysteine levels between the three genotypes, $p = 0.017$, and post Hoc analysis showed a significant difference between CC and TT genotypes, $p = 0.03$.

TABLE 4 Logistic regression analysis of the significant risk factors for PCAD.

Factor	OR	95% CI	P value
LDL-cholesterol	1.7	1.6...2.9	0.018
Triglycerides	0.07	0.01...0.42	0.004
Hcy Level	0.6	0.5...0.8	0.03
T allele	0.19	0.08...0.32	0.02
HDL-cholesterol	1.2	0.96...3.0	0.04
Smoking	0.2	0.1...0.7	0.02
Duration of Hypertension	0.9	0.7...1.1	0.6
Age	0.96	0.95...1.2	0.17
Duration of Diabetes	0.9	0.85...1.0	0.1
MTHFR polymorphism (TT)	2.9	2.3...3.9	0.001

Only risk factors for PCAD which were significantly different between the two groups were included in the analysis. All these factors remained significant for PCAD, except for age, duration of hypertension and duration of diabetes, which have no significant association with PCAD in Logistic regression analysis.

mutant T allele frequency was 36% in cases and 8% in controls (Table 2). In a study done on Zambian population, the T allele frequency was 1%, and no subject had the TT genotype (31). However, it is known that Sudanese population have a high level of genetic diversity and therefore are unlikely to be representative of African populations south of the Sahara (33, 34).

The frequency of the T allele was significantly different between T2DM patients with and without CAD, $p < 0.001$. This finding was consistent with other findings done in diabetic patients from different populations (30, 35).

Elevated homocysteine level (hyperhomocysteinemia) seems to predict cardiovascular events among diabetic patients (6, 36–41). However, many other studies failed to establish the connection and claimed that hyperhomocysteinemia is an innocent bystander among diabetic patients (42, 43). The genetic basis of hyperhomocysteinemia has also been known (44, 45) and MTHFR polymorphism MTHFR 677T allele has been associated with high homocysteine levels (46–48), while others failed to detect such a relationship (49, 50). Ethnicity differences could be a responsible parameter for at least some of these controversial results (28, 29). Hcy levels are also influenced by various environmental factors and, vitamin B₁₂ and folate (51, 52). The limitations of this study include the lack of data on drug treatments, not measuring lipoprotein (a) and that folate status and vitamin B₁₂ levels were not measured.

Plasma homocysteine levels are influenced by age, diet, genetic background and the use of medications like metformin and fibrates which are used frequently in T2DM patients (53). In the presence of these confounding factors, a single test of plasma homocysteine, in a relatively small number of study subjects, cannot provide enough information for us to establish a causal relationship between homocysteine and PCAD. In addition, the participants in the study came from one city capital Khartoum and might not be

representative of the whole country. We therefore cannot recommend universal measurement of homocysteine levels in our T2DM patients especially that homocysteine test is relatively expensive for our patients who are mostly working class. However, health professionals are encouraged to advise patients with T2DM to increase intake of vegetables and fruits, within a balanced diabetic diet. Improvement of lifestyle factors like cessation of smoking, physical exercise and maintaining ideal body weight will decrease the overall risk for cardiovascular disease and may have a positive effect on plasma total homocysteine levels (54).

In conclusion, homocysteine levels are increased in our T2DM with PCAD compared to diabetic patients without CAD. The increase in the homocysteine levels is associated with T allele in MTHFR gene, presence of T allele will increase risk for CAD significantly.

Our study suggested that hyperhomocysteinemia is an important factor for PCAD in our patients with T2DM and more studies are required to confirm these findings and to study the effect of nutritional advice and supplementation with the relevant vitamins on homocysteine levels and prevention of PCAD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by The National Research Ethics Committee, Ministry of Health, Sudan. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BS: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. NM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Writing – original draft. IA: Methodology, Supervision, Validation, Writing – review & editing. BE: Methodology, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2.1.7. Estudio No.7



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A systematic review and meta-analysis expounding the relationship between methylene tetrahydrofolate reductase gene polymorphism and the risk of intracerebral hemorrhage among populations

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Background: The relationship between methylenetetrahydrofolate reductase (MTHFR) gene C677T and A1298C polymorphism with the risk of intracerebral hemorrhage (ICH) has remained to be controversial in recent years. This meta-analysis is aimed to confirm the association of these.

Methods: Systematically searching the related studies from the PubMed, Embase, Cochrane Library, China national knowledge internet database from 1 January 1990 to 1 June 2022. The odd ratio (ORs) and 95% confidence interval (CIs) of gene-disease correlation in various gene models were calculated by fixed or random effect model of meta-analysis. We included 20 case-control studies in this meta-analysis with a total of 1,989 ICH patients and 4,032 health controls originated from Asian, Caucasian, and African populations.

Results: The statistical analysis demonstrated the association of MTHFR C677T gene polymorphism with ICH in allele model [OR_{T vs. C} = 1.20 (95%CI: 1.06–1.36)]; homozygote model [OR_{TT vs. CC} = 1.50 (95%CI: 1.20–1.88)]; dominant model [OR_{CT+TT vs. CC} = 1.23 (95%CI: 1.03–1.48)] and recessive model [OR_{TT vs. CT+CC} = 1.37 (95%CI: 1.17–1.60)]. Besides, we also found the relationship of MTHFR C677T gene polymorphism with Asian in four comparison model (OR_{T vs. C} = 1.19, 95%CI: 1.09–1.37, OR_{TT vs. CC} = 1.46, 95%CI: 1.15–1.85, OR_{CT+TT vs. CC} = 1.25, 95%CI: 1.01–1.54, OR_{TT vs. CT+CC} = 1.34, 95%CI: 1.54–1.17) and Caucasian in four comparison model (OR_{T vs. C} = 1.90, 95%CI: 1.22–2.97, OR_{TT vs. CC} = 2.67, 95%CI: 1.42–5.00, OR_{CT+TT vs. CC} =

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; ICH, intracerebral hemorrhage; OR, odd ratio; CI, confidence interval; CNKI, China national knowledge internet; HWE, hardy weinberg equilibrium; REM, random effect model; FEM, fixed effect model.

1.56.95%CI: 1.05–2.32, $OR_{TT \text{ vs. } CT+CC} = 2.25.95\%CI: 1.46–4.00$). But no statistically significant correlation between A1298C polymorphism and the occurrence of ICH was detected in four studies.

Conclusion: MTHFR C677T gene polymorphism increases the risk of ICH in Asian and Caucasian populations but has no impact on the incidence in African communities. More importantly, the risk of ICH increases in TT genotype individuals in comparison to CT and CC genotype individuals in Asian and Caucasian populations.

KEYWORDS

methylenetetrahydrofolate reductase, C677T, A1298C, gene polymorphism, intracerebral hemorrhage, meta-analysis

Introduction

ICH is a common acute cerebrovascular disease with much higher mortality rate than that of ischemic stroke (van Asch et al., 2010; Krishnamurthi et al., 2013; Krishnamurthi et al., 2020), accounted for 10–30% of strokes with incidence about 24/100,000 people per year (van Asch et al., 2010; Krishnamurthi et al., 2020) and prevalence about 300/100,000 people per year (Krishnamurthi et al., 2020). ICH is a serious threat to physical and mental health which brings huge economic burden to healthcare systems across human societies (Feigin et al., 2009; Krishnamurthi et al., 2013). Generally accepted risk factors for ICH include hypertension, use of anticoagulants or antiplatelet agents, cerebral arteriovenous malformations, cerebral amyloidosis, smoking, excessive drinking, and other environmental and generic factors (An et al., 2017). Commensurate with its implication in health and economy, more and more studies on the occurrence mechanism of ICH have shown that genes play a very important role in the pathogenesis of ICH (Chen et al., 2018; Wahab et al., 2019). However, the effect of gene polymorphism on ICH remains controversial.

MTHFR is an important enzyme in the regulation of plasma homocysteine level. Under normal physiological conditions, MTHFR catalyzes the reduction of 5, 10-methylene tetrahydrofolic acid to 5-methyl tetrahydrofolic acid and the resulting 5-methylenetetrahydrofolate is a source of methyl for the conversion of homocysteine to methionine (Goyette et al., 1994; Ogino and Wilson, 2003). The encoded gene of MTHFR is located at 1p36.3. MTHFR C677T gene polymorphism causes valine to be replaced by alanine, leading to the decrease of MTHFR activity and the increase of plasma homocysteine concentration (Munshi et al., 2008; Zhu et al., 2015; Chen et al., 2018). High plasma homocysteine concentration in humans contributes to accelerated atherosclerosis, as well as excessive inflammation, long-term endothelial pressure, and increased plaque rupture, all of which increase susceptibility to ICH (Lai and Kan, 2015; Vacek et al., 2015; Zhou et al., 2018). In addition, previous studies have indicated that

hyperhomocysteinemia is a risk factor for coronary artery disease, peripheral vascular disease, venous thrombosis and other vascular diseases (Veeranna et al., 2011; Vacek et al., 2015; Zhu et al., 2015; Zhou et al., 2018). In addition to C677T, A1298C is another common gene polymorphism in MTHFR with locus at rs1801131. The main pathological change of exon seven is the replacement of glutamate by alanine, which leads to the decrease of MTHFR activity in human body. In comparison to the C677T gene polymorphism, the MTHFR activity resulting from A1298C polymorphism is relatively higher (Viel et al., 1997; Weisberg et al., 1998).

Previously, some meta-analyses explored the relationship between MTHFR C677T or A1298C gene polymorphism and ischemic stroke, hemorrhagic stroke or coronary artery disease (Lv et al., 2013; Luo et al., 2018; Wang et al., 2021). However, there are only few meta-analyses to explore the relationship between MTHFR C677T and A1298C gene polymorphisms and ICH. Moreover, with the continuous emergence of emerging studies, there are some disputes between MTHFR C677T gene polymorphism and ICH. Recent studies in Morocco (Abidi et al., 2018), India (Somarajan et al., 2011; Sagar et al., 2018) and other countries reported contradictory results that the genetic MTHFR C677T polymorphism is not related to ICH. Interestingly, a Zambian study showed no C677TTT genotype in their population (Atadzhazov et al., 2013). Related to C677T polymorphism, significant association between the A1298C polymorphism of MTHFR and the risk of ischemic stroke in Asian population (Kang et al., 2014; Zhang et al., 2014; Kumar et al., 2020) has also been reported although no correlation studies on the susceptibility of A1298C polymorphism to ICH has been published. Therefore, we included emerging studies to update the relationship between MTHFR C677T gene polymorphism and intracerebral hemorrhage, and analyzed the impact of MTHFR C677T gene polymorphism on intracerebral hemorrhage among different populations and different regions. Besides, we also explore the relationship between MTHFR A1298C gene polymorphism with intracerebral hemorrhage.

Methods

Database search

This systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement guidelines. We systematically searched the studies published in PubMed, Embase, Cochrane Library, China national knowledge internet (CNKI) and other databases from 1 January 1990 to 1 June 2022 regarding the correlation between MTHFR C677T and A1298C polymorphism and ICH. The retrieval strategies: “methylene tetrahydrofolate reductase” or “methyl tetrahydrofolate reductase” or “MTHFR” and “polymorphism” or “mutation” or “genotype” or “A1298C” or “C677T” and “cerebral hemorrhage” or “intracerebral hemorrhage” or “hemorrhagic stroke” or “ICH” or “cerebrovascular disease” were executed for the search. The research population is limited to “human”. And the publication language is only allowed to include Chinese and English. Moreover, manual searches of the reference lists of retrieved study, review articles, and previous meta-analyses were performed to collect more relevant studies that were omitted during electronic database retrieval. Our review of literature inclusion consists of three main steps, first on the title of the study, then on the abstract, and finally on the complete text. If there is a dispute, discuss it in depth. As all data in this study are from published studies, no additional ethical approval is required.

Eligibility criteria

Inclusion criteria: 1) a case-control study investigating the correlation between MTHFR C677T or A1298C gene polymorphism and ICH; 2) ICH was clinically confirmed by clinical and computed tomography or magnetic resonance imaging scans; 3) Genotype frequencies are available to estimate the odds ratio of the 95% confidence interval. Exclusion criteria were: 1) Duplicate publications with overlapping topics in the same study; 2) No available data were reported; 3) low-quality studies with a quality score (Newcastle-Ottawa Scale) below 4.

Data extraction

According to the above inclusion and exclusion criteria, two investigators independently extracted data from the included literature. The extracted information includes the first author's name, published journal, published year, country, race, an average age of population, number of case group and control group, genotyping method, A1298C and C677T genotype distribution and allele frequency. The allele frequency distribution was calculated by Hardy Weinberg equilibrium (HWE) (Wang and Shete,

2017) in studies which did not provide allele frequency information.

Quality assessment

The quality of the included study was evaluated by Newcastle-Ottawa Scale (Stang, 2010) and was assessed from three aspects: population selection, comparability between groups, and measurement of exposure factors. The score ranged from 0 to 9, a score of 0–3 is considered as in poor quality, and a score of seven or above is considered as in high quality. Investigators strictly control the quality of articles included.

Statistical analysis

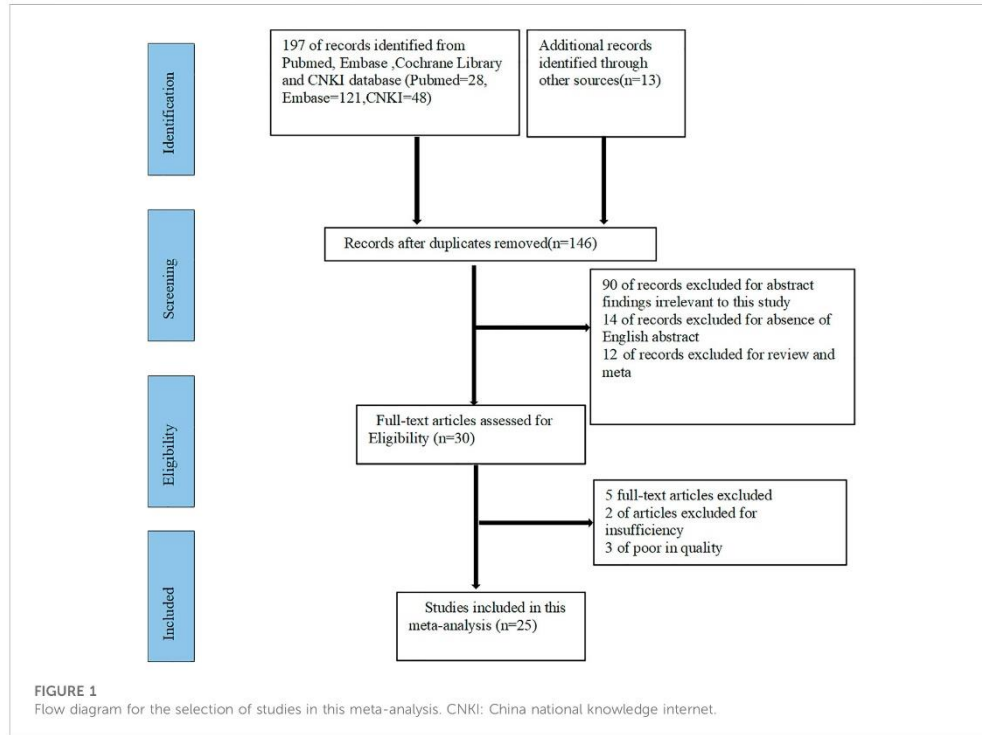
We assessed the association between the allelic model (T versus C), homozygous model (TT versus CC), heterozygous model (CT versus CC), recessive model (TT versus TC + CC), and dominant model (TC + TT versus CC) alleles of MTHFR C677T and A1298C and the hazard of ICH. Using a fixed effect model (Mantel-Haenszel method) or random effect model (DerSimonian and Laird's method) to calculate the odds ratio (ORs) of 95%CI (Mantel and Haenszel, 1959; DerSimonian, 1996). Heterogeneity between studies was compared using Cochran's Q statistics and I^2 measures (Higgins and Thompson, 2002; Higgins et al., 2003). I^2 was used to assess the degree of heterogeneity between the included studies, in which 0–25% indicated no heterogeneity, a higher value indicated increased heterogeneity, 25–50% was considered as low, 50–75% medium, and 75–100% high. If the heterogeneity is high, we will conduct subgroup analysis and meta regression to analyze the source of heterogeneity.

In order to verify the reliability of the results of the meta-analysis, sensitivity analysis was performed by case-by-case exclusion (Tobias, 1999). Furthermore, we conducted HWE in the control group and observed changes in sensitivity by excluding studies that did not conform to HWE. In terms of publication bias, the Begg funnel graph was used for evaluation and the Egger graph for verification (Egger et al., 1997). All statistical analyses were performed with STATA 14.0, and all p values were bilateral. When $p < 0.05$, it was considered statistically significant.

Results

Literature search

146 studies were preliminarily searched in PubMed, Embase, Cochrane Library and CNKI databases. After screening, we excluded 90 studies that were not strongly relevant to this study, 14 studies that did not have abstracts in English, and 12 meta-analyses and reviews. Then thirty studies were left.



Furthermore, we conducted an in-depth review of the full text of the 30 studies, excluding five of them with insufficient information or low quality. Finally, 25 studies were included for this meta-analysis (as showed in Figure 1).

Study characteristics

The basic features of the 25 case-control studies (Nakata et al., 1998; Zheng et al., 2000; Yingdong et al., 2002; Li et al., 2003; Zhang et al., 2004; Yan et al., 2004; Fang and Wu, 2004; Ye et al., 2004; Żur-Wyrozumska et al., 2017; Fang et al., 2005; Dikmen et al., 2006; Sazci et al., 2006; Hu et al., 2007; Shen et al., 2007; Zhang et al., 2008; Ruigrok et al., 2010; Hultdin et al., 2011; Somarajan et al., 2011; Atadzhanov et al., 2013; Rui-Rui et al., 2013; Das et al., 2015; Shao et al., 2016; Hu et al., 2016; Abidi et al., 2018; Jiang et al., 2018) included are shown in Table 1. The incorporated studies ranged from 1998 to 2018, involving mainly Asian populations and some European and African communities. Three different races were involved

in these 25 selected studies: two (Atadzhanov et al., 2013; Abidi et al., 2018) African, three (Dikmen et al., 2006; Sazci et al., 2006; Hultdin et al., 2011) Caucasian and the remaining 17 (Nakata et al., 1998; Zheng et al., 2000; Yingdong et al., 2002; Zhang et al., 2004; Yan et al., 2004; Fang and Wu, 2004; Ye et al., 2004; Żur-Wyrozumska et al., 2017; Hu et al., 2007; Zhang et al., 2008; Rui-Rui et al., 2013; Shao et al., 2016; Hu et al., 2016; Jiang et al., 2018) Asian. The control population in three of the 25 studies (Li et al., 2003; Shen et al., 2007; Das et al., 2015) did not meet Hardy-Weinberg equilibrium and be excluded. One study due to high heterogeneity (Fang et al., 2005) and one study of rebleeding after secondary subarachnoid hemorrhage (Ruigrok et al., 2010) were excluded (Table 2). All the 20 included studies had moderately high-quality scores. In four of the 20 studies (Dikmen et al., 2006; Sazci et al., 2006; Hultdin et al., 2011; Abidi et al., 2018), A1298C and C677T gene polymorphism and ICH were simultaneously studied, and a total of 3,280 patients with ICH and 9,324 controls were included in this meta-analysis.

TABLE 1 The characteristics of all studies in the meta-analysis of the association between MTHFR gene C677T and A1298C polymorphisms with the risk of intracerebral hemorrhage. PCR-RFLP = Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. SNP = Single Nucleotide Polymorphism. HWE = Hardy Weinberg Equilibrium. QS = Quality score.

Author	Year	Origin	Ethnicity	Size (Case/Control)	Mutation	Technology	HWE	QS
Nakata Y Nakata et al., 1998	1998	Japan	Asian	35/105	C677T	PCR-FRLP	Yes	5
Zheng Y Z Zheng et al., 2000	2000	China	Asian	30/122	C677T	PCR-FRLP	Yes	5
Zhao Y Żur-Wyrozumska et al., 2017	2001	China	Asian	202/190	C677T	PCR	Yes	4
Fang L Fang and Wu, (2004)	2004	China	Asian	27/96	C677T	PCR-FRLP	Yes	4
Zhang J Zhang et al., 2004	2004	China	Asian	94/100	C677T	PCR-FRLP	Yes	5
Zhang Y Yan et al., 2004	2004	China	Asian	156/239	C677T	PCR-FRLP	Yes	4
Ye H Ye et al., 2004	2004	China	Asian	100/300	C677T	PCR-FRLP	Yes	5
Fu Y Yingdong et al., 2002	2005	China	Asian	26/29	C677T	PCR-RFLP.	Yes	4
Sazci A Sazci et al., 2006	2006	Turkey	Caucasian	28/259	C677T	PCR-RFLP	Yes	5
Dikmen M Dikmen et al., 2006	2006	Turkey	Caucasian	49/55	A1298C	PCR-FRLP	Yes	6
					A1298C			
Hu RL Hu et al., 2007	2007	China	Asian	32/115	C677T	Sequenom	Yes	5
Zhang Y Zhang et al., 2008	2008	China	Asian	222/282	C677T	PCR-FRLP	Yes	4
Somarajan BI Somarajan et al., 2011	2011	India	Asian	215/188	C677T	PCR	Yes	6
Hultdin J Hultdin et al., 2011	2011	Sweden	Caucasian	60/778	C677T	PCR-FRLP	Yes	7
					A1298C			
Atadzhanov M Atadzhanov et al., 2013	2013	Zambian	African	18/116	C677T	TaqMan	Yes	5
Rui-Rui L I Rui-Rui et al., 2013	2013	China	Asian	182/200	C677T	PCR-RFLP	Yes	5
Shao D (Shao et al., 2016)	2015	China	Asian	96/206	C677T	PCR-FRLP	Yes	4
Hu X Hu et al., 2016	2016	China	Asian	180/180	C677T	PCR-RFLP.	Yes	5
Abidi O Abidi et al., 2018	2018	Morocca-n	African	113/323	C677T	PCR-PFLP	Yes	6
					A1298C			
Jiang D Jiang et al., (2018)	2018	China	Asian	124/149	C677T	SNa Pshot	Yes	5

TABLE 2 Information for some strong correlation research not included in the data collection process.

Author	Year	Origin	Ethnicity	Mutation	HWE	Delete reason
Zhaohui Li (Li et al., 2003)	2003	China	Asian	C677T	No	HWE not satisfied
Fang X (Fang et al., 2005)	2005	Japan	Asian	C677T	Yes	Large heterogeneity
Shen C D (Shen et al., 2007)	2007	China	Asian	C677T	No	HWE not satisfied
Ruigrok YM (Ruigrok et al., 2010)	2010	Dutch	Caucasian	C677T	Yes	Secondary to SAH, does not meet the requirements
Das (Das et al., 2015)	2015	India	Asian	C677T	NO	HWE not satisfied
Wenjing Ou (Ou et al., 2014)	2014	China	Asian	C677T	Yes	Unable to obtain sufficient raw data
Cai (Wang et al., 2021)	2005	China	Asian	C677T	NO	Unable to obtain sufficient raw data
Xiao (Wang et al., 2021)	2006	China	Asian	C677T	NO	Unable to obtain sufficient raw data
Sagar R (Sagar et al., 2018)	2018	India	Asian	C677T	NA	Unable to obtain sufficient raw data

The association of methylenetetrahydrofolate reductase C677T gene polymorphism with the risk of intracerebral hemorrhage

The forest plot of the relationship between MTHFR C667T gene polymorphism and ICH in various gene models were

demonstrated in Figure 2 and Supplementary Figure S1 with the OR and *p*-values of each gene model shown in Table 3. In each gene model, a significant association was observed between MTHFR C677T gene polymorphism and the risk of ICH. The results of subgroup analysis by ethnicity varied with the risk of ICH in the study populations. MTHFR C677T gene polymorphism in the dominant model [OR_{CT+TT} vs. CC =

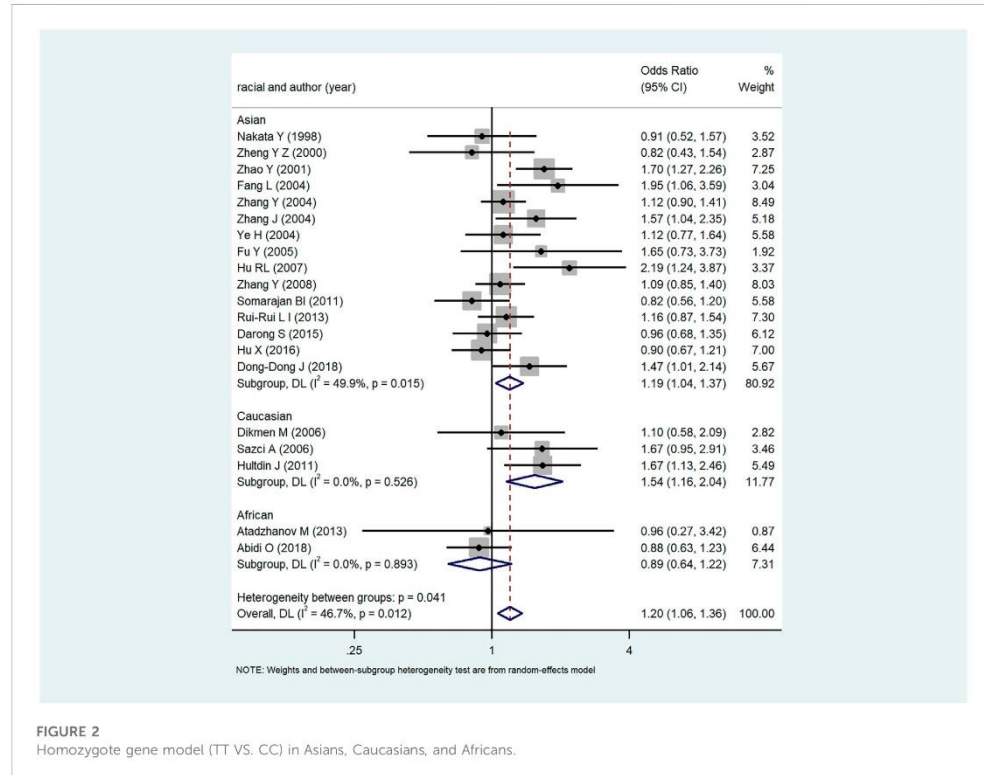


FIGURE 2
Homozygote gene model (TT VS. CC) in Asians, Caucasians, and Africans.

1.23 (95%CI: 1.03–1.48)], allele model [OR = 1.20 (95%CI: 1.06–1.36)], homozygous model [OR = 1.50 (95%CI: 1.20–1.88)] and recessive model [OR = 1.37 (95%CI: 1.17–1.60)] all revealed significant association with the risk of ICH. In addition, four comparison model ($OR_{TT \text{ vs. } C} = 1.19$, 95%CI: 1.09–1.37, $OR_{TT \text{ vs. } CC} = 1.46$, 95%CI: 1.15–1.85, $OR_{CT+TT \text{ vs. } CC} = 1.25$, 95%CI: 1.01–1.54, $OR_{TT \text{ vs. } CT+CC} = 1.34$, 95%CI: 1.54–1.17) also significantly related to Asian population. Likewise, in Caucasian population, allele model [(OR = 1.90 (95%CI: 1.22–2.97)] homozygous model [OR = 2.67 (95%CI: 1.42–5.00)], recessive model [OR = 2.25 (95%CI: 1.46–4.00)] and dominant model [OR $_{CT+TT \text{ vs. } CC} = 1.56$, 95%CI: 1.05–2.32] showed significant association with the risk of ICH, while the association in heterozygous model [OR = 1.39 (95%CI: 0.91–2.13)], were not evidenced. In African population, no substantial correlation between MTHFR C667T gene polymorphism and ICH was observed in any one of the five models with heterozygote model [OR = 0.86 (95%CI: 0.56–1.32)], dominant model [OR = 0.85 (95%CI: 0.57–1.28)], allele model

[OR = 0.89 (95%CI: 0.64–1.22)], homozygous model [OR = 0.82 (95%CI: 0.39–1.73)] and recessive model [OR = 0.89 (95%CI: 0.43–1.81)].

Publication bias

In this paper, Begg and Egger funnel plots were applied to assess the publication bias of the 20 included studies. No significant asymmetry was observed in any gene model by either of our two researchers (Figure 3 and Supplementary Figure S2). The p -values of Begg and Egger's test in all the five gene models were all over 0.05 with the dominant model ($P_{Begg} = 0.098$, $P_{Egger} = 0.149$), homozygous model ($P_{Begg} = 0.294$, $P_{Egger} = 0.183$), heterozygous model ($P_{Begg} = 0.098$, $P_{Egger} = 0.200$), allelic model ($P_{Begg} = 0.381$, $P_{Egger} = 0.398$), recessive model ($P_{Begg} = 0.162$, $P_{Egger} = 0.264$), which confirmed the absence of obvious publication bias in the 20 included studies.

TABLE 3 Results of association between C677T and A1298C with intracerebral hemorrhage in this meta-analysis.

	Comparison	Number of studies	p	I ² (%)	OR (95%CI)	Comparison Model
C677T overall						
	T VS C	20	0.012	46.7	1.20 (1.06–1.36)	REM
	TT VS CC	20	0.097	31.1	1.50 (1.20–1.88)	REM
	CT VS CC	20	0.256	15.9	1.09 (0.94–1.26)	REM
	CT+TT VS CC	20	0.006	49.4	1.23 (1.03–1.48)	REM
	TT VS CC+CT	20	0.398	4.80	1.37 (1.17–1.60)	REM
Asian						
	T VS C	15	0.015	49.9	1.19 (1.09–1.37)	REM
	TT VS CC	15	0.134	29.6	1.46 (1.15–1.85)	REM
	CT VS CC	15	0.212	21.8	1.08 (0.92–1.28)	REM
	CT+TT VS CC	15	0.04	54.9	1.25 (1.01–1.54)	REM
	TT VS CC+CT	15	0.461	0	1.34 (1.54–1.17)	REM
Caucasian						
	T VS C	3	0.526	0	1.90 (1.22–2.97)	REM
	TT VS CC	3	0.842	0	2.67 (1.42–5.00)	REM
	CT VS CC	3	0.352	4.20	1.39 (0.91–2.13)	REM
	CT+TT VS CC	3	0.406	0	1.56(1.05–2.32)	REM
	TT VS CC+CT	3	0.693	0	2.25 (1.46–4.00)	REM
African						
	T VS C	2	0.893	0	0.89 (0.64–1.22)	REM
	TT VS CC	2	...	0	0.82 (0.39–1.73)	REM
	CT VS CC	2	0.86	0	0.86 (0.56–1.32)	REM
	CT+TT VS CC	2	0.853	0	0.85 (0.57–1.28)	REM
	TT VS CC+CT	2	...	0	0.89 (0.43–1.81)	REM
A1298C						
	C VS A	4	0.452	0	0.86 (0.69–1.09)	FEM
	CC VS AA	4	0.084	54.9	0.72 (0.24–2.18)	REM
	AC VS AA	4	0.652	0	0.94 (0.70–1.26)	FEM
	CC+AC VS AA	4	0.810	0	0.89 (0.67–1.18)	FEM
	CC VS AC+AA	4	0.037	64.5	0.79 (0.23–2.65)	REM

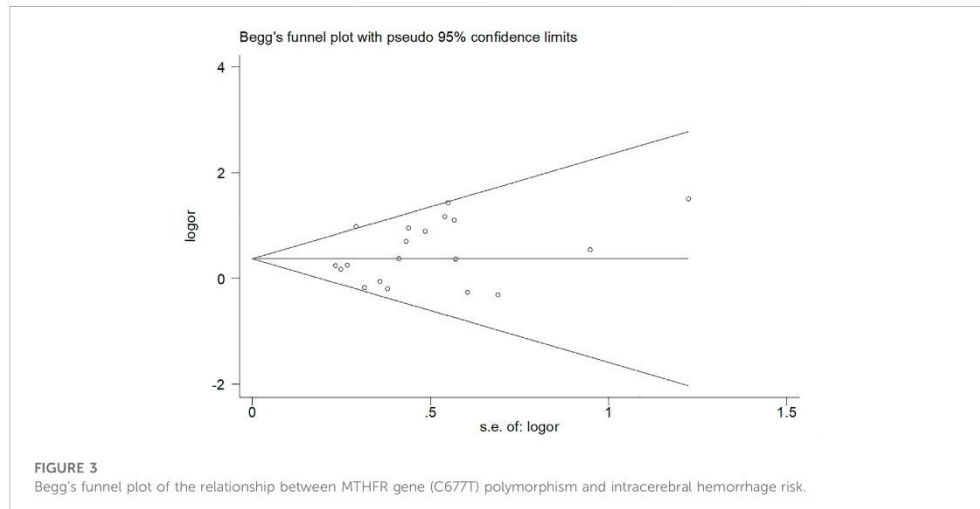
REM: random effect model.

Sensitivity analyses

A sensitivity analysis of the 20 included studies was performed to assess the impact of each study on ORs inclusion by sequentially omitting individual inclusion studies. No significant change in OR value was observed in the process of removing the study one by one. Therefore, overall sensitivity analysis reinforced that the results of this meta-analysis are statistically reliable. The correlation of MTHFR gene C677T polymorphism with the risk of ICH in this meta-analysis was validated by the following meta regression analysis of the selected 21 studies, i.e., published year ($p = 0.086$), the quality score ($p = 0.795$), race [Caucasian populations ($p = 0.100$), African populations ($p = 0.243$), the Asian population ($p = 0.931$)] as a covariate meta regression analysis, with all the p -values above 0.05 which indicated no statistically significant. And the subgroup analysis did not find obvious source of heterogeneity.

The association of methylenetetrahydrofolate reductase A1298C gene polymorphism with the risk of intracerebral hemorrhage

At present, there are still few studies on the relationship between MTHFR A1298C gene polymorphism and the risk of ICH only in Caucasian and African populations. Therefore, only four related studies (Dikmen et al., 2006; Sazci et al., 2006; Hultdin et al., 2011; Abidi et al., 2018) were included in the current paper. The meta-analysis results of the four studies were shown in Table 3 and Supplementary Figure S3 with corresponding OR and 95%CI for the five gene models as follows—heterozygous model [OR = 0.94 (95%CI: 0.7–1.26)], dominant model [OR = 0.87 (95%CI: 0.67–1.18), homozygous model [OR = 0.72 (95%CI: 0.24–2.18), recessive model [OR = 0.64 (95%CI: 0.34–1.2)] and allele model [OR = 0.86 (95%CI: 0.69–1.09)]. Hence, no significant correlation between MTHFR



A1298C allele polymorphism and ICH was detected in any one of the five gene models in Caucasian and African communities. Furthermore, no obvious publication bias was found in Begg funnel chart (Supplementary Figure S4). Considering the absence of adequate studies, sensitivity analysis, subgroup analysis or meta-regression was not conducted.

Discussion

In this meta-analysis, we included a total of 20 case-control studies, all 20 of which examined the correlation of the polymorphism of the MTHFR C677T gene with the risk of ICH (including 1,989 cases of ICH patients and 4,032 controls), while only four of the 20 studies interrogated the relationship of MTHFR A1298C gene polymorphism with the risk of ICH (with a total of 250 cases of ICH and 1,415 controls included). The results of the meta-analysis revealed significant association between MTHFR C677T gene polymorphism and risk of ICH under all the four genetic models in Asian populations. Funnel figure, subgroup analysis and sensitivity analysis all confirmed the reliability of the results. A subgroup analysis and a meta regression was performed to analyze the source of the heterogeneity, but no covariate was found to attribute to the source of heterogeneity.

Subgroup analysis based on ethnic classification uncovered a strong association between MTHFR C677T gene polymorphism and the risk of ICH in Asian, and Caucasians populations, while no association was found in Africans. What's more, compared with genotype CC or, CT, TT

genotype substantially increases the susceptibility to ICH with $OR_{TT \text{ vs. } CC} = 1.50$ (95%CI: 1.20–1.88) and $OR_{TT \text{ vs. } CT+CC} = 1.37$ (95%CI: 1.17–1.60). Considering the influence of MTHFR C677T gene polymorphism on homocysteine level, individuals with TT genotype have a higher risk of ICH which may be related to the significantly increased level of homocysteine. The revealed correlation provides a logic scientific basis for further in-depth study of gene polymorphism and molecular epidemiology of ICH.

The T-allele of methylene tetrahydrofolate reductase C677T can increase homocysteine levels in human body to a mild to moderate level (Cronin et al., 2005; Holmes et al., 2011). Artificially induced hyperhomocysteinemia mice were significantly more vulnerable to vascular inflammation, atherosclerosis, and hypercoagulability (Hofmann et al., 2001; Zhou et al., 2008; Veeranna et al., 2011; Vacek et al., 2015; Zhou et al., 2018). Likewise, MTHFR gene polymorphism also increases the risk of ICH by affecting the blood clotting function *via* hyperhomocysteinemia which accelerates the activation of coagulation factors V, X, and XII and rises the risk of arterial thrombosis and atherosclerotic cerebrovascular diseases of large and small arteries (Diaz-Arrastia, 2000; Kelly et al., 2002; Dikmen et al., 2006). Thinking on, investigations on reduction the level of homocysteine *via* intentionally ingestion of additional folic acid to mitigate the risk of ICH and coronary heart disease have been implemented. However, the clinical efficacy of folic acid on alleviation of ICH risk remains contentious (Lewis et al., 2005; Aléssio et al., 2011). Considering the common occurrence of T allele of C677T in Asian and Caucasian populations, the importance of correlation

investigations for the prediction, prevention and treatment of ICH in these populations cannot be overlooked.

The current and previous studies (Gao et al., 2012; Kang et al., 2013; Zhao and Jiang, 2013; Kang et al., 2014) on the association between MTHFR gene polymorphism and the risk of ICH were mainly concentrated on C677T polymorphism in Asian populations. No research on the influence of A1298C polymorphism on the danger of ICH has been published in Asian populations. Consequently, only four studies were included in this paper involving the association of MTHFR A1298C polymorphism with ICH in Caucasian and African populations. Considering the potential correlation of A1298C gene polymorphism with the susceptibility to ICH, further studies are warranted, especially for the Asian populations with high frequency of occurrence.

Compared with the previous meta-analysis (Gao S, et al., 2012), which only focused on MTHFR C677T, this systematic review and meta-analysis analyzed the relationship between MTHFR A1298C gene polymorphism and intracerebral hemorrhage. We also included emerging studies to update the relationship between MTHFR C677T gene polymorphism and intracerebral hemorrhage, and analyzed the impact of MTHFR C677T gene polymorphism on intracerebral hemorrhage among different populations and different regions. In our study, we found that MTHFR C677T gene polymorphism increases the risk of ICH in Asian and Caucasian populations but has no impact on the incidence in African communities. More importantly, the risk of ICH increases in TT genotype individuals in comparison to CT and CC genotype individuals in Asian populations. Therefore, our study not only revealed the important role of MTHFR A1298C in the prevention and treatment of ICH, but also indicated that the populational specific strategies for ICH prevention *via* MTHFR C677T and A1298C should be considered in Asians and Caucasians instead of Africans firstly.

Due to availability of data, there are several deficiencies in this research. First of all, only a small number of studies were included, mainly on A1298C genotype, which would affect the representativeness of the results of the meta-analysis in this part. Although we searched a large number of databases, only four relevant literatures were found. Secondly, all the included studies were case-control studies, which were basically retrospective, and there was probable bias caused by partial design when comparing with prospective studies. Moreover, some gene models showed moderate to low degree of heterogeneity, but meta-regression and subgroup analysis based on publication year, ethnicity, and quality score could not designate the source of heterogeneity, which may be related to study design, genetic and environmental interaction, and the number of included patients. Finally, we have not registered this search protocol in an online database such as PROSPERO.

In summary, this meta-analysis confirmed that MTHFR C677T gene polymorphism is related to the risk of ICH,

mainly in Asian populations, and that TT genotype individuals have a higher risk of ICH than CC and CT genotypes. No substantial correlation of MTHFR A1298C polymorphisms with the risk of ICH were found. The high incidence of ICH in Asian population may be related to the polymorphism of MTHFR C677T gene, which may be a predictor for the susceptibility of ICH in Asian. The detection of MTHFR C677T gene polymorphism in clinical will help to predict, prevent and reduce the pathological cases of cerebral hemorrhage in Asian population. Further studies are needed to clarify the prevention of ICH in Asian population targeting MTHFR C677T gene polymorphism.

Main messages

- 1) MTHFR is an important enzyme in the regulation of plasma homocysteine level. High plasma homocysteine concentration in humans contributes to increase susceptibility to ICH.
- 2) MTHFR C677T gene polymorphism is a biomarker gene of ICH in Asian and Caucasian populations, which has guiding significance for the prevention of ICH.
- 3) The risk of ICH increases in C677TTT genotype individuals in comparison to CT and CC genotype individuals in Asian and Caucasian populations.

Current research questions

- 1) Are C677T and A1298C gene polymorphisms of MTHFR associated with the risk of ICH?
- 2) Is there population difference in the effect of MTHFR gene polymorphism on the risk of ICH?
- 3) Are the different alleles of C677T and A1298C responsible for the difference in the risk of ICH in different populations? Can these allele differences become biomarkers for the prevention and treatment of ICH?
- 4) What is the potential mechanism of MTHFR C677T and A1298C gene polymorphisms on ICH?

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author Contributions

LZ and X-LZ designed the research and determined the structure of the paper. X-LZ, T-XY, LD, LC, and YL selected the references and contributed to the writing. X-LZ and T-XY

collected the data. X-LZ, T-XY, and LZ helped to analyze the results of this meta-analysis. LZ contributed to the revision and finalization of the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2022.829672/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

Forest plot of the association between MTHFR C677T gene polymorphism and the risk of ICH in Asians, Caucasians, and Africans. (A) Allele model (T VS C) in Asians, Caucasians, and Africans. T and C are the case group and T1 and C1 are the control group. (B) Recessive gene model (TT VS CC+CT) in Asians, Caucasians, and Africans. where R=CC+CT, R1=CT1+CC1, R and CC are the case group, and R1 and CC1 are the control group. (C) Dominant gene model (TT+CT VS CC) in Asians, Caucasians, and Africans, in which TT and CC are case groups and TT1 and CC1 are control groups. (D) Heterozygote gene model (CT VS CC) in Asians, Caucasians, and Africans, in which CT and CC are case groups and CT1 and CC1 are control groups.

SUPPLEMENTARY FIGURE S2

Sensitivity analysis of MTHFR C677T polymorphism and risk of ICH in a dominant gene model.

SUPPLEMENTARY FIGURE S3

Forest plot of the relationship between MTHFR A1298C gene polymorphism and the risk of ICH. (A) Allele model (C VS A), where C and A are case groups and C1 and A1 are control groups. (B) Homozygous model (CC VS AA), in which CC and AA are case groups and CC1 and AA1 are control groups. (C) Dominant model (CC+AC VS AA), where F=CC+AC, F1=CC1+AC1 and AA are case groups, and F1 and AA1 are control groups. (D) Recessive model (CCVS AA+AC), where B=AA+AC, B1=AA1+AC1, AA is the case group, and B1, AA1 are the control group. (E) Heterozygous model (AC VS AA), in which AC and AA are case groups and AC1 and AA1 are control groups.

SUPPLEMENTARY FIGURE S4

The Begg funnel plot of the relationship between MTHFR A1298C gene polymorphism and the risk of ICH.

SUPPLEMENTARY TABLE S1

Differences between other meta-analyses similar to this study.

SUPPLEMENTARY TABLE S2

Information for some strong correlation research not included in the data collection process.

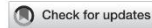
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2.1.8. Estudio No.8



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Association of methylentetrahydrofolate reductase (MTHFR) rs1801133 (677C>T) gene polymorphism with ischemic stroke risk in different populations: An updated meta-analysis

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Background: Recently, increasing evidence has implicated methylentetrahydrofolate reductase (MTHFR) gene mutation as a risk factor for ischemic stroke (IS) in the general population. However, studies have been inconclusive and lack evidence on specific populations. We aim to determine whether the rs1801133 (NC_000001.11 (MTHFR):g. 677C>T (p.Ala222Val) variant, we termed as MTHFR rs1801133 (677C>T), is linked to an increased risk of IS in different age groups and ancestry groups.

Methods: The literature relevant to our study was found by searching the PubMed, Cochrane Library, Web of Science, EMBASE, and CNKI databases. A random effect model analysis was used to calculate the pooled odds ratio (OR) and 95% confidence interval (CI) to evaluate any possible association. We conducted a subgroup analysis based on the age and ancestry groups of the included populations.

Results: As of March 2022, 1,925 citations had been identified in electronic databases, of which 96 studies involving 34,814 subjects met our eligibility criteria. A strong link was found between IS and the MTHFR gene rs1801133 (677C>T) polymorphism in all genetic models [dominant genetic model (OR = 1.47; 95%CI = 1.33–1.61; $p < 0.001$), recessive genetic model (OR = 1.52; 95%CI = 1.36–1.71; $p < 0.001$), heterozygous model (OR = 1.36; 95%CI = 1.24–1.48; $p < 0.001$), homozygous model (OR = 1.82; 95%CI = 1.58–2.11; $p < 0.001$), and T allelic genetic model (OR = 1.37; 95%CI = 1.27–1.48; $p < 0.001$)]. Further subgroup analyses indicated that the MTHFR rs1801133 (677C>T) variant may increase the risk of IS in Asian, Hispanic, or Latin population, middle-aged, and elderly populations ($p < 0.001$).

Conclusion: Our results implied that mutation of the T allele of MTHFR rs1801133 (677C>T) could be a risk factor for IS. A significant association was found among Asian, Hispanic, or Latin population, middle-aged, and elderly people.

KEYWORDS

polymorphism, ischemic stroke, meta-analysis, risk, MTHFR rs1801133 (677C>T)

1 Introduction

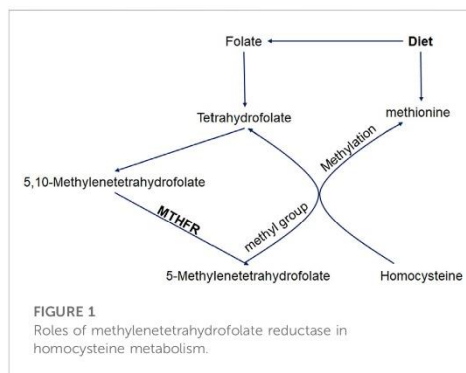
Ischemic stroke (IS) is an acute neurological deficit caused by vascular occlusion. It is one of the leading causes of death and disability worldwide (Francis et al., 2007; Malik and Dichgans, 2018; Phipps and Cronin, 2020) and is caused by a combination of environmental and genetic factors (Black et al., 2015; Prabhakaran et al., 2015; Malik et al., 2019). Several pathophysiological mechanisms are involved in the development of this condition. Hyperhomocysteinemia is reported to be independently associated with the risk of stroke (Linnebank et al., 2012). The 5,10-methylenetetrahydrofolate reductase (MTHFR) locus is mapped to chromosome 1 (1p36.3) which encodes for the dimeric proteins of 70–77 kDa subunits (Goyette et al., 1994). Folate metabolism is largely controlled by MTHFR, which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate. 5-Methylenetetrahydrofolate provides a methyl group in the methylation reaction that transforms homocysteine into methionine (Figure 1), as well as the DNA methylation process (Brattström et al., 1998). Thus, the MTHFR enzyme activity is important for homeostasis of the serum homocysteine level.

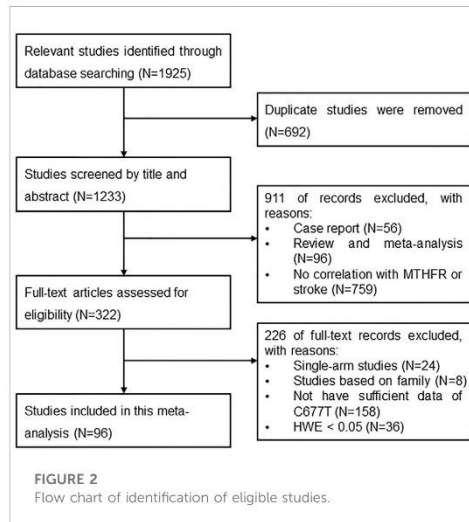
The previous study had demonstrated that approximately 40% of the intragenic coding CpG islands were hypermethylated, which had a higher C>T mutation rate. Also, the amino acid sequence of a protein and individual phenotypes could be changed by C>T substitutions at the CpG contexts in the protein-coding regions (Youk et al., 2020). The rs1801133 variant (NC_000001.11 (MTHFR):g. 677C>T (p.Ala222Val), also named MTHFR rs1801133 (677C>T), is a common mutant in MTHFR. The replacement of C with T at nucleotide 677 results in converting alanine to valine amino acid residue in the enzyme (Sharp and Little, 2004). Missense mutations cause a 50%–60% decrease in enzyme activity in patients who have the homozygous variant (TT) (Rozen, 1997), which contributes to hyperhomocysteinemia (Castro et al., 2004). In addition, reduction of the MTHFR enzymatic activity would cause deficiency of folate, which is also an independent risk factor of IS (Qin et al., 2020). Moreover, when folic acid is inadequate, the removal of homocysteine would be affected, leading to hyperhomocysteinemia and forming a vicious cycle (Liew and Gupta, 2015). Thus, it is important to determine the association between MTHFR rs1801133 (677C>T) polymorphism and the risk of IS for primary and secondary prevention of IS.

Many researchers have examined the relationship between MTHFR rs1801133 (677C>T) polymorphism and IS risk. However, there have been no definitive conclusions because different populations were examined and inconsistent results were obtained (Herak et al., 2017; Jiménez-González et al., 2021; Huang et al., 2022). Two meta-analyses were performed separately in 2016 and 2019 that reported a correlation between MTHFR rs1801133 (677C>T) polymorphism and IS (Song et al., 2016; Chang et al., 2019). However, only 22 studies were included in Song et al. (2016). Since then, many studies have been conducted in different populations. Moreover, the meta-analysis by Song et al. focused on the general population and did not consider whether MTHFR rs1801133 (677C>T) polymorphism might have varying effects on the risk of IS in different populations. Furthermore, Guilin Chang et al.'s study, published in 2019, included only nine studies on the elderly population and did not consider young and middle-aged IS patients (Chang et al., 2019). Therefore, past meta-analyses were updated to investigate whether MTHFR rs1801133 (677C>T) polymorphism and stroke risk are related across age and ancestry groups in this study.

2 Materials and methods

The study follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (Moher et al., 2009).





2.1 Literature search

A systematic search of the PubMed, EMBASE, Cochrane Library, Web of Science, and CNKI databases for relevant observational studies published until 15 March, 2022, was undertaken independently by two reviewers (Zhao and Li). We used the following search terms to identify eligible studies: ("methylentetrahydrofolate reductase" OR "MTHFR" OR "C677T" OR "rs1801133"), ("ischemic stroke" OR "cerebral infarction" OR "stroke"), AND ("single nucleotide polymorphism" OR "SNP" OR "genetic polymorphism" OR "mutation" OR "variation"). We reviewed the full text of each study when abstracts and titles were insufficient to make a final determination regarding study inclusion. The reference lists of included studies and existing reviews were screened to identify additional eligible studies. Any disagreements in the study selection process were resolved by a third person (Dang).

2.2 Selection criteria

We included the studies according to the following inclusion criteria: 1) the full text could be searched in electronic databases; 2) the studies were case-control or cohort studies examining MTHFR rs1801133 (677C>T) and stroke susceptibility; 3) the study population was limited to patients diagnosed with stroke for the first time; 4) the MTHFR rs1801133 (677C>T) genotype frequency was provided; and 5) articles were published in English or Chinese. The main exclusion criteria included the following: 1) the studies were duplicate articles or non-original research

(letters, commentaries, editorials, reviews, and meta-analyses); 2) the studies were case reports or involved animal experiments; 3) the genotype frequency of MTHFR rs1801133 (677C>T) was not provided; and 4) the *p*-value of the Hardy-Weinberg equilibrium (HWE) test was <0.05.

2.3 Data extraction and quality assessment

A pre-designed extraction form was used to extract the data. The extracted data included the name of the first author, type of stroke, publication date, ancestry groups, sample size (case and control), study design, mean or median age of the population, HWE, and the Newcastle-Ottawa scale score. Disagreements regarding data extraction were resolved by discussions among the two investigators (Zhao and Li), and a third reviewer (Dang) was consulted if necessary. The HWE *p*-value was also calculated using the genotypic frequencies of MTHFR polymorphisms, and the threshold of HWE deviation was set at 0.05. The quality of eligible publications was assessed using the Newcastle-Ottawa scale (Stang, 2010), and studies with a score of 7–9 were considered to be of good quality.

2.4 Statistical analysis

We used five genetic comparison models, the T allelic model (T vs. C), dominant model (TT + TC vs. CC), recessive model (TT vs. CC + TC), heterozygous model (TC vs. CC), and homozygous model (TT vs. CC), to estimate the relationship between MTHFR rs1801133 (677C>T) polymorphism and stroke susceptibility by calculating the odds ratio (OR) and 95% confidence interval (CI). The I^2 statistic was used to evaluate heterogeneity among genetic comparison models, and the pooled OR was estimated via the Mantel-Haenszel random effect model. Heterogeneity between studies is indicated by an $I^2 > 50\%$. Moreover, the Bonferroni method was utilized to adjust for multiple comparisons to control the false positive error rate. As we performed multiple comparisons in this meta-analysis for 45 times, the *p*-value which was less than 0.05/50 (0.001) indicated statistical significance after Bonferroni correction. To determine the possible causes of heterogeneity, the ancestry groups (Asian, European, African, Hispanic, or Latin American (HLA), and other and not reported ancestries (ONR)) and the study population (young: <18 years; middle-aged: 18–60 years; elderly: >60 years) were analyzed in subgroups. In addition, we examined the impact of a single study on the pooled OR by performing sensitivity analyses on different genetic comparison models. Egger's test, Begg's test, and funnel plots were used to evaluate the potential publication bias in our study (Peters et al., 2006). Stata 17.0 was used to perform the statistical analysis of all genetic comparison models.

TABLE 1 Characteristics of studies included in the meta-analysis.

Author	Year	Disease	Ancestry	Case			Control			Age	Design	HWE	NOS
				CC	CT	TT	CC	CT	TT				
Huang et al., (2022)	2022	IS	Asian	94	72	32	101	62	5	>18	Case-control	0.21	8
Salomi et al., (2021)	2021	IS	Asian	69	32	4	171	40	4	32.7±7.7	Case-control	0.36	8
Cernera et al., (2021)	2021	IS	European	64	140	76	138	206	85	8	Case-control	0.61	7
Jiménez-González et al., (2021)	2021	IS	HLA	35	105	38	60	83	40	34.1±5.4	Case-control	0.27	8
Mazdeh et al., (2021)	2021	IS	ONR	157	124	37	219	155	26	48±0.10	Case-control	0.84	7
Hou et al., (2018)	2018	IS	Asian	1063	793	138	1427	988	150	68.6±12.1	Case-control	0.22	9
Mao and Han, (2018)	2018	IS	Asian	73	189	27	82	100	16	68.42±13.26	Case-control	0.06	8
Li et al., (2017)	2017	IS	Asian	71	134	95	106	110	45	64.2±13.2	Case-control	0.08	8
Kamberi et al., (2016)	2017	IS	European	15	21	3	27	55	20	2.83–12.59	Case-control	0.40	8
Ma et al., (2017)	2017	IS	Asian	36	106	94	88	183	119	Media 66.0	Case-control	0.27	7
Lu et al., (2017)	2017	IS	Asian	31	97	89	41	109	73	NA	Case-control	0.8	7
Jiang et al., (2017)	2017	IS	Asian	36	52	18	40	54	12	57.8±10.7	Case-control	0.33	8
Kumar et al., (2016)	2016	IS	Asian	161	84	5	183	65	2	52.83±12.5	Case-control	0.14	7
Vijayan et al., (2016)	2016	IS	Asian	164	35	1	185	8	0	57.74±13.84	Case-control	0.77	8
Zhang et al., (2016)	2016	IS	Asian	13	27	10	36	18	5	NA	Case-Control	0.23	8
Herak et al., (2017)	2017	IS	European	29	34	10	41	51	8	4.3(0.01–16.7)	Case-control	0.15	7
Ranellou et al., (2015)	2015	IS	European	16	28	7	26	36	8	37.3±8.0	Case-control	0.40	8
Wei et al., (2015)	2015	IS	Asian	177	98	26	226	65	6	52.6±68.8	Case-control	0.60	8
Luo et al., (2015)	2015	IS	Asian	55	269	388	52	299	423	65.2±13.9	Case-control	0.93	8
Lv et al., (2015)	2015	IS	Asian	70	98	31	88	116	37	68.78 ± 10.63	Case-control	0.90	8
Zhou et al., (2014)	2014	IS	Asian	160	270	112	242	308	104	Media 66	Case-control	0.72	8
Atadzhanov et al., (2013)	2014	IS	African	15	8	0	96	20	0	54 ± 16	Case-control	0.31	8
Shi, (2014)	2014	IS	Asian	18	36	31	33	35	14	77.89 ± 8.85	Case-control	0.38	8
Supanc et al., (2014)	2014	IS	European	41	78	36	75	59	16	Middle-aged	Case-control	0.40	8
Fekih-Mrissa et al., (2013)	2013	IS	ONR	35	43	6	60	35	5	56.06 ± 12.5	Case-control	0.97	8
Zhang and Guo, (2012)	2012	IS	Asian	9	19	12	10	20	10	39.25 ± 4.08	Case-control	1.00	8
Djordjevic et al., (2012)	2012	IS	ONR	24	50	6	33	54	13	6.7 ± 4.9	Case-control	0.21	8
Djordjevic et al., (2012)	2012	IS	ONR	39	23	11	59	47	14	40.3 ± 11.6	Case-control	0.33	88
Somarajan et al., (2011)	2011	IS	Asian	137	65	5	129	54	5	54 ± 15.9	Case-control	0.82	8
Mohamed et al., (2011)	2011	IS	Asian	69	60	21	85	48	9	61.0 ± 10.1	Case-control	0.53	8
Hultdin et al., (2011)	2011	IS	ONR	172	114	27	401	306	59	55.0 ± 8.0	Nested	0.95	8
Salem-Berrabah et al., (2010)	2011	IS	ONR	33	15	2	57	35	5	Mean 57.62	Case-control	0.90	7
Isordia-Salas et al., (2010)	2010	IS	HLA	35	105	38	60	83	40	33.1 ± 5.8	Case-control	0.27	8

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TABLE 1 (Continued) Characteristics of studies included in the meta-analysis.

Author	Year	Disease	Ancestry	Case			Control			Age	Design	HWE	NOS
				CC	CT	TT	CC	CT	TT				
Giusti et al., (2010)	2010	IS	European	130	240	131	572	529	110	Media 44.0	Case-control	0.437	7
Tatarskyy et al., (2010)	2010	IS	ONR	73	87	23	50	45	5	64.6 ± 9.1	Case-control	0.20	8
Al-Allawi et al., (2009)	2009	IS	ONR	26	30	14	27	20	3	Media 60	Case-control	0.78	7
Sun et al., (2009)	2009	IS	Asian	45	36	38	49	37	10	>60	Case-control	0.45	7
Sabino et al., (2009)	2009	IS	HLA	12	9	0	24	12	1	33.3 ± 16.3	Case-control	0.73	7
Biswas et al., (2009)	2009	IS	Asian	24	32	2	48	10	0	<15	Case-control	0.47	7
Biswas et al., (2009))	2009	IS	Asian	67	49	4	90	30	0	Young	Case-control	0.12	8
(Morita et al., 2009)	2009	IS	European	5	6	4	48	37	5	Children	Case-control	0.53	7
Herak et al., (2009)	2009	IS	European	11	17	5	46	56	10	Children	Case-control	0.22	7
Goracy et al., (2009)	2009	IS	European	65	69	18	83	46	6	66.7 ± 12.5	Case-control	0.91	8
Zak et al., (2009)	2009	IS	European	25	30	9	32	25	2	Children	Case-control	0.27	8
Djordjevic et al., (2009)	2009	IS	ONR	9	16	1	23	22	5	Children	Case-control	0.94	8
Almawi et al., (2009)	2009	IS	ONR	54	26	38	90	26	4	NA	Case-control	0.23	7
Sawula et al., (2009)	2009	IS	European	70	50	8	35	19	5	18–101	Case-control	0.31	8
Yue et al., (2010)	2009	IS	Asian	8	19	35	14	11	5	67 ± 9	Case-control	0.29	8
Celiker et al., (2009)	2009	IS	ONR	158	4	0	63	37	6	Mean 69.8	Case-control	0.85	8
Shi et al., (2008)	2008	IS	Asian	23	45	29	20	45	34	Young	Case-control	0.07	8
Moe et al., (2008)	2008	IS	Asian	73	36	11	136	68	3	62.5 ± 1.1	Case-control	0.09	8
Gao et al., (2008)	2008	IS	Asian	4	20	18	6	11	13	≤45	Case-control	0.22	8
Zhang et al., (2008)	2008	IS	Asian	49	116	80	74	140	68	63.7 ± 10.4	Case-control	0.91	8
Berge et al., (2007)	2007	IS	European	29	125	182	25	141	163	Old	Case-control	0.47	7
Nan et al., (2007)	2007	IS	Asian	14	55	31	28	53	19	<45	case-control	0.49	8
Kim et al., (2007)	2007	IS	Asian	81	113	43	66	119	38	61.18 ± 11.09	Case-control	0.21	8
Komitopoulou et al., (2006)	2006	IS	European	36	46	8	46	39	18	5.55 ± 0.48	Case-control	0.06	8
Sazci et al., (2006)	2006	IS	ONR	42	41	9	115	119	25	53.45 ± 9.21	Case-control	0.47	8
Li et al., (2006)	2006	IS	Asian	235	184	35	190	128	16	65.20 ± 12.75	Case-control	0.34	8
Dikmen et al., (2006)	2006	IS	ONR	75	57	14	32	21	2	63.4 ± 0.87	Case-control	0.52	8
Gao et al., (2006)	2006	IS	Asian	30	49	21	32	44	24	61.08 ± 10.77	Case-control	0.25	8
Yanqun et al., (2006)	2006	IS	Asian	49	73	40	49	41	10	55 ± 6	Case-control	0.74	7
Hermans et al., (2006)	2006	IS	ONR	4	13	6	62	58	22	71.7 ± 9.3	Case-control	0.18	9
Pezzini et al., (2005)	2005	IS	European	46	83	34	60	75	23	35.0 ± 7.5	Case-control	0.96	9
En et al., (2005)	2005	IS	Asian	88	43	5	51	17	2	65 ± 10	Case-control	0.69	8
Yan et al., (2006)	2005	IS	Asian	34	24	3	37	17	3	Old	Case-control	0.58	8
Jinhuan et al., (2005)	2005	IS	Asian	55	28	4	53	24	3	Mean 65	Case-control	0.89	8
Alluri et al., (2005)	2005	IS	ONR	47	21	1	48	1	0	7–78	Case-control	0.94	8

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TABLE 1 (Continued) Characteristics of studies included in the meta-analysis.

Author	Year	Disease	Ancestry	Case			Control			Age	Design	HWE	NOS
				CC	CT	TT	CC	CT	TT				
Kawamoto et al., (2005)	2005	IS	Asian	33	43	21	91	110	40	78 ± 8.3	Case-control	0.49	8
Yi et al., (2005)	2005	IS	Asian	27	42	9	22	25	3	68.3 ± 7.6	Case-control	0.23	8
Lingling et al., (2004)	2004	IS	Asian	26	18	3	12	15	5	67.16 ± 10.11	Case-control	0.93	8
Choi et al., (2003)	2003	IS	Asian	62	97	36	73	100	25	61.4 ± 10.9	Case-control	0.30	7
Jin et al., (2004)	2003	IS	Asian	21	59	14	40	49	11	69.68 ± 8.9	Case-control	0.48	8
Jin et al., (2004)	2003	IS	Asian	15	23	21	15	11	7	NA	Case-control	0.09	7
Yan et al., (2003)	2003	IS	Asian	55	28	4	53	24	3	66.13 ± 12.54	Case-control	0.89	8
Li et al., (2002)	2002	IS	Asian	58	65	20	97	49	8	40–90	Case-control	0.58	8
Chuanqing et al., (2002)	2002	IS	Asian	24	35	10	25	35	7	62.3 ± 12.9	Case-control	0.30	8
Wenping et al., (2003)	2002	IS	Asian	8	34	19	25	46	15	61–79	Case-control	0.43	8
Huang et al., (2002)	2002	IS	Asian	11	25	13	16	24	10	55.5 ± 13.0	Case-control	0.85	8
Guangsen and Chongwen, (2002)	2002	IS	Asian	40	47	15	37	47	16	65	Case-control	0.87	8
Akar et al., (2001)	2001	IS	ONR	24	18	4	39	23	6	Children	Case-control	0.34	7
Wu et al., (2001)	2001	IS	Asian	23	40	14	92	113	24	61.4 ± 6.8	Case-control	0.21	8
Yaqin et al., (2001)	2000	IS	Asian	15	23	21	15	11	7	NA	Case-control	0.09	7
Zheng et al., (2000)	2000	IS	Asian	43	62	10	62	45	15	Mean 59	Case-control	0.14	8
Cumming et al., (1999)	1999	IS	ONR	41	6	1	42	6	0	7–36	Case-control	0.64	7
Harmon et al., (1999)	1999	IS	European	74	73	27	86	78	19	>60	Case-control	0.83	7
Gaustadnes et al., (1999)	1999	IS	European	97	88	22	545	449	90	25–68	Case-control	0.85	8
Akar et al., (1999)	1999	IS	ONR	14	10	4	63	37	6	Children	Case-control	0.85	8
Press et al., (1999)	1999	IS	ONR	72	85	10	50	57	8	65 ± 8	Case-control	0.12	8
Lalouschek et al., (1999)	1999	IS	ONR	35	37	9	32	40	9	64.76 ± 13.2	Case-control	0.50	8
Xinliang et al., (1999)	1999	IS	Asian	10	45	25	58	32	20	60.2 ± 6.1	Case-control	0.48	8
De Stefano et al., (1998)	1998	IS	European	28	27	17	65	98	35	Mean 33.9	Case-control	0.85	8
Kostulas et al., (1998)	1998	IS	ONR	50	40	10	50	40	10	NA	Case-control	0.63	7
Salooja et al., (1998)	1998	IS	European	81	76	16	114	107	21	68 (22–94)	Case-control	0.56	8
Nakata et al., (1998)	1998	IS	Asian	19	23	6	35	51	19	30–80	Case-control	0.96	8
Markus et al., (1997)	1997	IS	European	162	146	37	76	63	22	65.7 (10.5)	Case-control	0.13	7

3 Results

3.1 Literature search and characteristics of the included studies

There were 1,925 articles initially identified after searching the databases. Among them, 692 articles were removed because of duplication, and 911 articles were removed after the titles and

abstracts were screened. In total, 322 articles were further screened for eligibility by reviewing their full texts, and eventually, 96 articles that met the criteria were selected (Figure 2).

Of the 96 studies included, 95 were case-control studies, and one was a nested case-referent study. In total, 52 studies were conducted in the Asian population, 19 were in the European population, 1 was in the African population, 3 were in the

TABLE 2 Pooled odds ratios (ORs) and 95% confidence intervals (CIs) of the association between C677T polymorphism and stroke in the dominant model.

Model	OR (95% CI)	P _Z	I ²	P _H
Dominant				
All IS	1.47 (1.33–1.61)	<0.001*	69.9%	<0.001
Ancestry				
Asian	1.59 (1.41–1.80)	<0.001*	67.2%	<0.001
European	1.35 (1.10–1.65)	0.004	69.9%	<0.001
African	2.56 (0.96–6.85)	0.061	—	—
Hispanic or Latin American	1.93 (1.39–2.67)	<0.001*	0.0%	0.824
Other and not reported ancestries	1.20 (0.91–1.57)	0.190	76.4%	<0.001
Age				
Young	1.46 (1.12–1.91)	0.005	52.2%	0.012
Middle-aged	1.59 (1.33–1.90)	<0.001*	69.8%	<0.001
Elderly	1.34 (1.17–1.53)	<0.001*	71.3%	<0.001
NA	1.77 (1.30–2.41)	<0.001*	69.3%	<0.001

Bonferroni correction for multiple testing was applied (*p*-value threshold, 0.001);P_Z, *p*-value for the Z-test; P_H, *p*-value for heterogeneity;

*Association was still significant after Bonferroni correction for multiple testing.

TABLE 3 Pooled odds ratios (ORs) and 95% confidence intervals (CIs) of the association between C677T polymorphism and stroke in the recessive model.

Model	Or (95% CI)	P _Z	I ²	P _H
Recessive				
All IS	1.52 (1.36–1.71)	<0.001*	55.7%	<0.001
Ancestry				
Asian	1.58 (1.38–1.81)	<0.001*	47.1%	<0.001
European	1.48 (1.11–1.97)	0.007	73.1%	<0.001
African	—	—	—	—
Hispanic or Latin American	0.96 (0.68–1.37)	0.839	0.0%	0.949
Other and not reported ancestries	1.47 (1.05–2.05)	0.026	49.6%	0.005
Age				
Young	1.25 (0.81–1.92)	0.313	53.7%	0.009
Middle-aged	1.55 (1.22–1.96)	<0.001*	57.5%	<0.001
Elderly	1.47 (1.28–1.68)	<0.001*	46.8%	0.001
NA	1.99 (1.29–3.07)	<0.001*	64.7%	0.001

Bonferroni correction for multiple testing was applied (*p*-value threshold 0.001);P_Z, *p*-value for the Z-test; P_H, *p*-value for heterogeneity;

*Association was still significant after Bonferroni correction for multiple testing.

Hispanic or Latin American population, and 21 were in other and not reported ancestry population. In total, 14 studies examined children, 27 examined middle-aged people, and 42 examined the

elderly population. The other 13 studies examined the general population. The main features of the included studies are shown in Table 1.

TABLE 4 Pooled odds ratios (ORs) and 95% confidence intervals (CIs) of the association between C677T polymorphism and stroke in the heterozygous model.

Model	OR (95% CI)	P _Z	I ²	P _H
Heterozygote				
All IS	1.36 (1.24–1.48)	<0.001*	60.4%	<0.001
Ancestry				
Asian	1.46 (1.30–1.63)	<0.001*	58.2%	<0.001
European	1.27 (1.08–1.51)	0.005	50.4%	0.006
African	2.56 (0.96–6.85)	0.061	—	—
Hispanic or Latin American	2.09 (1.50–2.95)	<0.001*	0.0%	0.825
Other and not reported ancestries	1.10 (0.86–1.40)	0.453	67.6%	<0.001
Age				
Young	1.44 (1.13–1.84)	0.003	38.7%	0.069
Middle-aged	1.50 (1.27–1.78)	<0.001*	62.5%	<0.001
Elder	1.23 (1.08–1.40)	0.001*	62.8%	0.001
NA	1.48 (1.14–1.92)	0.003	50.2%	0.020

Bonferroni correction for multiple testing was applied (*p*-value threshold 0.001);P_Z, *p*-value for the Z-test; P_H, *p*-value for heterogeneity;

*Association was still significant after Bonferroni correction for multiple testing.

TABLE 5 Pooled odds ratios (ORs) and 95% confidence intervals (CIs) of the association between C677T polymorphism and stroke in the homozygous model.

Model	Or (95% CI)	P _Z	I ²	P _H
Homozygote				
All IS	1.82 (1.58–2.11)	<0.001*	64.2%	<0.001
Ancestry				
Asian	1.98 (1.66–2.37)	<0.001*	59.4%	<0.001
European	1.65 (1.14–2.39)	0.008	79.3%	<0.001
African	—	—	—	—
Hispanic or Latin American	1.60 (1.05–2.46)	0.030	0.0%	0.863
Other and not reported ancestries	1.59 (1.09–2.32)	0.017	56.2%	0.001
Age				
Young	1.43 (0.88–2.33)	0.146	55.6%	0.006
Middle-aged	1.92 (1.45–2.53)	<0.001*	62.7%	<0.001
Elder	1.74 (1.44–2.11)	<0.001*	62.3%	<0.001
NA	2.45 (1.47–4.01)	0.001*	69.1%	<0.001

Bonferroni correction for multiple testing was applied (*p*-value threshold 0.001);P_Z, *p*-value for the Z-test; P_H, *p*-value for heterogeneity;

*Association was still significant after Bonferroni correction for multiple testing.

3.2 Meta-analysis results

There were 34,814 participants (15,569 cases and 19,245 controls) in the 96 studies included in the meta-analysis.

3.2.1 Dominant model

The TT + CT genotype showed significant heterogeneity compared with the CC genotype in the dominant genetic model ($I^2 = 69.9\%$; $p < 0.001$) (Table 2). The MTHFR rs1801133

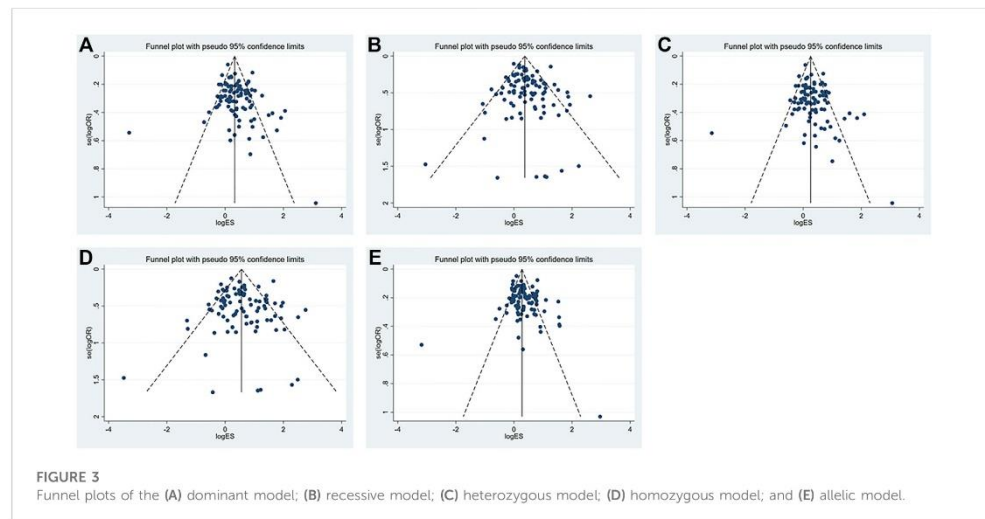
TABLE 6 Pooled odds ratios (ORs) and 95% confidence intervals (CIs) of the association between C677T polymorphism and stroke in the allelic model.

Model	Or (95% CI)	P _Z	I ²	P _H
Allele				
All IS	1.37 (1.27–1.48)	<0.001*	75.7%	<0.001
Ancestry				
Asian	1.46 (1.33–1.60)	<0.001*	72.6%	<0.001
European	1.29 (1.09–1.53)	0.003	79.5%	<0.001
African	2.51 (1.06–5.93)	0.036	—	—
Hispanic or Latin American	1.28 (1.05–1.57)	0.016	0.0%	0.981
Other and not reported ancestries	1.26 (0.96–1.52)	0.116	81.4%	<0.001
Age				
Young	1.31 (1.05–1.64)	0.016	65.5%	<0.001
Middle-aged	1.42 (1.24–1.64)	<0.001*	73.7%	<0.001
Elder	1.30 (1.18–1.43)	<0.001*	75.0%	<0.001
NA	1.70 (1.28–2.26)	<0.001*	80.6%	<0.001

Bonferroni correction for multiple testing was applied (p -value threshold 0.001);

P_Z, p -value for the Z-test; P_H, p -value for heterogeneity;

*Association was still significant after Bonferroni correction for multiple testing.



(677C>T) mutation significantly increased the IS risk under the dominant genetic model (OR = 1.47; 95%CI = 1.33–1.61; $p < 0.001$). In the ancestry subgroup analysis, the rs1801133 (677C>T) polymorphism of MTHFR was evidently linked to an increased risk of IS in Asian (OR = 1.59; 95%CI = 1.41–1.80; $p < 0.001$) and

Hispanic or Latin population (OR = 1.93; 95%CI = 1.39–2.67; $p < 0.001$). MTHFR rs1801133 (677C>T) gene polymorphism was associated with IS susceptibility in all age groups except young populations (middle-aged: OR = 1.59, 95%CI = 1.33–1.90, and $p < 0.001$; elderly: OR = 1.34, 95%CI = 1.17–1.53, and $p < 0.001$).

TABLE 7 Publication bias of included studies.

IS	Egger's test			Begg's test	
	Co-efficiency	Standard error	p-value	Z	p-value
Dominant model	0.83	0.39	0.04*	1.56	0.12
Recessive model	0.37	0.29	0.20	1.28	0.20
Heterozygote model	0.74	0.34	0.03*	1.83	0.07
Homozygote model	0.28	0.36	0.43	1.18	0.24
Allele model	0.76	0.44	0.09	1.71	0.09

* $p < 0.05$.

3.2.2 Recessive model

The TT genotype showed significant heterogeneity compared with the CC + CT genotype in the recessive genetic model ($I^2 = 55.7\%$; $p < 0.001$) (Table 3). MTHFR rs1801133 (677C>T) polymorphism was associated with an increased risk of stroke under the recessive model (OR = 1.52; 95%CI = 1.36–1.71; $p < 0.001$). The ancestry subgroup analysis showed a significant difference in the Asian populations, with combined ORs of 1.58 (95% CI = 1.38–1.81; $p < 0.001$). The middle-aged and elderly people had an increased risk of stroke according to the subgroup analysis (middle-aged: OR = 1.55, 95%CI = 1.22–1.96, and $p < 0.001$; elderly: OR = 1.47, 95%CI = 1.28–1.68, and $p < 0.001$).

3.2.3 Heterozygous model

The TC genotype showed significant heterogeneity compared with the CC genotype in the heterozygous genetic model ($I^2 = 60.4\%$; $p < 0.001$) (Table 4). There was an obvious association between MTHFR rs1801133 (677C>T) polymorphism and increased risk of stroke under the heterozygous model (OR = 1.36; 95%CI = 1.24–1.48; $p < 0.001$). In the subgroup analysis, MTHFR rs1801133 (677C>T) gene polymorphism was associated with stroke susceptibility in Asian (OR = 1.46; 95% CI = 1.30–1.63; $p < 0.001$), Hispanic or Latin populations (OR = 2.09; 95%CI = 1.50–2.95; $p < 0.001$), middle-aged (OR = 1.50; 95%CI = 1.27–1.78; $p < 0.001$), and elderly groups (OR = 1.23; 95%CI = 1.08–1.40; $p = 0.001$).

3.2.4 Homozygous model

The TT genotype showed significant heterogeneity compared with the CC genotype in the homozygous genetic model ($I^2 = 64.2\%$; $p < 0.001$) (Table 5). There was also a significant association between MTHFR rs1801133 (677C>T) polymorphism and an increased risk of stroke under this model (OR = 1.82; 95%CI = 1.58–2.11; $p < 0.001$). However, the stratification analysis results were similar to those of the recessive model. Significant correlation was detected between MTHFR rs1801133 (677C>T) polymorphisms and the increased

risk of stroke in the Asian population (OR = 1.98; 95%CI = 1.66–2.37; $p < 0.001$). Furthermore, the middle-aged and elderly people had an increased risk of stroke in the subgroup analysis (middle-aged: OR = 1.92, 95%CI = 1.45–2.53, and $p < 0.001$; elderly: OR = 1.74, 95%CI = 1.44–2.11, and $p < 0.001$).

3.2.5 Allelic model

The T allele showed significant heterogeneity compared with the C allele in the allelic genetic model ($I^2 = 75.7\%$; $p < 0.001$) (Table 6). There was an obvious association between MTHFR rs1801133 (677C>T) polymorphism and an increased risk of stroke under the allelic model (OR = 1.37; 95%CI = 1.27–1.48; $p < 0.001$). In the subgroup analysis, MTHFR rs1801133 (677C>T) gene polymorphism was associated with stroke susceptibility in Asian populations (OR = 1.46; 95%CI = 1.33–1.60; $p < 0.001$). The middle-aged and elderly people with T allele mutation had a higher risk of stroke (middle-aged: OR = 1.42, 95%CI = 1.24–1.64, and $p < 0.001$; elderly: OR = 1.30, 95%CI = 1.18–1.43, and $p < 0.001$).

3.3 Sensitivity analysis

A sensitivity analysis was conducted to compare the pooled ORs after individually excluding each included study. There was no significant change in the results (Supplementary Figure S1).

3.4 Publication bias

The funnel plot is shown in Figure 3. All research studies included in this study distributed above the funnel plots, which indicated that variability of the effect size was low and the results were reliable. The Egger's funnel plots for these five models were basically symmetrical though Egger's test, indicating there was publication bias in the dominant ($p = 0.04$) and heterozygous models ($p = 0.03$) (Table 7). Nonetheless, we did not find any publication bias in all

genetic models using Begg's tests. The correlation between the $\ln OR$ and its variance and the level of heterogeneity across studies might contribute to the discrepancy between Egger's and Begg's tests. Actually, Begg's test is more robust and has the appropriate type I error rates despite the sample size, the number of included studies, and the level of heterogeneity. Furthermore, when the summary estimates are ORs or RRs and there is obvious heterogeneity between studies (Schwarzer et al., 2002; Peters et al., 2006), type I error rates for Egger's test are higher than those for Begg's test.

4 Discussion

This meta-analysis demonstrates associations between MTHFR rs1801133 (677C>T) genetic polymorphism and susceptibility to IS under all genetic models. Our results were consistent with a previous meta-analysis performed in 2016, wherein this polymorphism was found to be potentially involved in the development of IS (Song et al., 2016). This suggests the MTHFR C677T mutation is a genetic risk factor of IS, and primary and secondary prevention should be initiated in a timely manner in population with this mutation.

Several factors might explain the association of the MTHFR rs1801133 (677C>T) mutation and increased IS risk. Most importantly, the MTHFR rs1801133 (677C>T) mutation leads to decreased MTHFR activity and elevated homocysteine levels (Castro et al., 2004). Hyperhomocysteinemia is linked to the overproduction of free radicals (Xi et al., 2016), induction of oxidative stress (Esse et al., 2019; Tchantchou et al., 2021), endothelial injury (Salvio et al., 2021), coagulation, and lipid metabolism disturbance (Herrmann, 2001), which all contribute to the incidence of IS. Meanwhile, previous studies demonstrated that people with the MTHFR rs1801133 (677C>T) mutation show a poor response to homocysteine-lowering treatment (Qin et al., 2020). Furthermore, a meta-analysis published in the current year showed that MTHFR rs1801133 (677C>T) polymorphism is related to susceptibility to H-type hypertension (Liao et al., 2022), which is a traditional risk factor for IS.

We also conducted a subgroup analysis on the basis of age and ancestry of the study population to further understand the significance of the MTHFR rs1801133 (677C>T) mutation in various populations. The result of subgroup analysis showed increased IS risk in populations with the MTHFR rs1801133 (677C>T) mutation in middle-aged and elderly groups. It was consistent with the fact that older people were more susceptible to atherosclerosis, which was an important cause of IS. Previous studies had demonstrated that the MTHFR rs1801133

(677C>T) mutation increased the risk of IS in patients with large-artery atherosclerosis (Cui, 2016) and adults (Xin et al., 2009).

Another important finding of this meta-analysis was the stable association between IS risk and MTHFR rs1801133 (677C>T) polymorphism in Asian populations in all genetic models and Hispanic or Latin American population in dominant and heterozygous models. Similar trends were also found among other populations, although no statistically significant difference was found in some genetic models. This may be related to the following reasons: 1) the frequency of the MTHFR 677T gene variant differs among ethnic groups due to different genetic backgrounds. Previous studies indicated that the frequency of the MTHFR rs1801133 T allele was 24–40% in Europeans, 40% in Koreans, and 26–37% in Japanese (Shao et al., 2017); 2) MTHFR rs1801133 (677C>T) was associated with increased coronary heart disease only when the folate level was low (Klerk et al., 2002). Thus, various dietary habits and differences in folate intake may also contribute to this difference; 3) the difference in the power of included studies may be another cause of this result. Nonetheless, the results for these ancestry groups need to be interpreted with caution, and more high-quality studies are still required to explore the correlation between MTHFR rs1801133 (677C>T) polymorphism and IS risk in these ancestry groups.

This study has several strengths. First, we included the most recent and relevant studies in this meta-analysis. In addition, we further analyzed the association between MTHFR rs1801133 (677C>T) polymorphism and IS risk in different populations. Finally, this meta-analysis included high-quality observational studies using real-world data with a large number of patients.

However, this meta-analysis also has some limitations. First, the study was based on the secondary study-level data. Age groups were defined according to the mean or median age of study subjects, and some studies did not provide clear information on age. Thus, the age stratification of subgroups might not be accurate. Second, the findings of this meta-analysis were mainly based on case-control studies and lacked prospective research; therefore, they should be interpreted with caution.

5 Conclusion

Our findings showed that the MTHFR rs1801133 (677C>T) variant may contribute to an increased risk of IS. This association was statistically significant in the Asian and Hispanic or Latin American cohorts and showed a similar trend in the populations

of other ancestries. For middle-aged and elderly people, MTHFR rs1801133 (677C>T) might be a promising biomarker for early detection and prediction of the prognosis of IS. However, high-quality, prospective studies are needed in the future.

Author contributions

All authors contributed to the data analysis and drafting or revision of the manuscript, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2022.1021423/full#supplementary-material>

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RESEARCH ARTICLE

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A prospective cohort study of MTHFR C677T gene polymorphism and its influence on the therapeutic effect of homocysteine in stroke patients with hyperhomocysteinemia

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Abstract

Background: Hyperhomocysteinemia (HHCY) is a risk factor for cardiovascular and cerebrovascular diseases. The C677T 5, 10-methylenetetrahydrofolate reductase (MTHFR) gene polymorphism increases homocysteine (HCY) levels. This study analyzed the relationship between C677T MTHFR polymorphism and the therapeutic effect of lowering HCY in stroke patients with HHCY.

Methods: Baseline data were collected from stroke patients with HHCY for this prospective cohort study. The C677T MTHFR genotype was detected by polymerase chain reaction-restriction fragment length polymorphism and the therapeutic effect to reduce HCY was compared.

Results: Of 200 stroke patients 162 (81.0%) completed follow-up and were evaluated. Most of them responded well to treatment (103 cases, 63.5%), but 59 (36.4%) patients were in the poor efficacy group. There was a significant difference in terms of age ($P < 0.001$), hypertension ($P = 0.041$), hyperuricemia ($P = 0.042$), HCY after treatment ($P < 0.001$), and MTHFR genotype ($P < 0.001$) between the poor efficacy and effective groups, with increased frequency of the TT genotype in the poor efficacy group. Logistic regression showed that the T allele was associated with poor efficacy (OR = 0.733, 95%CI: 0.693, 0.862, $P < 0.001$). In the codominant model the TT genotype was associated with poor outcome (OR = 0.862, 95%CI: 0.767, 0.970, $P = 0.017$) and this was also the case in the recessive model (OR = 0.585, 95%CI: 0.462, 0.741, $P < 0.001$) but there was no association between CT and TT in the dominant model.

Conclusions: The T allele and TT genotype of the MTHFR C677T polymorphism was associated with poor HCY reduction treatment efficacy in stroke patients with HHCY.

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Trial registration: The registration number of the clinical trial is ChiCTR1800020048. Registration date: December 12, 2018.

Keywords: Homocysteine, Stroke, Intracerebral hemorrhage, Methylene tetrahydrofolate reductase, Single nucleotide polymorphism

Background

The global burden of stroke is extremely high, 16.9 million people suffer a stroke each year, and the aging and increasing number of the world's population means stroke is increasing [1]. The traditional risk factors of stroke, such as hypertension, diabetes mellitus, hyperlipidemia, and atrial fibrillation have been studied extensively, and their underlying mechanisms for stroke are evident [2]. This has led to primary prevention measures that have been implemented to decrease the incidence of stroke in high income countries [1]. As stroke can lead to long-term disability, an early detection of risk factors and active control to effectively preventing the occurrence of stroke is particularly important [3]. Effective prevention is an important strategy to reduce the overall burden of stroke worldwide.

However, many other risk factors are less well understood. Homocysteine (HCY) is a less well studied non-traditional risk factor for stroke [4, 5]. Framingham's offspring cohort study found that the predictive power of the score will be improved if four biomarkers, including homocysteine, were included in the Framingham Stroke Risk Score [6, 7]. Our previous studies found that elevated HCY was a risk factor for stroke in young people [8, 9]. The correlation between high HCY and stroke is stronger in younger individuals [9]. Patients with an abnormally high level of HCY, or hyperhomocysteinemia (HHCY), are considered to be at risk of cardiovascular and cerebrovascular diseases [10–12], but its mechanism is rather complex, and not fully understood. 60.6% of stroke patients have HHCY, which is associated with a low level of serum B12 [13]. There is indeed a significant positive correlation between HCY levels and ischemic stroke [14–17]. Intracerebral hemorrhage (ICH), which accounts for 10–15% of cases, has the highest mortality and morbidity rate among all strokes. HCY level may be an aggravating factor in atherosclerosis, which indirectly contributes to a high risk of ICH [18, 19]. High HCY concentrations may impair endothelial function, increase oxidative stress, impair methylation reactions, and alter protein structure [20]. Elevated HCY is affected by diet, nutrition, heredity, disease and drug factors [21, 22]. The contribution of genetic factors to the pathogenesis of stroke is demonstrated by the association between specific gene variants and stroke risk. However, due to conflicting results from different studies [13, 23, 24], the

effects of these polymorphisms on the risk of stroke development are remain uncertain. It has been suggested that differences between studies are related to the heterogeneity of cerebral infarction [25–27].

Among genetic factors, the C677T gene polymorphism site of methylenetetrahydrofolate reductase (MTHFR) gene has become an important research locus [28]. MTHFR encodes methylenetetrahydrofolate reductase which is a rate-limiting enzyme for folic acid metabolism. Dietary folate converts to its active cofactor in HCY catabolism and catalyze the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Thus, it plays an important role in folic acid metabolism, DNA methylation and repair [29]. Some studies have shown that individuals with C677T MTHFR genotype have doubled HCY levels in plasma compared with normal individuals [29]. The C677T mutation apparently modifies the association between HCY and stroke [30]. However it is not clear whether the C677T MTHFR polymorphism influences efforts to decrease HCY in patients with stroke and HHCY.

This study aimed to compare the relationship among C677T MTHFR gene polymorphism and the risk of stroke, and the therapeutic effect of lowering HCY in stroke patients with HHCY. The results should help reduce the family burden and economic burden of the stroke through standardized prevention and treatment strategies in stroke patients with HHCY.

Methods

This prospective cohort study (ChiCTR1800020048) was approved by the institutional review board of China Rehabilitation Research Center (CRRC) (2016–018-1). Written informed consent was obtained from all the patients. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patients

From March 2016 to December 2018, we recruited stroke patients with HHCY in the outpatients clinic and wards of the Department of Neurorehabilitation of CRRC. The inclusion criteria were as follows: 1) age $\geq$ 18 years; 2) patients with ischemic stroke (consistent with diagnostic criteria in the 2010 guidelines for the diagnosis and treatment of acute ischemic stroke in China [31]); patients with cerebral hemorrhage

(diagnostic criteria at the Fourth National Conference on Cerebrovascular Diseases [32]); 3) patients with HHcy (HCY $\geq 15 \mu\text{mol/L}$). The exclusion criteria were as follows: 1) patients with subarachnoid hemorrhage; patients with traumatic intracerebral hemorrhage; 2) pregnant or lactating women; 3) patients participated in or were participating in other clinical studies within 3 months; 4) patients with malignant tumors. The exit and termination criteria were: patients who could not complete the examination items as required or could not be followed up.

The clinical data of the patients were collected, including gender, age, history of hypertension, history of diabetes, history of lipid metabolism disorder, history of heart disease, and type of stroke.

Detection of HCY and C677T polymorphism of the MTHFR gene

All the subjects were tested for HCY and MTHFR C677T gene polymorphism in the Laboratory Department of CRRC.

The detection and reference values of plasma HCY were as follows: all patients were tested for baseline HCY in the morning, 5 ml of fasting elbow venous blood was collected (heparin anticoagulation), and centrifuged for 10 min at 3000 r/min, and the plasma HCY concentration was measured by a chemiluminescence with a BIO-RAD automatic biochemical analyzer (S1000 PCR instrument) using the mindray reagent. The normal reference range set by the kit manual was 5–15 $\mu\text{mol/L}$ [33], and if HCY is more than 15 $\mu\text{mol/L}$, HHcy should be considered. After 1 month of treatment, the patients were tested again.

The MTHFR C677T gene polymorphism was tested with the Baio MTHFR (C677T) gene detection kit using a BS-800 M S1000TM Thermal cycler. The C677T locus genotyping of MTHFR gene was performed by polymerase chain reaction-restriction fragment length polymorphism.

Treatment

In this study, patients were given folic acid tablets 0.4 mg/day, vitamin B6 100mg with 3 times/day, methylcobalamin 500 μg with 3 times/day to reduce HCY. The data of Baseline levels and HCY levels after 1 month of treatment were collected. Patients with poor efficacy were those who failed to reach the normal range or whose reduction was less than 20%. The rest of the patients were considered as control groups. Patients with other diseases (hypertension, diabetes, etc.) were treated according to their original treatment plan.

Statistical analysis

SPSS 21.0 (IBM Corp., Armonk, NY, USA) statistical software was used to analyze the research data. The

measurement data were expressed as $\bar{x} \pm s$. T-test was used for comparison between the two groups, and analysis of variance (ANOVA) was used for multi-group comparison. Least significant difference (LSD) test was used for pairwise comparison, and the paired t-test was used for paired data. The count data were expressed as examples (percentages) and was compared by chi-square test. Regarding effective treatment as the outcome, logistic regression was used to analyze the correlation between different genotypes and outcomes (indicators with $P < 0.05$ of univariate analysis were selected for multivariate analysis). $P < 0.05$ was accepted as a significant difference.

Results

Baseline characteristics and HCY change after treatment

A total of 200 stroke patients were enrolled in the study. Of these, 11 cases (5.5%) withdrew from the study due to renal insufficiency, and 27 cases (13.5%) withdrew from the study due to hepatic insufficiency without a full course of regular treatment. A total of 162 patients (81.0%) completed the follow-up. Most of them responded well to folic acid tablets, vitamin B6 and methylcobalamin treatment, with a reduction of more than 20% or a reduction to the normal range and so they were grouped into the effective efficacy group (103 cases, 63.5%). The remaining 59 (36.4%) patients were grouped in the poor efficacy group (Table 1). There were significant differences between the groups in age ($P < 0.001$), hypertension ($P = 0.041$), hyperuricemia ($P = 0.042$), HCY after treatment ($P < 0.001$), and MTHFR genotype ($P < 0.001$).

MTHFR polymorphism and HCY changes

The P value of the Hardy-Weinberg equilibrium test was 0.56, which indicated the genetic balance of the population. In the poor efficacy group of 59 cases, there were 48 cases of TT, 10 cases of CT; 1 case of CC. Within this group there were 12 patients with unstable HCY levels which are increased instead of being decreased, in which 11 cases were TT, and 1 case was CT (Table 2). HCY levels before and after treatments for each genotype are shown in Table 3. These results showed that there were significant differences in HCY levels among the genotype groups before ($P = 0.049$) and after ($P = 0.002$) treatment.

Logistic regression analysis was carried out with adjustment for age, history of hypertension and hyperuricemia for the outcome of poor efficacy, and the results showed that the T allele was associated with poor efficacy (OR = 0.733, 95%CI: 0.693, 0.862, $P < 0.001$). In the codominant model the TT genotype was associated with poor outcome (OR = 0.862, 95%CI: 0.767, 0.970, $P = 0.017$) and this was also the case in the recessive model (OR = 0.585, 95%CI: 0.462, 0.741, $P < 0.001$) but there

Table 1 Baseline characteristics and homocysteine levels before and after treatment in stroke patients grouped according to treatment efficacy

	Poor efficacy group (n = 59)	Effective efficacy group (n = 103)	P value
Gender, male (%)	51 (86.4%)	87 (84.5%)	0.734
Age, years	46.51 ± 13.7	57.7 ± 13.3	< 0.001
Stroke types			
Hemorrhagic, n (%)	34 (57.6%)	45 (43.7%)	0.088
Ischemic, n (%)	25 (42.4%)	58 (56.3%)	
Hypertension, n (%)	55 (93.2%)	84 (81.6%)	0.041
Diabetes mellitus, n (%)	17 (28.8%)	38 (36.9%)	0.269
Abnormal lipid metabolism, n (%)	34 (57.6%)	75 (72.8%)	0.070
Hyperuricemia, n (%)	10 (16.9%)	7 (6.8%)	0.042
HCY, μmol/L			
Before treatment	26.49 ± 15.75	21.88 ± 18.99	0.116
After treatment	21.64 ± 10.81	12.42 ± 5.99	< 0.001
MTHFR C677T genotype			
CC, n (%)	1 (1.7%)	9 (8.7%)	< 0.001
CT, n (%)	10 (16.9%)	45 (43.7%)	
TT, n (%)	48 (81.4%)	49 (47.6%)	

was no association of between CT and TT in the dominant model (Table 4).

Discussion

MTHFR involved in folate metabolism has two variants, C677T and A1298C [34]. Racial-ethnic differences in the distribution of the C677T polymorphism are well-described [35, 36]. Carrier rates of the 677 T polymorphism differ across white, Asian, and black ethnic groups. This study aimed to analyze the relationship between C677T MTHFR polymorphism and the therapeutic effect of lowering HCY in stroke patients with HHcy. Results from previous studies have shown that the TT genotype of the MTHFR gene in Asian countries had a greater impact on HCY levels [15, 37, 38]. The TT genotype of C677T polymorphism in the MTHFR gene contributes to the genetic susceptibility of acute ischemic stroke in population of Singapore [39]. The frequency of TT genotype of MTHFR gene in this group of stroke patients was up to 57.92% (117). TT genotype is more frequently seen in Asian compared with that of white individuals, and least common in those of African ethnic origin [29]. However, ethnic subgroups may in turn

exhibit different carrier rates or environmental susceptibility to disease-specific morphisms, which may in part explain ethnic and regional differences in stroke risk [35]. These results suggest that race and genetic factors influence the incidence of disease. The results of this study showed that overall 59.9% of the stroke patients had the TT genotype. There were no significant differences between ischemic stroke group and hemorrhagic stroke group in the comparison of TT genotype (60.9% vs 57.5%), but the percentage of patients with the TT genotype was much higher in the poor treatment efficacy group than that of patients who achieved a good reduction in HCY levels (81.4 vs 47.6%). This shows the importance of early detection of HCY in the Chinese population with the TT genotype of MTHFR gene.

Vitamin B, especially methylcobalamin, can effectively reduce plasma HCY. The most commonly used regimen is the combined supplementation of folic acid (vitamin B9), vitamin B12 and vitamin B6 [40]. Folic acid needs to be combined with vitamin B12 and vitamin B6 to reduce the risk of stroke effectively [41–43]. Previous studies suggested that 0.8 mg/d of folic acid have the best effect on reducing HCY [31]. The Framingham investigators and others have demonstrated reductions in total HCY following population folic acid grain fortification programs [44]. In areas where grain is not fortified, folic acid can slightly reduce stroke risk. However, there was no evidence that long-term use of larger doses of folic acid could further improve efficacy, and its safety is worthy of attention. In clinical practice, folic acid tablets, vitamin B6 and methylcobalamin are used to treat

Table 2 Distribution of the three MTHFR C677T genotypes in patients with poor efficacy

Patients with poor efficacy	CC (n = 1)	TT (n = 49)	CT (n = 9)
Decrease < 20% (n = 47)	1	38	8
Unstable increase (n = 12)	0	11	1

Table 3 Homocysteine levels before and after treatment according to MTHFR C677T genotype

	CC (n = 10)	CT (n = 55)	TT (n = 97)	P value ^a
HCY before treatment, $\mu\text{mol/L}$	20.26 $\pm$ 6.60	18.90 $\pm$ 6.37	21.75 $\pm$ 8.19	0.049
HCY after treatment, $\mu\text{mol/L}$	14.02 $\pm$ 3.53	12.98 $\pm$ 5.01 ^{ab}	19.45 $\pm$ 11.32 ^{ab}	0.002
P value ^{**}	0.535	0.328	0.211	

^a Analysis of variance was used for comparison among genotypes^{**} Paired sample t-test was used for comparison between baseline and 1 month after treatment^{ab} Different letters indicate statistical significance. Least significant difference (LSD) test was used for pairwise comparison. CCvsCT $p = 0.816$; CC vs TT $p = 0.215$; CT vs TT $p = 0.001$

HHCY. According to the US guidelines, the supplementation of three kinds of compound vitamin B could reduce HCY and reduce the risk of stroke in HHcy [41, 45]. A daily multivitamin preparations with adequate vitamin B6 and vitamin B12, and folate (400 $\mu\text{g/d}$) are reasonable to reduce the level of homocysteine [46]. The Chinese guidelines for Secondary Prevention of Ischemic Stroke and Transient Ischemic Attack (TIA) recommend supplementation of folic acid, vitamin B6 and vitamin B12 to be used to reduce HCY in patients with recent ischemic stroke or TIA and mild to moderate homocysteine elevation [31]. Most of the patients in this study responded well to folic acid tablets, vitamin B6 and methylcobalamin therapy. Patients affected more by genetic factors were less responsive to folic acid tablets, vitamin B6 and methylcobalamin and needed longer medication cycles. Literatures have shown that HHcy patients with TT genotype could still benefit from folic acid supplementation despite normal folic acid and vitamin B12 levels [47], but there are few studies on how to adjust treatment in a few patients with elevated levels. Elevated HCY is influenced by many factors such as diet, heredity, and drugs [48]. Further study is required to

analyze genetic subgroups within large clinical trials of HHcy therapy. Young individuals with elevated HCY and people with high blood pressure may particularly benefit from lowering HCY [49].

Several limitations of this study must be acknowledged. This study was a single-center study with a relatively small sample size and only one-month follow-up time. For HHcy patients with TT genotype, methods to controlling the abnormal increases of HCY (the dosage, method, type and cycle of the drugs) need to be studied, and these are our future research direction. Multi-center clinical trials using larger sample sizes and better design will be needed to validate our results.

Conclusions

In conclusion, the TT genotype of MTHFR C677T was common in this Chinese stroke population and was an independent risk factor for poor efficacy of HCY lowering treatment.

Abbreviations

HHcy: Hyperhomocysteinemia; HCY: Homocysteine; ICH: Intracerebral hemorrhage; CRRC: China Rehabilitation Research Center; MTHFR: The 5, 10-methylenetetrahydrofolate reductase; OR: Odds ratio; CI: Confidence interval; LSD: Least significant difference

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Authors' contributions

XXD conceived and coordinated the study, designed, performed and analyzed the experiments, wrote the paper. LX, RS, KPL, LL, LPS and ZZL carried out the data collection, data analysis, and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This prospective cohort study was approved by the institutional review board of CRRC (2016-018-1). Written informed consent was obtained from all the patients. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Table 4 Logistic regression analysis of MTHFR C677T in poor efficacy cases and controls

	Cases, n (%)	Controls, n (%)	OR (95% CI)	P value
Allele				
C	63 (30.6)	12 (10.2)	1.000 (reference)	–
T	143 (69.4)	106 (89.8)	0.773 (0.693, 0.862)	< 0.001
Model type				
Codominant				
CC	9 (8.7)	1 (1.7)	1.000 (reference)	–
CT	45 (43.7)	10 (16.9)	0.917 (0.734, 1.144)	0.460
TT	49 (47.6)	48 (81.4)	0.862 (0.767, 0.970)	0.017
Dominant				
CC	9 (8.7)	1 (1.7)	1.000 (reference)	–
CT + TT	94 (91.3)	58 (98.3)	0.928 (0.867, 0.994)	0.095
Recessive				
CC + CT	54 (52.4)	11 (18.6)	1.000 (reference)	–
TT	49 (47.6)	48 (81.4)	0.585 (0.462, 0.741)	< 0.001

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Prevalence of MTHFR C677T polymorphism and its association with serum homocysteine and blood pressure among different ethnic groups: insights from a cohort study of Nepal

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Abstract

Background The risk of hypertension varies based on ethnicity, environmental factors, and genetic predispositions. Studies have reported a higher risk of cardiovascular diseases (CVD) and hypertension among the *Newar* ethnic groups in Nepal. However, the genetic analysis for Methylene tetrahydrofolate reductase (MTHFR C677T) gene mutations, serum homocysteine, and high-sensitivity C-reactive protein (hs-CRP) levels across different ethnicities remains unexplored.

Methods Sociodemographic information and baseline data of 489 participants were obtained from the first phase of the Dhulikhel Heart Study. Preserved blood samples were analyzed for MTHFR C677T polymorphism using real-time polymerase chain reaction (TaqMan assay), and serum homocysteine was measured through immunoassay techniques. Descriptive analysis, the Hardy-Weinberg equilibrium test, and multinomial regression were performed.

Results The prevalence of homozygous mutation (TT) was 19.8% in the *Newar* group and 12.5% in the *Brahmin/Chhetri* ethnicity. The highest mean value of homocysteine (19.4 $\mu\text{mol/L}$) was observed in homozygous participants, followed by the heterozygous mutant group (17.4 $\mu\text{mol/L}$). A statistically significant association ($P < 0.001$) was found between homocysteine levels and blood pressure.

Conclusions The Dhulikhel Heart Study reveals a significant prevalence of the MTHFR C677T gene mutation among the *Newar* ethnicity compared to other groups. Elevated levels of homocysteine and high-sensitivity C-reactive protein (hs-CRP) were associated with increased blood pressure.

Clinical trial number Not applicable

Keywords Hyperhomocysteinemia, Hypertension, MTHFR C677T, Nepal, Polymorphism

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Introduction

Essential hypertension (EH) is a complicated condition that most likely arises from the interplay of several gene-environmental interaction and their identification is essential for the prevention and management of cardiovascular diseases (CVD) [1, 2]. Various environmental and clinical risk factors are linked to essential hypertension, such as high sodium intake, alcohol consumption, lack of physical activity, poor diet, obesity, insulin-resistant diabetes, and hyperlipidemia. While these factors significantly contribute to hypertension susceptibility, it is estimated that genetic factors account for up to 60% of the variation in hypertension risk [3–5].

Genome-wide association studies (GWASs) have identified common genetic variations linked to both normal and pathological traits, indicating that single nucleotide polymorphisms (SNPs) can serve as effective biomarkers for screening at-risk populations and facilitating genetic diagnosis and gene therapy for hypertension [6].

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme encoded by the MTHFR gene and is responsible for homocysteine (Hcy) and folate metabolism [7]. According to a meta-analysis of case-control studies, the C677T polymorphism in MTHFR is linked to 36–87% higher incidence of hypertension due to reducing catalytic activity by MTHFR enzyme resulting in hyperhomocysteinemia (HHcy) [8]. This C677T polymorphism occurs in exon 4, where cytosine is replaced by thymine at the 677th position, leads to the replacement of Alanine with Valine at position 222 in the protein (A222V) [9]. Hyperhomocysteinemia, induced by this genetic variation, is considered an independent risk factor for cardiovascular diseases (CVD), stroke, and venous thrombosis, which triggers the proliferation of vascular smooth muscle and endothelial injury and contributes to hypertension [10].

Currently, an estimated 1.28 billion people suffer from hypertension, and this number is projected to escalate to 1.56 billion by 2025 [11]. In the context of Nepal, hypertension affects 27.3% of the general population, with a notable increase to 31.3% in the age group of ≥ 35 years [12]. The prevalence of hypertension in the Nepalese population varies based on ethnicity and geography, with the lowest rates observed in mountainous regions and the highest in hilly regions. Gandaki Province ranks on the top with a prevalence of 27.5%, followed by Bagmati Province 24.1% and lowest (13.9%) in Far Western Province [13].

Nepal is currently undergoing rapid transformations in diet, demography, and epidemiology, potentially exerting a substantial impact on the distribution and risk of non-communicable diseases [14, 15]. In Dhulikhel, Nepal, a community-based longitudinal cohort study revealed that individuals belonging to the *Newar* ethnic group

faced a 5.6 times higher risk of hypertension attributable to disparities in dietary practices and other environmental factors. Numerous studies from Nepal indicated that 51–64.5% of patients with a history of hypertension or under medication struggle with inadequate blood pressure control [16, 17]. However, the genetic association behind the higher risk of hypertension and poor blood control among under medication patients remains elusive. Earlier meta-analyses have suggested a link between the MTHFR C677T polymorphism and a heightened risk of coronary artery disease [18, 19] and high levels of plasma homocysteine have been observed in hypertensive patients and are positively correlated with blood pressure and ischemic stroke [20]. A two-year, randomized, placebo-controlled trial demonstrated that participants assigned to homocysteine-lowering treatment experienced a reduction in blood pressure [21].

All these findings originated from developed countries or urban areas, but there was a lack of similar evidence among the Nepalese ethnic groups. This research could aid in identifying target populations of CVD and hypertension and formulating affordable treatment plans tailored to the diverse needs of ethnic groups with different genetic mutations on MTHFR C677T and levels of serum homocysteine for the prevention and management of hypertension.

Hence, the primary objective of this study was to investigate the prevalence of polymorphisms in the MTHFR C677T gene, assess plasma homocysteine levels and high-sensitive C-reactive protein (hs-CRP) in well-preserved blood specimens of Dhulikhel Heart Study participants, and examine their association with blood pressure.

Materials and methods

The Dhulikhel Heart Study (DHS) was conducted in 2013–2015 as cohort study in Dhulikhel municipality, Nepal aimed to investigate cardiovascular risk factors within a representative population. In the first phase, one third households of municipality were randomly selected and comprehensive baseline data were collected. Every household was invited to participate in the survey, including all eligible adults aged 18 or older who had resided there for at least six months. The study excluded pregnant women, individuals who declined to provide consent, those residing in institutionalized settings (such as hostels and hotels), and those facing communication difficulties due to medical or mental conditions [16, 17].

Building on this foundation, the second phase of the study adopted a cross-sectional design utilizing preserved blood samples from the initial cohort in 2023. The baseline data for this study were derived from the initial phase of the Dhulikhel Heart Study (DHS). A well-preserved whole blood and serum sample (stored at -80°C) from 535 DHS participants were used for the analysis of

the MTHFR C677T gene and homocysteine. However, only the results from 489 samples were deemed suitable for inclusion in this study because 27 blood sample had poor labelling, 19 were insufficient for analysis. Ethical approval for the study was granted by the Institutional Review Committee of Kathmandu University School of Medical Science (IRC-KUSMS), with approval number 35/23.

Genotyping and DNA extraction

DNA extraction from whole blood was carried out using a DNA extraction kit (Zymo Research Inc., Catalogue Number: D4068) [22]. Subsequently, qPCR was performed using the molecular genetics MTHFR (C677T) kit (Sacace Biotechnologies, Catalogue Number: T01103-5-T) [23] to identify mutations in the MTHFR C677T gene, utilizing the CFX96 Bio-Rad system (BioRad Laboratories). The entire process of extraction, elution, and analysis adhered to the manufacturer's instructions. For the quantitative determination of homocysteine, a chemiluminescent microparticle immunoassay (CMIA) [24] was employed, while hs-CRP was quantified using a fully automated biochemistry analyzer (BA 400, Biosystems, Spain). Both analyses were conducted according to the manufacturer's guidelines [25].

Interpretation of results

Following the completion of qPCR, (TaqMan assay) the baseline was established within cycles 3–15, and the threshold was set at 100. Regarding the results, if the sample exhibited signals for HEX, FAM, HEX, and FAM, both polymorphisms were interpreted as homozygous wild type, homozygous mutated, and heterozygous mutated, respectively. Samples with a Ct value > 32 was deemed invalid and subjected to qPCR repetition. If the results remained invalid in the second run, they were subsequently excluded [23]. Genotyping quality was assured by re-genotyping a randomly selected subset of 10% of the total samples. Additionally, standard quality control including (positive and negative controls) was run in each PCR run to minimize technical errors. The distribution of hs-CRP serum concentrations was classified into three groups. Cardiac risk was deemed low, average, and high when the hs-CRP value was < 1.0 mg/L, 1.0–3.0 mg/L, and > 3.0 mg/L, respectively [26–29]. Serum homocysteine level < 15.0 µmol/L considered normal while 15–30 µmol/L, 30–100 µmol/L and > 100.0 µmol/L is moderate, intermediate and severe high respectively [30].

Statistical analysis

Continuous variables were presented as mean and standard deviation, while categorical variables were expressed as frequency and proportions using SPSS version 23.0. The Hardy-Weinberg equilibrium test was employed to

calculate the Chi-square (χ^2) value, assessing the concordance between the observed and expected frequencies of different alleles. Descriptive analysis, one-way analysis of variance (ANOVA), and bivariate correlation were utilized to examine mean differences for continuous variables across categorical variables. Statistical significance was established when the *P*-value was < 0.05.

Results

Among all the participants, 60.5% were female, with the highest representation from the *Newar* ethnic group (39.1%), followed by *Brahmin/Chhetri* (35.8%) and *Tamang* (18.4%). The average age was 41.1 ($\pm$ 16.2) years. Over one-third (37.2%) of the participants were overweight, while 8.4% were obese. Additionally, 29.7% had hypertension, and 33.0% were pre-hypertensive (Table 1).

The Hardy-Weinberg equilibrium test identified 227 CC genotypes, 187 CT heterozygotes, and 75 TT homozygous mutants (Table 2). Applying Hardy-Weinberg equilibrium based on the expected values resulted in a statistically significant Chi-square value of 11.59 (*P* = 0.003). The overall prevalence of homozygous (TT) mutations was 15.3%, while that of heterozygous (CT) polymorphisms was 38.2%.

Table 3 displays the descriptive analysis assessing the prevalence of different genotypes across various ethnic groups and genders. The incidence of homozygous mutations (TT) in the *Newar* ethnic group was 7.3% higher than in the *Brahmin and Chhetri* ethnic groups.

Although a notable difference was noted in low-density lipoprotein levels and diastolic blood pressure, this difference was not statistically significant (Table 4). However, the serum homocysteine level exhibited statistically significant (*P* = 0.01).

In the normal group (no mutation on MTHFR C677T), the mean serum homocysteine level was 13.9 ± 8.8 (µmol/L), while the heterozygous and homozygous mutant groups exhibited levels of 17.4 ± 11.9 and 19.4 ± 11.4 (µmol/L), respectively. A positive Pearson correlation was observed between serum homocysteine levels and systolic blood pressure (*r* = 0.18) as well as diastolic blood pressure (*r* = 0.19). Furthermore, there was a positive correlation between homocysteine and hs-CRP (*r* = 0.47, *P* = < 0.001), Total cholesterol (*r* = 0.23, *P* = < 0.001), and LDL cholesterol (*r* = 0.20, *P* = < 0.001).

The outcomes of a multivariate multinomial logistic regression analysis (Table 5) revealed significant associations between various factors and distinct genotype categories, even after adjusting for age, sex, ethnicity, hypertension, homocysteine levels, and lipid profiles. In comparison to *Brahmin/Chhetri*, individuals of *Newar* ethnicity exhibited a 75% increase in the relative risk for Alleles 1 and 2 and heterozygous mutant compared to Allele 1 and homozygous wild type (RR = 1.75, 95%

Table 1 Baseline characteristics of the study population ($n=489$)

	Minimum	Maximum	Mean	Std. Deviation
Age (Years)	18.0	85.0	41.1	16.2
FBG (mg/dL)	38.0	324.0	79.2	19.1
Total Cholesterol (mg/dL)	89.0	352.0	166.7	41.0
HDL Cholesterol (mg/dL)	21.0	91.0	42.3	11.4
LDL Cholesterol (mg/dL)	45.0	231.0	108.3	29.3
Triacylglycerol (mg/dL)	42.0	674.0	125.0	73.8
HbA1c (%)	3.9	12.0	6.1	0.9
Homocysteine ($\mu\text{mol/L}$)	1.4	50.0	16.1	10.7
hs-CRP (mg/L)	0.10	13.5	1.8	1.3
Systolic BP (mmHg)	87.0	197.0	128.2	18.3
Diastolic BP (mmHg)	55.0	125.0	82.0	10.5
Pulse	53.0	121.0	78.4	10.6
Height (cm)	130.0	186.0	156.0	8.9
Weight (kg)	39.0	95.0	59.9	10.8
Waist (cm)	56.0	121.0	82.2	11.0
Hip (cm)	60.0	129.0	91.4	8.9
BMI	15.6	39.0	24.5	3.9

*FBG=Fasting Blood Glucose, HDL=High Density Lipoprotein, LDL=Low Density Lipoprotein

HbA1c=Glycated hemoglobin, hs-CRP=high sensitive C reactive protein, BMI=body mass index

Table 2 Hardy–Weinberg equilibrium test for the MTHFR polymorphisms

MTHFR C677T	Observed Number	Expected Number	Chi-Square (X^2)	P-Value
CC	227	210	11.59	0.003
CT	187	221		
TT	75	58		
Total	489	489		

*CC=normal genotype, CT=heterozygous mutant and TT=homozygous mutant

Table 3 Genotype and allele frequency of different ethnic groups and sex ($n=489$)

	Genotype CC ($n=227$) 46.4%	Genotype CT ($n=187$) 38.2%	Genotype TT ($n=75$) 15.3%
Ethnic group			
Brahmin and Chhetri (175)	95 (54.2%)	58 (33.1%)	22 (12.5%)
Newar (191)	73 (38.2%)	80 (41.8%)	38 (19.8%)
Tamang (90)	47 (52.2%)	35 (38.8%)	8 (8.8.6%)
Others (33)	12 (36.3%)	14 (42.4%)	7 (21.2%)
Sex			
Female (296)	145 (48.9%)	106 (35.8%)	45 (15.2%)
Male (193)	82 (42.4%)	81 (41.9%)	30 (15.5%)

*CC=normal genotype, CT=heterozygous mutant, and TT=homozygous mutant

CI: 1.09–2.81, $P=0.02$). Furthermore, participants with hypertension were more likely to fall into the Allele 2 homozygous mutant category ($RR=2.28$, 95% CI: 1.15–4.48, $P=0.01$), while keeping other variables in the model constant.

Discussion

This study pioneers the demonstration of polymorphisms in the MTHFR C677T gene across various ethnic groups and their association with homocysteine and blood

pressure in the participants of this cohort of Dhulikhel municipality and holds significance on two fronts. Firstly, it affirms that the extent of polymorphism in the MTHFR C677T gene varies among representative population of a suburban community of Nepal. Secondly, it establishes an association between the mutation of the MTHFR gene and hyperhomocysteinemia, as well as high blood pressure, particularly in the Newar ethnic groups in a suburban community in central Nepal. The genetic mutation causes decreased activity of MTHFR activity, increased

Table 4 Characteristics of the biochemical parameters with respective genotypes (ANOVA)

Biochemical Parameters	Genotype	Mean	Std. Deviation	Significance (P = < 0.05)
FBG (mg/dL)	CC Genotype (Normal)	78.6	21.3	0.38
	CT Genotype (Heterozygous mutant)	78.7	14.9	
	TT Genotype (Homozygous mutant)	82.0	20.9	
Total Cholesterol (mg/dL)	CC Genotype (Normal)	163.9	39.9	0.93
	CT Genotype (Heterozygous mutant)	166.5	38.5	
	TT Genotype (Homozygous mutant)	175.8	49.1	
HDL Cholesterol (mg/dL)	CC Genotype (Normal)	42.7	11.6	0.54
	CT Genotype (Heterozygous mutant)	42.4	11.1	
	TT Genotype (Homozygous mutant)	41.0	11.9	
LDL Cholesterol (mg/dL)	CC Genotype (Normal)	106.2	27.6	0.06
	CT Genotype (Heterozygous mutant)	107.0	27.3	
	TT Genotype (Homozygous mutant)	118.2	36.7	
Triacylglycerol (mg/dL)	CC Genotype (Normal)	122.7	74.3	0.59
	CT Genotype (Heterozygous mutant)	124.8	68.7	
	TT Genotype (Homozygous mutant)	132.7	84.2	
HbA1C (%)	CC Genotype (Normal)	6.1	0.9	0.05
	CT Genotype (Heterozygous mutant)	6.0	0.8	
	TT Genotype (Homozygous mutant)	6.3	1.1	
Homocysteine (μmol/L)	CC Genotype (Normal)	13.9	8.8	0.01
	CT Genotype (Heterozygous mutant)	17.4	11.9	
	TT Genotype (Homozygous mutant)	19.4	11.4	
hs-CRP (mg/L)	CC Genotype (Normal)	1.7	1.2	0.16
	CT Genotype (Heterozygous mutant)	1.9	1.3	
	TT Genotype (Homozygous mutant)	2.0	1.4	
Systolic BP (mmHg)	CC Genotype (Normal)	126.1	18.5	0.27
	CT Genotype (Heterozygous mutant)	129.1	17.8	
	TT Genotype (Homozygous mutant)	132.3	18.2	
Diastolic BP (mmHg)	CC Genotype (Normal)	81.0	10.2	0.09
	CT Genotype (Heterozygous mutant)	82.5	11.2	
	TT Genotype (Homozygous mutant)	83.8	9.6	

*FBG = Fasting blood glucose, HDL = High Density Lipoprotein, LDL = Low Density Lipoprotein

HbA1c = Glycated hemoglobin, hs-CRP = high sensitive C reactive protein, BP = blood pressure

Table 5 Multinomial logistic regression analysis to calculate factors associated with the genotype (n = 489)

	Allele 1 (CC) (Reference)	Allele 2 (TT) homozygous mutants			Allele 1 and 2 (CT) heterozygous mutants		
		RR*	95% CI	P-value	RR	95% CI	P-value
Age in years	1	1.00	0.97–1.01	0.72	1.00	0.98–1.01	0.61
Sex Male	1	0.86	0.47–1.54	0.60	1.28	0.84–1.94	0.25
Ethnicity							
Tamang	1	0.81	0.32–1.20	0.64	1.21	0.68–2.11	0.51
Newar	1	1.87	0.98–3.56	0.06	1.75	1.09–2.81	0.02
Others	1	2.55	0.86–7.48	0.08	1.77	0.75–4.15	0.19
Hypertension	1	2.28	1.15–4.48	0.01	1.45	0.84–2.48	0.18
Homocysteine	1	1.04	1.01–1.06	< 0.05	1.03	1.00–1.05	0.01
HDL	1	0.97	0.94–0.99	0.03	1.00	0.97–1.01	0.63
LDL	1	1.01	1.00–1.02	0.03	1.00	0.99–1.00	0.61

*RR stands for relative risk ratio. Brahmin/Chhetri ethnicity was used as the reference group for the ethnicity variable. Bold p-values indicate significance at the 5% level

plasma homocysteine and MTHFR activity in carriers of CT and TT genotype is 35% and 70% lower than in normal subjects respectively [31]. Although elevated homocysteine is a risk of cardiovascular illness, its connection to hypertension is still up for debate but studies has

clearly established causal relationship with blood pressure and HHcy [32].

Our analysis revealed a notable prevalence of the homozygous TT genotype among the Newar group (19.8%), significantly higher than in the Brahmin/Chhetri group (12.5%). This suggests a genetic predisposition that

might contribute to the higher incidence of hypertension observed in the *Newar* community. Analysis of the Hardy–Weinberg equilibrium indicated a notable deviation in the genotype frequencies for the MTHFR C677T polymorphism ($\chi^2 = 11.59$, $P = 0.003$). The observed genotype frequencies for CC, CT, and TT (227, 187, and 75, respectively) were different from the expected frequencies (210, 221, and 58, respectively). The mutations in the TT allele in our study closely resemble those observed in Europe, North America, and some Asian countries. In Europe and North America, the prevalence of the TT homozygous genotype ranges from 8 to 18%, though it is notably lower in individuals with dark skin (1.45%) [33]. The frequency of the TT genotype is 15% among the Japanese, 14% among Koreans, 8–32.8% among Chinese, and 0–3.7% among Indian populations [34]. The results indicate a possible genetic predisposition or evolutionary impact on the distribution of the MTHFR C677T polymorphism within this population.

The average homocysteine level in our study was 16.1 $\mu\text{mol/L}$, and the frequency of polymorphisms, particularly in the homozygous state (TT), was found to be comparatively higher than in other similar studies [35, 36]. This clearly indicates there is variation in polymorphisms in the MTHFR C677T gene and fluctuations in homocysteine levels among diverse ethnicities. And Homocysteine (Hcy), a demethylated derivative of methionine, could be the possible metabolic cause, recognized as a thrombogenic non-proteinogenic amino acid and is produced within the “1-carbon metabolism (methionine-Hcy-folate)” cycle [36].

The synthesis and removal of Hcy typically maintain a delicate balance, but in pathological states, such as hyperhomocysteinemia (HHcy), overall plasma Hcy levels tend to rise due to impaired Hcy metabolism. The primary causes of HHcy in individuals include: (1) regular consumption of an excessive methionine-rich protein diet; (2) insufficiency of vitamin B12 and/or folate; (3) the presence of heterozygous or homozygous cystathionine β -synthase (CBS) gene; and (4) insufficient clearance of Hcy from the kidney [37].

In our investigation, we identified a higher level of homocysteine (Hcy) in the *Newar* ethnic groups compared to other groups and a distinct dietary pattern, incorporating refined grains, meat, and alcohol as major components in their cuisine [17] could be the possible link. The dietary intake of methionine influences the rate of S-adenosylmethionine (SAM) production, which, in turn, could impact the homocysteine pathway [38].

These findings suggested that there was high prevalence of MTHFR C677T gene polymorphism and high level of homocysteine associated with high blood pressure in participated population and varies with ethnic groups. In spite of these genetic and biochemical

association with blood pressure, finding cannot be generalized for entire Nepalese population. So bigger sample size and multicenter study are recommended to establish more clear findings to address genetic and biochemical risk factors which could enhance the effectiveness of hypertension prevention and treatment programs for different ethnic groups in Nepal.

Conclusions

This cohort study highlights high prevalence of the MTHFR C677T gene mutation among selected ethnic groups. The *Newar* ethnic group exhibited the highest mutation frequency and TT homozygous genotype showed the strongest association with elevated serum homocysteine and increased blood pressure.

Abbreviations

ANOVA	Analysis of variance
BMI	Body mass index
BP	Blood pressure
CMIA	Chemiluminescent microparticle immunoassay
CVDs	Cardiovascular disease (s)
DHS	Dhulikhel heart study
FBG	Fasting blood glucose
Hcy	Homocysteine
HDL	High density lipoprotein
HHcy	Hyperhomocysteinemia
hs-CRP	High sensitive C reactive protein
IRC-KUSMS	Intitutional review committee-Kathmandu university school of medical sciences
LDL	Low density lipoprotein
LMICs	Low- and middle-income country (s)
MTHFR	Methylenetetrahydrofolate reductase
qPCR	Quantitative polymerase chain reaction
SPSS	Statistical package for social sciences

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Author contributions

Conceptualization: Rajendra Dev Bhatt, Biraj Man Karmacharya, Prabodh Rimal, Evaluation of results: Rajendra Dev Bhatt, Archana Shrestha, Surendra Madhup, Rajesh Shahi, Writing—original draft: Rajendra Dev Bhatt, Dinesh Timalsena, Nishan Katuwal, Supervision: Prabodh Rimal, Rajeev Shrestha, Proof reading: Annette L. Fitzpatrick, Prabodh Rimal, Biraj Man Karmacharya.

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Data availability

The data supporting this study's findings are available upon reasonable request from Dhulikhel Hospital-Kathmandu University Hospital or corresponding author. However, due to ethical guidelines and confidentiality agreements, raw participant data cannot be shared. Aggregated and anonymized data may be provided after obtaining approval from the relevant institutional and ethical review board.

Declarations

Ethics approval and consent to participate

Human ethics was approved from Kathmandu University School of Medical Science (IRC-KUSMS) and written consent was obtained from each participant

for blood sample collection, testing, preservation and publication. Ethical approval letter is attached separately.

Consent for publication

Yes, written consent from each participant was taken for blood collection, testing, analysis, preservation and publication. All author were agreeing to publish with written consent.

Competing interests

The authors declare no competing interests.

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2.2. Antecedentes históricos

La hipótesis de la homocisteína como factor de riesgo vascular se origina en la década de 1960. En 1969 McCully observó depósitos ateroscleróticos prematuros en niños con homocistinuria y postuló por primera vez una relación entre niveles elevados de homocisteína y enfermedad arterial. Durante los años 90, estudios epidemiológicos (p. ej. Boushey et al. 1995) reforzaron esta asociación al cuantificar la relación dosis-respuesta entre homocisteína y eventos cardiovasculares. En 1995 Frosst et al. identificaron el polimorfismo C677T del gen MTHFR como variante común (~38% de alelos en poblaciones estudiadas) que altera un aminoácido conservado, dando lugar a una enzima termolábil con actividad reducida. Los portadores homocigotos TT exhiben niveles significativamente elevados de homocisteína plasmática y, por ello, esta mutación ha sido propuesta como factor de riesgo genético importante para enfermedad cardiovascular. Este hallazgo histórico introdujo el análisis genético de MTHFR en el estudio de la aterosclerosis.

En paralelo, se identificaron otros polimorfismos comunes de MTHFR, como A1298C, aunque su efecto enzimático es menor. Desde entonces, se ha acumulado abundante evidencia sobre el metabolismo de la homocisteína y su posible papel en aterogénesis. No obstante, la relación causal entre estos polimorfismos y las enfermedades cardiovasculares ha sido muy debatida. De hecho, estudios posteriores han reportado resultados heterogéneos: por ejemplo, el metaanálisis de Mohammed et al. (2025) concluye que la asociación MTHFR–enfermedad coronaria permanece controvertida. Asimismo, recomendaciones de organismos genéticos señalan que la mayoría de las personas con los polimorfismos C677T/A1298C no presentan

trastornos clínicos atribuibles, por lo que no se recomienda su tamizaje rutinario para evaluar riesgo cardiovascular.

2.3. Homocisteína y su papel en el metabolismo

La homocisteína (Hcy) es un aminoácido sulfurado intermedio derivado del metabolismo de la metionina. Ésta se obtiene de la dieta, se convierte en S-adenosilmetionina (SAM) –donador universal de grupos metilo– y luego se transforma en S-adenosilhomocisteína (SAH), que se hidroliza liberando homocisteína. Bajo condiciones fisiológicas normales, la homocisteína es metabolizada a través de dos vías principales: la remetilación, que la convierte nuevamente en metionina mediante la acción del cofactor 5-metiltetrahidrofolato, y la transulfuración, que la transforma en cisteína utilizando vitamina B6 como cofactor. En la remetilación, la enzima metilentetrahidrofolato reductasa (MTHFR) cataliza la conversión de 5,10-metilentetrahidrofolato en 5-metiltetrahidrofolato, la forma activa del folato responsable de donar grupos metilo necesarios para la remetilación de la homocisteína.

La concentración plasmática normal de homocisteína en ayuno es 5–15 $\mu\text{mol/L}$. Los niveles plasmáticos de homocisteína elevados por encima de los 15 $\mu\text{mol/L}$, es conocida bajo la condición de hiperhomocisteinemia (HHCY), se han asociado con un incremento del riesgo de enfermedades cardiovasculares, trombosis venosa y complicaciones neurológicas (Kong et al., 2022). La homocisteína puede dañar el endotelio vascular mediante varios mecanismos: genera estrés oxidativo y especies de oxígeno reactivo, induce lesión de ADN en células endoteliales, promueve disfunción endotelial al interferir con la síntesis de óxido nítrico, y favorece la agregación plaquetaria y la formación de trombos. Así, niveles altos de homocisteína

pueden agravar la progresión de la aterosclerosis, lo que la convierte en un marcador metabólico de relevancia clínica, especialmente en pacientes con comorbilidades; ejemplo la DM2 (Denis de Armas et al., 2024; Kong et al., 2022).

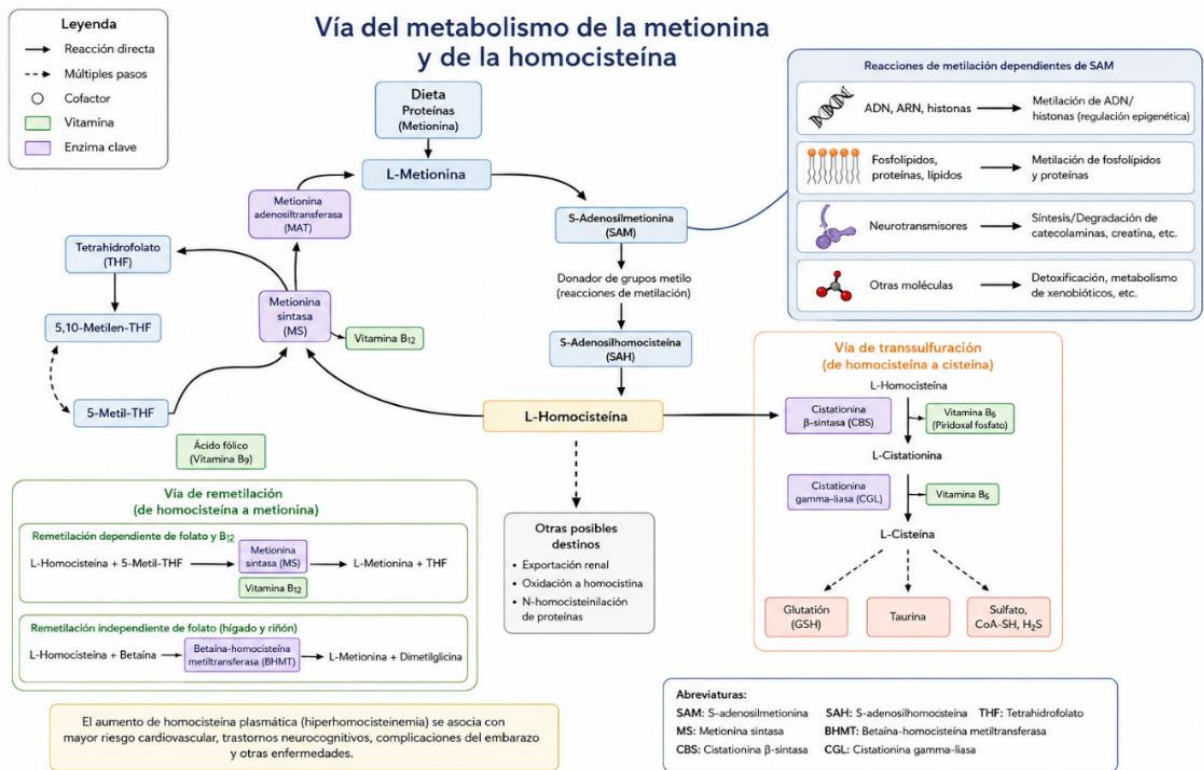


Figura 1. Metabolismo de la metionina y vía de la homocisteína

Diversos estudios han demostrado que la concentración plasmática de homocisteína está influenciada por múltiples factores: genéticos, nutricionales y ambientales. Entre los factores genéticos, los polimorfismos en el gen MTHFR representan un determinante crucial de la variabilidad de Hcy en la población, modulando la eficiencia de la remetilación. Por otro lado, una ingesta insuficiente de vitaminas del complejo B (B6, B12 y folato) puede limitar la conversión de Hcy a metionina y cisteína, incrementando su acumulación plasmática (Wrzosek & Ślusarczyk, 2022).

La evidencia reciente subraya que la identificación de factores genéticos y metabólicos que afectan los niveles de Hcy, como los polimorfismos C677T y A1298C del gen MTHFR, podría permitir la estratificación del riesgo individualizado, mejorando la prevención y el manejo de las complicaciones asociadas al riesgo cardiovascular (Kong et al., 2022; Meng et al., 2021; Wrzosek & Ślusarczyk, 2022).

2.4. Gen MTHFR y polimorfismos relevantes (C677T y A1298C)

El gen MTHFR está ubicado en el cromosoma 1 humano y codifica la enzima descrita (metilentetrahidrofolato reductasa), una enzima clave en el metabolismo del folato y la homocisteína, cuya función principal es catalizar la conversión de 5,10-metilentetrahidrofolato en 5-metiltetrahidrofolato, la forma activa que sirve como donante de grupos metilo para la remetilación de la homocisteína a metionina (Kong et al., 2022; Liu et al., 2024). La actividad de esta enzima determina la eficiencia del ciclo del folato y, por ende, regula indirectamente los niveles plasmáticos de homocisteína.

Entre los polimorfismos más estudiados, destacan C677T (rs1801133) y A1298C (rs1801131). El polimorfismo C677T consiste en una transición de citosina a timina en la posición 677 del exón 4, resultando en la sustitución del aminoácido alanina por valina. Esta variante produce una enzima termolábil con actividad reducida, particularmente en individuos homocigotos TT, quienes pueden experimentar una disminución de hasta el 70% en la función enzimática, mientras que los heterocigotos CT presentan una reducción aproximada del 35% (Meng et al., 2021; Kong et al., 2022).

El polimorfismo A1298C, por su parte, implica la sustitución de adenina por citosina en la posición 1298 (exón 7), generando un efecto más leve sobre la actividad enzimática. No obstante, cuando coexiste con el C677T en una condición de heterocigosis compuesta (CT/AC), la actividad de la MTHFR se reduce de manera significativa, alcanzando niveles comparables a los homocigotos TT para C677T (Liu et al., 2024; Mohammed et al., 2025).

Pese a la evidencia sólida, algunos estudios muestran resultados heterogéneos. Las discrepancias pueden explicarse por factores de confusión, como la ingesta de vitaminas del grupo B, edad, sexo, función renal y tratamientos farmacológicos, que pueden modular la relación entre los polimorfismos y los niveles de homocisteína (Planello et al., 2024; Pham et al., 2025).

2.5. Factores modificadores y variables de confusión

La relación entre los polimorfismos MTHFR y los niveles plasmáticos de homocisteína no se encuentra aislada de otros determinantes biológicos y ambientales. Diversos factores modificadores y variables de confusión pueden alterar la concentración de Hcy, impactando directamente la interpretación de los resultados en estudios clínicos y epidemiológicos.

2.6. Factores nutricionales

Una ingesta insuficiente de folato, vitamina B12 y vitamina B6 puede limitar la capacidad del organismo para convertir la homocisteína en metionina o cisteína, promoviendo la acumulación plasmática de Hcy (Wrzosek & Ślusarczyk, 2022; Kong et al., 2022). Los estudios recientes muestran que incluso individuos portadores de los polimorfismos C677T y A1298C pueden mantener niveles normales de Hcy si su dieta

es adecuada en vitaminas del grupo B, lo que enfatiza la importancia de considerar el estado nutricional como un modulador clave.

2.6.1. Edad y sexo

La concentración de homocisteína tiende a incrementar con la edad, lo que se ha atribuido a la disminución de la función renal y cambios en el metabolismo de vitaminas (Denis de Armas et al. 2024). Asimismo, se ha observado que los varones presentan niveles más altos de Hcy que las mujeres, probablemente debido a diferencias hormonales y de masa muscular, lo que constituye un factor de confusión que debe ser ajustado en los análisis estadísticos.

2.6.2. Función renal

La insuficiencia renal representa un determinante significativo de hiperhomocisteinemia, ya que la homocisteína se elimina principalmente por vía renal. Estudios recientes han demostrado la asociación de comorbilidades como pacientes con DM2 y función renal disminuida presentan Hcy elevada, independientemente de la presencia de polimorfismos genéticos (Pham et al., 2025; Mohammed et al., 2025).

2.6.3. Medicamentos y otras condiciones clínicas

El uso de ciertos fármacos, como metotrexato, anticonvulsivantes y diuréticos, puede interferir en el metabolismo del folato y aumentar los niveles de Hcy. Además, comorbilidades como hipertensión, dislipidemia y obesidad contribuyen a la variabilidad de Hcy en pacientes diabéticos (Meng et al., 2021; Kong et al., 2022).

2.7. Asociación MTHFR–Homocisteína–Enfermedad Cardiovascular

Las enfermedades cardiovasculares (ECV) comprenden un grupo de trastornos del corazón y vasos sanguíneos que incluyen la cardiopatía isquémica (infarto de

miocardio), la enfermedad cerebrovascular (ictus), la enfermedad arterial periférica, cardiopatía reumática, cardiopatía congénita y las tromboembolias venosas (trombosis/embolia). El modelo teórico que vincula MTHFR con estas enfermedades es el siguiente: los polimorfismos genéticos reducen la actividad de MTHFR, elevan la homocisteína sérica, y este exceso de homocisteína genera daño endotelial, hipercoagulabilidad y procesos ateroscleróticos. Así, se planteó que los portadores de 677T tendrían mayor hiperhomocisteinemia y, por ende, riesgo cardiovascular.

Cabe destacar que la elevación de la homocisteína puede actuar en sinergia con otros factores de riesgo. Por ejemplo, la combinación de diabetes mellitus con hiperhomocisteinemia tiene un efecto vascular adverso especialmente pronunciado. De ahí que en pacientes diabéticos –ya con riesgo elevado de enfermedad coronaria– se recomienda evaluar la homocisteína como un factor adicional de riesgo.

Similarmente, la hiperhomocisteinemia también ha sido implicada en la fisiopatología de ciertos tipos de hipertensión arterial, algunos autores han caracterizado la denominada “hipertensión tipo H” (HTA con hiperhomocisteinemia). Revisiones recientes sugieren que el genotipo TT de C677T podría estar significativamente asociado a esta forma de hipertensión resistente, actuando potencialmente como biomarcador predictivo. Por ejemplo, Kong et al. (2022) reportaron en un metaanálisis que el genotipo TT se asociaba con mayor susceptibilidad a la hipertensión H-HTN.

En suma, los fundamentos teóricos implican que los polimorfismos de MTHFR modifican la capacidad de reutilizar homocisteína, lo cual puede incrementar el daño endotelial y el riesgo aterotrombótico. Sin embargo, esta cascada depende también

de la disponibilidad de folato y otras vitaminas, así como de factores tradicionales (hipertensión, tabaquismo, obesidad, dislipidemia), que a su vez explican la mayoría de la variabilidad en riesgo cardiovascular.

2.8. Magnitud del problema y contexto epidemiológico

Las enfermedades cardiovasculares constituyen la principal causa de mortalidad global. La OMS reporta que en 2022 murieron $\approx 19,8$ millones de personas por ECV, esto es $\sim 32\%$ de todas las muertes mundiales; el 85% de estas defunciones se debieron a infarto de miocardio o ictus. La carga es particularmente alta en países de ingresos bajos y medios.

En América Latina las ECV también son muy prevalentes: cada año causan unos 5 millones de muertes ($\approx 37\%$ de la mortalidad total). Panamá no es excepción: los datos del Instituto Nacional de Estadística y Censos (INEC) indican que las enfermedades circulatorias fueron responsables de aproximadamente el 31% de las muertes panameñas entre 2001 y 2013. Ante esta realidad, en 2014 el Ministerio de Salud de Panamá impulsó un Plan Estratégico Nacional para la Prevención de Enfermedades No Transmisibles (ENT), enfocando esfuerzos en identificar y controlar los factores de riesgo cardiovascular. Este plan se sustenta legalmente en el Decreto Ejecutivo No. 1,510 del 19 de septiembre de 2014, que establece una estrategia nacional integral para ENT y sus factores de riesgo.

2.9. Controversias científicas

A pesar de los hallazgos indicados, la literatura registra controversias importantes. Por un lado, multitud de estudios observacionales y metaanálisis han encontrado asociaciones estadísticamente significativas entre el alelo T de MTHFR y el riesgo de

hipertensión, infarto o ictus en ciertas poblaciones. Por ejemplo, Meng et al. (2021) reportaron que el polimorfismo C677T estaba vinculado al riesgo de hipertensión esencial en varios análisis (aunque consideraron la evidencia global como “débil” tras ajustes). Asimismo, estudios de randomización mendeliana recientes (p. ej. Yuan et al., 2021) sugieren una posible relación causal de la homocisteína con el ictus: encontraron que un aumento genéticamente determinado de 1 SD en homocisteína elevaba el riesgo de ictus isquémico ($OR \approx 1.14$) y hemorragia subaracnoidea. Además, estos estudios señalan que niveles genéticamente más altos de folato y vitamina B6 parecen asociarse con menor riesgo de enfermedad coronaria y de ictus, lo que apoya la plausibilidad biológica de la vía folato-homocisteína-ECV.

Por otro lado, otros autores enfatizan la inconsistencia y la falta de robustez de los hallazgos. En consecuencia, los organismos científicos han tomado posiciones cautelosas. Por ejemplo, el American College of Medical Genetics and Genomics (ACMG) considera que en la mayoría de los casos no existe una razón médica para realizar pruebas de detección de MTHFR C677T/A1298C para evaluar riesgo de enfermedad, ya que la presencia de estas variantes no altera en forma demostrada el riesgo cardiovascular en la población general. En línea con esto, las guías clínicas actuales no recomiendan el cribado genético de MTHFR para prevención cardiovascular rutinaria. En cambio, se prioriza el control de los factores de riesgo establecidos (hipertensión, diabetes, lípidos, tabaquismo, etc.) y, en pacientes con hiperhomocisteinemia inexplicada, evaluar factores nutricionales y hábitos de vida antes que factores genéticos aislados.

3. CAPÍTULO III MARCO METODOLÓGICO

3.1. Tipo de estudio

La presente investigación corresponde a una revisión bibliográfica de carácter descriptivo y documental, orientada al análisis, síntesis e integración de la evidencia científica actual disponible sobre los polimorfismos del gen *MTHFR*, su relación con los niveles de homocisteína y su asociación con el riesgo cardiovascular.

3.2. Diseño metodológico

El tipo documental, descriptivo, retrospectivo y de enfoque cualitativo. Utiliza como fuente de información publicaciones científicas previamente elaboradas, expone y caracteriza los hallazgos reportados en la literatura, analiza información generada durante la investigación, se centra en la interpretación, comparación y síntesis de la evidencia científica disponible, sin manipular variables ni intervenir sobre poblaciones de estudio.

3.3. Metodología

3.3.1. Búsqueda en la Base de Datos

Se llevo a cabo una búsqueda sistemática en las bases de datos PubMed y Google Scholar para identificar estudios originales (casos y controles, cohortes y estudios transversales analíticos), revisiones sistemáticas y metaanálisis relevantes publicados entre los años 2016 y 2026.

Para identificar los estudios elegibles fueron utilizados los siguientes descriptores de búsqueda en inglés y en español:

- Español: *MTHFR*, *metilentetrahidrofolato reductasa*, *homocisteína*, *hiperhomocisteinemia*, *polimorfismo*, *riesgo cardiovascular*, *C677T*, *A1298C*.

- Inglés: *MTHFR, methylenetetrahydrofolate reductase, homocysteine, hyperhomocysteinemia, polymorphism, cardiovascular risk, C677T, A1298C.*

Con el propósito de optimizar la sensibilidad y especificidad de la búsqueda, se emplearon los operadores booleanos (“**AND**”, “**OR**”), paréntesis y comillas para priorizar los términos de interés.

La fórmula principal de búsqueda utilizada fue la siguiente:

("homocysteine" **OR** "hyperhomocysteinemia" **OR** "HCY") **AND** ("MTHFR" **OR** "Methylenetetrahydrofolate reductase") **AND** ("polymorphisms" **OR** "C667T" **OR** "A1298C") **AND** ("cardiovascular risk")

3.3.2. Criterios de Elegibilidad

Para garantizar la pertinencia de la evidencia científica analizada, se establecieron criterios de inclusión y exclusión que permitieron seleccionar los estudios más relevantes para los objetivos de la investigación (Tabla 1).

Tabla 1. Criterios de Inclusión y Exclusión

Criterios de inclusión
Estudios originales: Casos y Controles, Cohorte, Corte transversales analíticos, metaanálisis
Criterio geográfico: Internacional
Criterio de temporalidad: 10 años (2016-2026)
Publicaciones en inglés o español
Artículos que evalúen polimorfismos MTHFR (C677T y/o A1298C), medición de homocisteína y/o relación con ECV
Criterios de exclusión
Estudios que no aborden claramente los conceptos de interés
Estudios con población pediátrica o realizados en animales
Artículos sin acceso a texto completo
Reportes de caso, cartas al editor o resúmenes sin revisión por pares
Duplicados

Se identificó un total de 91 publicaciones, tras el proceso de cribado, se eliminaron 2 estudios duplicados. Posteriormente, 12 estudios fueron excluidos tras la revisión del título y resumen por no abordar los conceptos de interés.

Adicionalmente, se descartaron 31 estudios por no disponer de acceso a texto completo, 19 estudios por corresponder a investigaciones realizadas en animales y 17 por incluir población pediátrica.

Finalmente, 10 estudios cumplieron con los criterios de inclusión establecidos y fueron seleccionados para el análisis y síntesis de la evidencia científica.



Figura 2. Diagrama de flujo para la selección de estudios

Con el fin de facilitar la comparación de los estudios incluidos, se elaboró una matriz que reúne los aspectos más relevantes de cada publicación (Tabla 2).

Tabla 2. CARACTERÍSTICAS GENERALES DE LOS ESTUDIOS - MATRIZ DE LA REVISIÓN

Título	Autor / Año	País de origen / Idioma	Diseño	Población / Muestras	Polimorfismo	Nivel de Homocisteína	Hallazgos Principales
Asociación bioquímica del polimorfismo C677T del gen MTHFR con el infarto de miocardio en presencia de diabetes mellitus como factor de riesgo	Mallhi et al., 2023	Pakistán / Inglés	Estudio de Casos y Controles	300 participantes: Grupo control (n=100) Pacientes IM (n=100) Pacientes IM-DM (n=100)	C677T	Elevado, asociado al polimorfismo MTHFR C677T	Asociación entre polimorfismo C677T MTHFR y pacientes con infarto de miocardio (IM) y los pacientes con IM y diabetes mellitus.
Los niveles de homocisteína, la hipertensión y los genotipos C677T del gen MTHFR: una interacción compleja	Al Hageh et al., 2023	Líbano / Inglés	Estudio de casos y controles	2.640 participantes: Hipertensos: (n=1.589) Grupo control: (n=1.051)	C677T	Elevado, asociado al polimorfismo MTHFR C677T	Asociación de HHcy al polimorfismo C677T y a un alto riesgo de ictus como consecuencia de HTN.
Asociación entre los niveles de homocisteína y metionina y el polimorfismo MTHFR 677C>T con la tasa de desarrollo de multimorbilidad cardiovascular en personas mayores en Suecia	Calderón-Larrañaga et al., 2020	Suecia / Inglés	Estudio de cohorte prospectiva longitudinal poblacional	Datos de 1969 personas con información completa y sin enfermedades cardiovasculares basales, recopilados del 2001 al 2016. El análisis de los datos se llevó a cabo en 2019.	C677T	Elevado, asociado al polimorfismo MTHFR C677T	Niveles elevados de homocisteína y bajos de metionina asociados al desarrollo de multimorbilidad cardiovascular. El polimorfismo C677T incrementó este riesgo.

Asociación de los polimorfismos C677T y A1298C del gen MTHFR con los niveles séricos de homocisteína, folato y vitamina B12 en pacientes con enfermedad coronaria de aparición temprana	Shivkar et al., 2021	India / Inglés	Estudio de Casos y Controles	90 participantes: EAC: (n = 45) Grupo control: (n = 45)	C677T A1298C	Homocisteína elevada independiente de los polimorfismos MTHFR	Homocisteína elevada asociada a ECT, independiente de la presencia o ausencia de los polimorfismos C677T o A1298C.
El gen MTHFR y el riesgo de accidente cerebrovascular y enfermedad cardíaca en una población con bajos niveles de folato: un estudio prospectivo con 156 000 adultos chinos	Bennett et al., 2023	China / Inglés	Estudio de cohorte prospectiva longitudinal poblacional	Se determinó el genotipo del gen MTHFR en 156 253 participantes y 12 240 sufrieron un ictus durante los 12 años de seguimiento	C677T	Elevado, asociado al polimorfismo MTHFR C677T	MTHFR C677T aumenta el riesgo de accidente cerebrovascular en población con bajos niveles de folato.
El polimorfismo genético C677T de la 5,10-metilentetrahidrofolato reductasa como factor de riesgo de enfermedad coronaria prematura en pacientes con diabetes mellitus tipo 2	Mohammed et al., 2025	Sudan / Inglés	Estudio comparativo, técnica de muestreo selectivo no probabilístico (caso-control)	226 pacientes T2DM: Con EAC: (n = 113) Sin EAC: (n = 113)	C677T	Elevado, asociado al polimorfismo MTHFR C677T	El polimorfismo C677T MTHFR y la hiperhomocisteinemia son factores de riesgo de cardiopatía coronaria primaria en población con diabetes mellitus T2.

Una revisión sistemática y un metaanálisis que analizan la relación entre el polimorfismo del gen de la metilentetrahidrofolato reductasa y el riesgo de hemorragia intracerebral en la población	Zou et al., 2022	China / Inglés	Metaanálisis: 20 estudios caso-control	1.989 pacientes con hemorragia intracerebral y 4.032 controles sanos procedentes de poblaciones asiáticas, caucásicas y africanas.	C677T A1298C	Elevado, asociado al polimorfismo MTHFR C677T	Hhcty y Polimorfismo MTHFR C677T asociado a un mayor riesgo de hemorragia intracerebral. polimorfismo A1298C sin asociación significativa.
Asociación del polimorfismo genético rs1801133 (677C>T) de la metilenotetrahidrofolato reductasa (MTHFR) con el riesgo de ictus isquémico en diferentes poblaciones: un metaanálisis actualizado	Zhao et al., 2023	China / Inglés	Metaanálisis: 96 estudios caso-control	34,814 participantes: 15,569 casos 19,245 controles	C677T	Elevado, asociado al polimorfismo MTHFR C677T	El polimorfismo C677T MTHFR contribuye a un mayor riesgo de accidente isquémico cerebral.
Estudio prospectivo de cohorte sobre el polimorfismo genético C677T MTHFR y su influencia en el efecto terapéutico de la homocisteína en pacientes con ictus e hiperhomocisteinemia	Du et al., 2020	China / Inglés	Estudio de Cohorte Prospectivo	162 participantes (han sufrido un ictus) - grupos según eficacia al tratamiento: eficacia buena: (n=103) eficacia deficiente: (n=59)	C677T	Elevado, asociado al polimorfismo MTHFR C677T	C677T constituye un factor de riesgo independiente de la escasa eficacia del tratamiento para reducir los niveles de homocisteína.
Prevalencia del polimorfismo C677T del gen MTHFR y su asociación con los niveles séricos de homocisteína y la presión arterial en diferentes grupos étnicos: conclusiones de un estudio de cohorte realizado en Nepal	Bhatt et al., 2025	Nepal / Inglés	Estudio transversal	489 muestras de participantes: 227 genotipos CC 187 heterocigotos CT 75 mutantes homocigotos TT	C677T	Elevado, asociado al polimorfismo MTHFR C677T	Según la etnia mayor frecuencia de mutaciones; los homocigotos TT se asocian con niveles elevados de homocisteína sérica y aumento de la presión arterial.

Elaborada por el autor

Los artículos seleccionados fueron publicados entre los años 2020 y 2025; en cuanto a la distribución geográfica, la mayoría de los estudios fueron realizados en población asiática, mientras que uno se llevó a cabo en población africana y otro en población europea. Adicionalmente, el metaanálisis de Zhao et al. para determinar las posibles causas de la heterogeneidad, incluyó diferentes grupos de ascendencia asiática, europea, africana e hispana.



Tabla 3. distribución continente–país

Africa	Sudan	Mohammed et al., 2025
Asia	China	Bennett et al., 2023
		Zou et al., 2022
		Zhao et al., 2023
		Du et al., 2020
	India	Shivkar et al., 2021
	Nepal	Bhatt et al., 2025
Europa	Pakistán	Malhi et al., 2023
	Líbano	Al Hageh et al., 2023
	Suecia	Calderón et al., 2020

3.3.3. Evaluación de la calidad

La valoración de la calidad de los estudios se realizó mediante la aplicación de las plantillas del **Programa de Habilidades en Lectura Crítica en Español (CASPe)** y **Herramientas de evaluación crítica de JBI** (Instituto Joanna Briggs), de acuerdo con el diseño metodológico de cada artículo.

Los estudios de casos-contrroles y de cohorte fueron evaluados mediante las plantillas CASPe de 11 preguntas, mientras que las revisiones sistemáticas y metaanálisis se valoraron con la plantilla CASPe de 10 preguntas. Por su parte, los estudios transversales fueron evaluados utilizando la herramienta JBI (Joanna Briggs Institute), compuesta por 8 preguntas.

Para garantizar la evaluación, cada respuesta afirmativa recibió 1 punto, mientras que las respuestas “No” y “No sé” recibieron 0 puntos. Una puntuación de 0 a 3 se considera de baja calidad, mientras que una puntuación igual o mayor a 7 se considera de alta calidad.

Tabla 4. Resultados de la evaluación crítica de las evidencias científicas

Autor-año	Plantilla aplicada	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	Calidad metodológica
Mallhi et al., 2023	CASPe: caso-control	1	1	1	1	1	0	1	1	1	1	1	Alta
Al Hageh et al., 2023	CASPe: caso-control	1	1	1	1	1	1	1	1	1	1	1	Alta
Calderón et al., 2020	CASPe: cohorte	1	1	1	1	1	1	1	1	1	1	1	Alta
Shivkar et al., 2021	CASPe: caso-control	1	1	1	1	1	1	1	1	1	1	1	Alta
Bennett et al., 2023	CASPe: cohorte	1	1	1	1	1	1	1	1	1	1	1	Alta
Mohammed et al., 2025	CASPe: caso-control	1	1	1	1	0	0	1	1	1	1	1	Alta
Zou et al., 2022	CASPe: metaanálisis	1	1	1	1	1	1	1	1	1	1	N/A	Alta
Zhao et al., 2023	CASPe: metaanálisis	1	1	1	1	1	1	1	1	1	1	N/A	Alta
Du et al., 2020	CASPe: cohorte	1	1	1	1	1	1	1	1	1	1	1	Alta
Bhatt et al., 2025	JB1: transversal	1	1	1	1	1	1	1	1	N/A	N/A	N/A	Alta

Elaborada por el autor

4. CAPÍTULO IV RESULTADOS

4.1. Características y participantes de los estudios seleccionados

De los 10 estudios seleccionados, cuatro correspondieron a estudios de casos y controles, tres a estudios de cohorte prospectiva, un estudio de diseño transversal y dos metaanálisis.

El tamaño de las muestras oscilaba desde 90 hasta 156,253 participantes en estudios de caso-control y cohorte poblacional. En los metaanálisis, las muestras alcanzaron un rango de 6,021 hasta 34,814 participantes.

Además, las poblaciones analizadas abarcaron distintos grupos etarios, desde adultos jóvenes con enfermedad coronaria prematura hasta adultos mayores seguidos longitudinalmente.

Esta diversidad de los diseños metodológicos, heterogeneidad en el tamaño muestral y características poblacionales permitió analizar desde distintas perspectivas epidemiológicas la asociación entre los polimorfismos del gen *MTHFR* y el riesgo cardiovascular.

4.2. Asociación entre los polimorfismos MTHFR y los niveles de homocisteína

4.2.1 MTHFR y C677T

Los estudios analizados demostraron la existencia de asociaciones entre el polimorfismo genético MTHFR C677T (rs1801133) y el incremento de los niveles séricos de homocisteína. Esto sucede debido a que la mutación C677T reduce la actividad de la enzima MTHFR, lo que provoca una alteración en el metabolismo del folato y, en consecuencia, un aumento de los niveles de homocisteína (Castro et al., 2004).

En general, se observaron niveles más elevados de homocisteína en los pacientes portadores del alelo T en comparación con los portadores del alelo C. Asimismo, en comparación con el genotipo CC y CT, los TT homocigotos mutantes presentaron niveles de homocisteína notablemente más elevados (Frosst et al., 1995). Independiente de la patología o desenlace cardiovascular, el genotipo TT mostro una asociación más consistente con la hiperhomocisteinemia.

4.2.2 MTHFR y A1298C

De los estudios que evaluaron el polimorfismo MTHFR A1298C, la evidencia difiere de lo observado con el polimorfismo C677T, no se encontraron asociaciones significativas a los niveles plasmáticos de homocisteína, o folato.

Los estudios que abordaban el polimorfismo A1298C MTHFR presentaron diversas limitaciones, entre ellas el reducido tamaño muestral y la escasez de investigaciones publicadas, dificultando establecer conclusiones definitivas sobre la asociación del polimorfismo A1298C, niveles de homocisteína y riesgo cardiovascular.

4.3. Homocisteína y riesgo cardiovascular

Los estudios analizados mostraron resultados heterogéneos, la mayoría de los estudios incluidos respaldan la hipótesis de que el polimorfismo MTHFR C677T, particularmente el genotipo homocigoto TT, favorece el desarrollo de hiperhomocisteinemia y contribuye al incremento del riesgo cardiovascular.

En contraste, Shivkar et al. (2021) encontraron que los niveles elevados de homocisteína sérica se asociaron con un mayor riesgo de enfermedad coronaria de aparición temprana, independiente de los polimorfismos MTHFR C677T y A1298C.

De manera similar, varios estudios señalaron que la relación entre polimorfismos MTHFR, los niveles de homocisteína y el riesgo cardiovascular pueden ser modulados por otros factores, como la edad, etnia y niveles de folato.

4.4. Influencia de la etnia y otros factores modificadores

4.4.1. Etnia

A nivel mundial, la frecuencia de las mutaciones del gen MTHFR varía entre los distintos grupos raciales y étnicos (Reyes et al., 2021; Botto et al., 2000). En África, el

polimorfismo del gen MTHFR es notablemente bajo (por debajo del 10 %) para el alelo 677 T (Raghubeer et al., 2021; Atadzhanov et al., 2014). En europeos y norteamericanos, la frecuencia oscila entre el 10 % y el 18 %; por el contrario, la frecuencia del alelo T es notablemente alta entre las poblaciones asiáticas, situándose en torno al 45 % (Zhang et al., 2019).

Se corrobora como la asociación entre el polimorfismo C677T y el riesgo cardiovascular mostró variaciones según la población estudiada. Por ejemplo, el estudio de Bhatt et al., 2025 en Nepal, evidencio como de las diferentes etnias, Brahmin, Chhetri, Newar y Tamang, la etnia Newar era la que presentaba una mayor prevalencia de la mutación genética MTHFR C677T. Asimismo, los metaanálisis Zou et al., 2022; Zhao et al., 2023, demostraron asociaciones más consistentes en poblaciones asiáticas y caucásicas, mientras que en poblaciones africanas los resultados fueron menos concluyentes.

4.4.2. Folato

Los homocigotos TT con niveles bajos de folato presentaban una mayor probabilidad de padecer enfermedad coronaria, mientras que los homocigotos TT con niveles altos de folato presentaban una menor probabilidad de padecerla (Mallhi et al., 2023).

Los efectos adversos de los homocigotos TT, caracterizados por un aumento de los niveles de homocisteína plasmática, se ven atenuados por suplementación con ácido fólico (Bennett et al., 2023).

No obstante, Du et al., 2020 determinó que los pacientes afectados por el alelo T y el genotipo TT del polimorfismo C677T MTHFR, tenían una baja respuesta a los

comprimidos de ácido fólico, vitamina B6 y metilcobalamina, y necesitaban ciclos de tratamiento más prolongados.

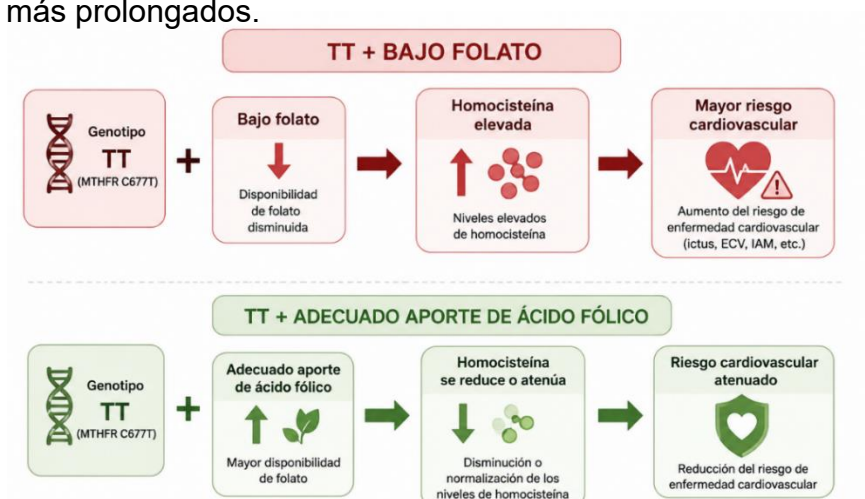


Figura 4. Diagrama de la interacción MTHFR C677T (TT), folato y homocisteína.

4.4.3 Edad

Calderón-Larrañaga et al. (2020) observaron que los niveles elevados de homocisteína y las concentraciones bajas de metionina se asociaron con un desarrollo más acelerado de multimorbilidad cardiovascular en adultos mayores.

Además, los autores encontraron que la asociación entre el polimorfismo MTHFR C677T y las bajas concentraciones de metionina era más evidente en los participantes de mayor edad, sugiriendo que el efecto metabólico del polimorfismo sobre la vía de la metionina-homocisteína puede manifestarse con mayor intensidad en edades avanzadas.

Para facilitar la comparación de los hallazgos entre los estudios analizados, se presenta una síntesis de las asociaciones observadas entre los polimorfismos MTHFR C677T y A1298C, los niveles de homocisteína, los distintos desenlaces cardiovasculares evaluados y comorbilidades, factores confusores y covariables (Tabla 5). Además, se resumen los principales resultados reportados por cada estudio.

Tabla No. 5 Resultados de la asociación entre las mutaciones C677T y A1298C, nivel de homocisteína y riesgo (desenlace cardiovascular).

Estudio	Desenlace cardiovascular	Polimorfismo	Nivel de homocisteína	Comorbilidades, factores confusores y covariables	Resultado
Mallhi et al., 2023	Infarto de miocardio	C677T	Elevado en genotipo homocigoto T/T	Diabetes mellitus	factor de riesgo significativo entre los pacientes con infarto de miocardio
Al Hageh et al., 2023	Hipertensión tipo H	C677T	Elevado en genotipo homocigoto T/T	Historial de hipertensión, fumadores, diabetes, ictus, edad, uso de medicamentos.	asociación entre los niveles elevados de homocisteína y la hipertensión, el tratamiento farmacológico para la hipertensión y el riesgo de accidente cerebrovascular en pacientes con el alelo T del gen MTHFR, especialmente en el genotipo TT.
Calderón Larrañaga et al., 2020	Multimorbilidad cardiovascular en adultos mayores	C677T	El genotipo TT se asoció con niveles más elevados de homocisteína en comparación con el genotipo CC.	Edad, sexo, nivel educativo, hipertensión, diabetes, hipercolesterolemia, enfermedad renal crónica, IMC, tabaquismo, consumo de alcohol, actividad física, uso de vitaminas B.	A mayor concentración de tHcy, menor concentración de Met, evidenciando una actividad de metilación alterada, asociada con una mayor tasa de desarrollo de multimorbilidad cardiovascular. El polimorfismo C677T contribuyó aún más a acelerar el desarrollo de multimorbilidad cardiovascular en los participantes con concentraciones bajas de Met.
Shivkar et al., 2021	Enfermedad coronaria prematura	C677T A1298C	Homocisteína elevada independiente de los polimorfismos MTHFR	Edad, dislipidemia, niveles de folato y B12.	Los niveles elevados de homocisteína sérica se asociaron con un mayor riesgo de EAC en el grupo de edad joven (<45 años) independientemente de los polimorfismos MTHFR C677T y A1298C.

Bennett et al., 2023	Accidente cerebrovascular y cardiopatías	C677T	Niveles elevados asociado al polimorfismo C677T	Edad, sexo, poblaciones fortificadas y no fortificadas con ácido fólico.	El polimorfismo C677T del gen MTHFR se asoció con un mayor riesgo de accidente cerebrovascular. Los hallazgos se corroboraron mediante ensayos de ácido fólico y la implementación de programas obligatorios de fortificación con ácido fólico para la prevención del accidente cerebrovascular en poblaciones con bajos niveles de folato.
Mohammed et al., 2025	Enfermedad coronaria prematura	C677T	Niveles más elevados de homocisteína en el genotipo TT que en el genotipo CC	Diabetes mellitus tipo 2, edad,	El polimorfismo C677T del gen MTHFR y la hiperhomocisteinemia son factores de riesgo para la ECP.
Zou et al., 2022	Hemorragia intracerebral	C677T A1298C	El alelo T se correlaciona con niveles elevados de homocisteína	Etnia, edad, número de casos y controles, método de genotipado.	El polimorfismo C677T está relacionado con el riesgo de hemorragia intracerebral (HIC), principalmente en poblaciones asiáticas; y las personas con el genotipo TT presentan un mayor riesgo de HIC que las que tienen los genotipos CC y CT. No se encontró una correlación significativa entre A1298C y el riesgo de HIC.

Zhao et al., 2023	Ictus isquémico	C677T	Niveles elevados asociado al polimorfismo C677T	Etnia, edad, número de casos y controles, método de genotipado.	Asociación significativa en la población asiática, hispana o latina, así como en personas de mediana edad y de edad avanzada, del polimorfismo C677T y el ictus isquémico.
Du et al., 2020	Influencia terapéutica sobre la homocisteína en pacientes con ictus	C677T	Niveles elevados asociado al polimorfismo C677T	Edad, hipertensión, hiperuricemia, hct tras el tratamiento, y genotipo MTHFR	El alelo T y el genotipo TT del polimorfismo C677T del gen MTHFR se asociaron con una baja eficacia del tratamiento para reducir los niveles de homocisteína en pacientes con ictus.
Bhatt et al., 2025	Presión arterial	C677T	Hiperhomocisteinemia inducido por el polimorfismo C677T	Edad, etnia, patrón alimentario.	Prevalencia significativa de la mutación genética MTHFR C677T entre las etnias. Relación entre la mutación del gen MTHFR, la hiperhomocisteinemia, y la hipertensión arterial,

Elaborada por el autor

DISCUSIÓN

A lo largo de los años, las enfermedades cardiovasculares (ECV) se han identificado como una de las principales causas de muerte.

La homocisteína es un aminoácido intermediario en la conversión de la metionina en cisteína. Su nivel depende de la ingesta alimentaria y de la actividad de la enzima metilenotetrahidrofolato reductasa (MTHFR) (Doevendans et al., 2001).

Los polimorfismos del gen MTHFR, C677T y A1298C, son los más comunes y los más estudiados. Estos son los únicos dos polimorfismos de los que se sabe actualmente que tienen un efecto confirmado sobre la actividad enzimática (Leclerc et al., 2025).

La mutación MTHFR rs1801133 (C677T) conduce a una disminución de la actividad de la MTHFR y a niveles elevados de homocisteína (Castro et al., 2004). La hiperhomocisteinemia está relacionada con la sobreproducción de radicales libres (Xi et al., 2016), la inducción de estrés oxidativo (Esse et al., 2019; Tchantchou et al., 2021), la lesión endotelial (Salvio et al., 2021), la coagulación y la alteración del metabolismo lipídico (Herrmann, 2001), factores que contribuyen a la incidencia del riesgo cardiovascular.

Esta revisión bibliográfica respalda la existencia de asociaciones entre el polimorfismo genético MTHFR rs1801133 (C677T) y la hiperhomocisteinemia. En la mayoría de los estudios analizados, los portadores del alelo T, particularmente el genotipo homocigoto TT, presentaron concentraciones más elevadas de homocisteína, atribuibles a una disminución de la actividad enzimática de la MTHFR.

Asimismo, diversos estudios observaron una asociación positiva entre el polimorfismo C677T (rs1801133) y las enfermedades cardiovasculares (Mallhi et al., 2023; Mohammed et al., 2025; Bennett et al., 2023; Calderón-Larrañaga et al., 2020; Al Hageh et al., 2023; Zou et al., 2022; Zhao et al., 2023; Bhatt et al., 2025), mientras que otros no encontraron ninguna asociación entre el polimorfismo C677T y el riesgo de ECV (Shivkar et al., 2021; Du et al., 2020).

Se evidencio que la asociación entre el polimorfismo MTHFR, la homocisteína y el riesgo cardiovascular puede verse modificada por factores como la edad, la etnia y el estado nutricional.

El folato también emergió como un factor modulador relevante. Los estudios revisados sugieren que la suplementación o fortificación con ácido fólico puede atenuar el efecto del genotipo TT sobre los niveles de homocisteína, reduciendo parcialmente el riesgo cardiovascular asociado.

La escasa evidencia disponible respecto a los polimorfismos A1298C no encontró asociaciones significativas con los niveles de homocisteína ni con el riesgo cardiovascular. Sin embargo, la baja cantidad de investigaciones publicadas y los tamaños muestrales reducidos limitan la solidez de estas conclusiones.

Entre las fortalezas de esta revisión destaca la inclusión de diferentes diseños epidemiológicos, así como la evaluación metodológica de todos los estudios mediante herramientas validadas de lectura crítica. Sin embargo, también hubo limitaciones como la heterogeneidad de las poblaciones, la variabilidad en los desenlaces cardiovasculares y el predominio de estudios realizados en poblaciones asiáticas, lo que limita la generalización de los resultados a otras regiones.

CONCLUSIÓN

1. La evidencia analizada indica que el polimorfismo C677T, se asocia con una disminución de la MTHFR, la información sobre el polimorfismo A1298C y su impacto funcional sobre la actividad de la MTHFR es limitada.

2. Se asocio que el polimorfismo MTHFR C677T, particularmente el genotipo homocigoto TT, constituye un factor genético relevante en la regulación de los niveles de homocisteína.

3. A su vez, la hiperhomocisteinemia se relaciona con diversos desenlaces cardiovasculares.

4. La asociación entre los polimorfismos del gen MTHFR, los niveles de homocisteína y el riesgo cardiovascular parece estar influenciada por factores genéticos, bioquímicos y clínicos, entre ellos la edad, la etnia, la presencia de comorbilidades y el estado nutricional de folato.

5. La literatura revisada evidenció una predominancia de estudios realizados en población asiática, y una escasez de investigaciones en poblaciones latinoamericanas. Asimismo, la evidencia disponible para el polimorfismo A1298C es limitada e insuficiente para establecer conclusiones definitivas sobre su papel en la hiperhomocisteinemia y el riesgo cardiovascular.

Lagunas de conocimiento y recomendaciones para futuras investigaciones

1. Realizar más estudios sobre el polimorfismo **A1298C**.
2. Evaluar simultáneamente los polimorfismos MTHFR, los niveles de homocisteína, folato y vitamina B12 en una misma población.

3. Investigar con mayor profundidad el efecto de la suplementación y fortificación con ácido fólico en portadores del genotipo TT.
4. Desarrollar estudios en poblaciones latinoamericanas
5. Evaluar el potencial de estrategias terapéuticas personalizadas basadas en el genotipo MTHFR para la prevención del riesgo cardiovascular.

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ANEXOS



PROGRAMA DE LECTURA CRÍTICA CASPe Leyendo críticamente la evidencia clínica

11 preguntas para ayudarte a entender un estudio de Casos y Controles

Comentarios generales

- Hay tres aspectos generales a tener en cuenta cuando se hace lectura crítica de un estudio de Casos y Controles:

¿Son válidos los resultados del estudio?

¿Cuáles son los resultados?

¿Pueden aplicarse en tu medio?

Las 11 preguntas contenidas en las siguientes páginas están diseñadas para ayudarte a pensar sistemáticamente sobre estos temas.

- Las dos primeras preguntas son “de eliminación” y pueden contestarse rápidamente. Sólo si la respuesta a estas dos preguntas es afirmativa, merece la pena continuar con las restantes.
- Hay un cierto grado de solapamiento entre algunas de las preguntas.
- En la mayoría de las preguntas se te pide que respondas “sí”, “no” o “no sé”.
- En itálica y debajo de las preguntas encontrarás una serie de pistas para contestar a las preguntas. Están pensadas para recordarte por qué la pregunta es importante. ¡En los pequeños grupos no suele haber tiempo para responder a todo con detalle!

A/ ¿Son los resultados del estudio válidos?

Preguntas de eliminación

1 ¿El estudio se centra en un tema claramente definido? <i>PISTA: Una pregunta se puede definir en términos de</i> - La población estudiada. - Los factores de riesgo estudiados. - Si el estudio intentó detectar un efecto beneficioso o perjudicial.	☐ SÍ ☐ NO SÉ ☐ NO
2 ¿Los autores han utilizado un método apropiado para responder a la pregunta? <i>PISTA: Considerar</i> - ¿Es el estudio de Casos y Controles una forma adecuada para contestar la pregunta en estas circunstancias? (¿Es el resultado a estudio raro o perjudicial?). - ¿El estudio está dirigido a contestar la pregunta?	☐ SÍ ☐ NO SÉ ☐ NO

¿Merece la pena continuar?

Preguntas de detalle

3 ¿Los casos se reclutaron/incluyeron de una forma aceptable? <i>PISTA: Se trata de buscar sesgo de selección que pueda comprometer la validez de los hallazgos</i> - ¿Los casos se han definido de forma precisa? - ¿Los casos son representativos de una población definida (geográfica y/o temporalmente)? - ¿Se estableció un sistema fiable para la selección de todos los casos? - ¿Son incidencia o prevalencia? - ¿Hay algo “especial” que afecta a los casos? - ¿El marco temporal del estudio es relevante en relación a la enfermedad/exposición? - ¿Se seleccionó un número suficiente de casos? - ¿Tiene potencia estadística?	☐ SÍ ☐ NO SÉ ☐ NO
4 ¿Los controles se seleccionaron de una manera aceptable? <i>PISTA: Se trata de buscar sesgo de selección que pueda comprometer la generalizabilidad de los hallazgos.</i> - ¿Los controles son representativos de una población definida (geográfica y/o temporalmente)? - ¿Hay algo “especial” que afecta a los controles? - ¿Hay muchos no respondedores? - ¿Podrían ser los no respondedores de alguna manera diferentes al resto? - ¿Han sido seleccionados de forma aleatorizada, basados en una población? - ¿Se seleccionó un número suficiente de controles?	☐ SÍ ☐ NO SÉ ☐ NO

5 ¿La exposición se midió de forma precisa con el fin de minimizar posibles sesgos? <i>PISTA: Estamos buscando sesgos de medida, retirada o de clasificación:</i> - ¿Se definió la exposición claramente y se midió ésta de forma precisa? - ¿Los autores utilizaron variables objetivas o subjetivas? - ¿Las variables reflejan de forma adecuada aquello que se suponen que tiene que medir? (han sido validadas). - ¿Los métodos de medida fueron similares tanto en los casos como en los controles? - ¿Cuando fue posible, se utilizó en el estudio cegamiento? - ¿La relación temporal es correcta (la exposición de interés precede al resultado/variable de medida)?	☐ SÍ ☐ NO SÉ ☐ NO
6 A. ¿Qué factores de confusión han tenido en cuenta los autores? <i>Haz una lista de los factores que piensas que son importantes y que los autores han omitido (genéticos, ambientales, socioeconómicos).</i> B. ¿Han tenido en cuenta los autores el potencial de los factores de confusión en el diseño y/o análisis? <i>PISTA: Busca restricciones en el diseño y técnica, por ejemplo, análisis de modelización, estratificación, regresión o de sensibilidad para corregir, controlar o ajustar los factores de confusión.</i>	Lista: ☐ SÍ ☐ NO SÉ ☐ NO

B/ ¿Cuáles son los resultados?

7 ¿Cuáles son los resultados de este estudio?

PISTA:

- ¿Cuáles son los resultados netos?
- ¿El análisis es apropiado para su diseño?
- ¿Cuán fuerte es la relación de asociación entre la exposición y el resultado (mira los odds ratio (OR))?
- ¿Los resultados se han ajustado a los posibles factores de confusión y, aun así, podrían estos factores explicar la asociación?
- ¿Los ajustes han modificado de forma sustancial los OR?

8 ¿Cuál es la precisión de los resultados?

¿Cuál es la precisión de la estimación del riesgo?

PISTA:

- Tamaño del valor de P.
- Tamaño de los intervalos de confianza.
- ¿Los autores han considerado todas las variables importantes?
- ¿Cuál fue el efecto de los individuos que rechazaron el participar en la evaluación?

9 ¿Te crees los resultados?

PISTA:

- ¡Un efecto grande es difícil de ignorar!
- ¿Puede deberse al azar, sesgo o confusión?
- ¿El diseño y los métodos de este estudio son lo suficientemente defectuosos para hacer que los resultados sean poco creíbles?
- Considera los criterios de Bradford Hills (por ejemplo, secuencia temporal, gradiente dosis-respuesta, fortaleza de asociación, verosimilitud biológica).

☐
SÍ

☐
NO SÉ

☐
NO

¿Merece la pena continuar?

C/ ¿Son los resultados aplicables a tu medio?

10 ¿Se pueden aplicar los resultados a tu medio?	☐ SÍ	☐ NO SÉ	☐ NO
<i>PISTA: Considera si</i>			
- Los pacientes cubiertos por el estudio pueden ser suficientemente diferentes de los de tu área. - Tu medio parece ser muy diferente al del estudio. - ¿Puedes estimar los beneficios y perjuicios en tu medio?			
11 ¿Los resultados de este estudio coinciden con otra evidencia disponible?	☐ SÍ	☐ NO SÉ	☐ NO
<i>PISTA:</i>			
<i>Considera toda la evidencia disponible: Ensayos Clínicos aleatorizados, Revisiones Sistemáticas, Estudios de Cohorte y Estudios de Casos y Controles, así como su consistencia.</i>			



PROGRAMA DE LECTURA CRÍTICA CASPe

Leyendo críticamente la evidencia clínica

11 preguntas para ayudarte a entender un estudio de cohortes

Comentarios generales

- Hay tres aspectos generales a tener en cuenta cuando se hace lectura crítica de un estudio de Cohortes:

¿Son válidos los resultados del estudio?

¿Cuáles son los resultados?

¿Pueden aplicarse a tu medio?

Las 11 preguntas contenidas en las siguientes páginas están diseñadas para ayudarte a pensar sistemáticamente sobre estos temas.

- Las dos primeras preguntas son “de eliminación” y pueden contestarse rápidamente. Sólo si la respuesta a estas dos preguntas es afirmativa, merece la pena continuar con las restantes.
- Puede haber cierto grado de solapamiento entre algunas de las preguntas.
- En *itálica* y debajo de las preguntas encontrarás una serie de pistas para contestar a las mismas. Están pensadas para recordarte por qué la pregunta es importante. ¡En los pequeños grupos no suele haber tiempo para responder a todo con detalle!

El marco conceptual necesario para la interpretación y el uso de estos instrumentos puede encontrarse en la referencia de abajo o/y puede aprenderse en los talleres de CASPe:

Juan B Cabello por CASPe. Lectura crítica de la evidencia clínica. Barcelona: Elsevier; 2015. (ISBN 978-84-9022-447-2)

Esta plantilla debería citarse como:

Cabello, J.B. por CASPe. Plantilla para ayudarte a entender Estudios de Cohortes. En: CASPe. Guías CASPe de Lectura Crítica de la Literatura Médica. Alicante: CASPe; 2005. Cuaderno II. p.23-27.

1

A/ ¿Son los resultados del estudio válidos?

Preguntas de eliminación

1 ¿El estudio se centra en un tema claramente definido? <i>PISTA: Una pregunta se puede definir en términos de</i> - La población estudiada. - Los factores de riesgo estudiados. - Los resultados "outcomes" considerados. - ¿El estudio intentó detectar un efecto beneficioso o perjudicial?	☐ SÍ ☐ NO SÉ ☐ NO
2 ¿La cohorte se reclutó de la manera más adecuada? <i>PISTA: Se trata de buscar posibles sesgos de selección que puedan comprometer que los hallazgos se puedan generalizar.</i> - ¿La cohorte es representativa de una población definida? - ¿Hay algo "especial" en la cohorte? - ¿Se incluyó a todos los que deberían haberse incluido en la cohorte? - ¿La exposición se midió de forma precisa con el fin de minimizar posibles sesgos?	☐ SÍ ☐ NO SÉ ☐ NO

¿Merece la pena continuar?

Preguntas de detalle

3 ¿El resultado se midió de forma precisa con el fin de minimizar posibles sesgos?

☐ SÍ ☐ NO SÉ ☐ NO

PISTA: Se trata de buscar sesgos de medida o de clasificación:

- ¿Los autores utilizaron variables objetivas o subjetivas?
- ¿Las medidas reflejan de forma adecuada aquello que se supone que tiene que medir?
- ¿Se ha establecido un sistema fiable para detectar todos los casos (por ejemplo, para medir los casos de enfermedad)?
- ¿Se clasificaron a todos los sujetos en el grupo exposición utilizando el mismo tratamiento?
- ¿Los métodos de medida fueron similares en los diferentes grupos?
- ¿Eran los sujetos y/o el evaluador de los resultados ciegos a la exposición (si esto no fue así, importa)?

4 ¿Han tenido en cuenta los autores el potencial efecto de los factores de confusión en el diseño y/o análisis del estudio? <i>PISTA: Haz una lista de los factores que consideras importantes</i> - <i>Busca restricciones en el diseño y en las técnicas utilizadas como, por ejemplo, los análisis de modelización, estratificación, regresión o de sensibilidad utilizados para corregir, controlar o justificar los factores de confusión.</i> <i>Lista:</i>	☐ SÍ ☐ NO SÉ ☐ NO
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5 ¿El seguimiento de los sujetos fue lo suficientemente largo y completo? PISTA: - Los efectos buenos o malos deberían aparecer por ellos mismos. - Los sujetos perdidos durante el seguimiento pueden haber tenido resultados distintos a los disponibles para la evaluación. - En una cohorte abierta o dinámica, ¿hubo algo especial que influyó en el resultado o en la exposición de los sujetos que entraron en la cohorte?	☐ SÍ ☐ NO SÉ ☐ NO
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B/ ¿Cuáles son los resultados?

6 ¿Cuáles son los resultados de este estudio? PISTA: - ¿Cuáles son los resultados netos? - ¿Los autores han dado la tasa o la proporción entre los expuestos/no expuestos? - ¿Cómo de fuerte es la relación de asociación entre la exposición y el resultado (RR)?	
7 ¿Cuál es la precisión de los resultados?	

C/ ¿Son los resultados aplicables a tu medio?

8 ¿Te parecen creíbles los resultados? <i>PISTA: ¡Un efecto grande es difícil de ignorar!</i> - ¿Puede deberse al azar, sesgo o confusión? - ¿El diseño y los métodos de este estudio son lo suficientemente defectuosos para hacer que los resultados sean poco creíbles? <i>Considera los criterios de Bradford Hill (por ejemplo, secuencia temporal, gradiente dosis-respuesta, fortaleza de asociación, verosimilitud biológica).</i>	☐ SÍ ☐ NO SÉ ☐ NO
9 ¿Los resultados de este estudio coinciden con otra evidencia disponible?	☐ SÍ ☐ NO SÉ ☐ NO
10 ¿Se pueden aplicar los resultados en tu medio? <i>PISTA: Considera si</i> - Los pacientes cubiertos por el estudio pueden ser suficientemente diferentes de los de tu área. - Tu medio parece ser muy diferente al del estudio. - ¿Puedes estimar los beneficios y perjuicios en tu medio?	☐ SÍ ☐ NO SÉ ☐ NO
11 ¿Va a cambiar esto tu decisión clínica?	



PROGRAMA DE LECTURA CRÍTICA CASPe

Leyendo críticamente la evidencia clínica

10 preguntas para ayudarte a entender una revisión

Comentarios generales

- Hay tres aspectos generales a tener en cuenta cuando se hace la lectura crítica de una revisión:

¿Son válidos esos resultados?

¿Cuáles son los resultados?

¿Son aplicables en tu medio?

- Las 10 preguntas de las próximas páginas están diseñadas para ayudarte a pensar sistemáticamente sobre estos aspectos. Las dos primeras preguntas son preguntas "de eliminación" y se pueden responder rápidamente. Sólo si la respuesta es "sí" en ambas, entonces merece la pena continuar con las preguntas restantes.
- Puede haber cierto grado de solapamiento entre algunas de las preguntas.
- En *itálica* y debajo de las preguntas encontrarás una serie de pistas para contestar a las preguntas. Están pensadas para recordarte por que la pregunta es importante. ¡En los pequeños grupos no suele haber tiempo para responder a todo con detalle!
- Estas 10 preguntas están adaptadas de: Oxman AD, Guyatt GH et al, Users' Guides to The Medical Literature, VI How to use an overview. (JAMA 1994; 272 (17): 1367-1371)

El marco conceptual necesario para la interpretación y el uso de estos instrumentos puede encontrarse en la referencia de abajo o/y puede aprenderse en los talleres de CASPe:

Juan B Cabello por CASPe. Lectura crítica de la evidencia clínica. Barcelona: Elsevier; 2015. (ISBN 978-84-9022-447-2)

A/ ¿Los resultados de la revisión son válidos?

Preguntas "de eliminación"

1 ¿Se hizo la revisión sobre un tema claramente definido? <i>PISTA: Un tema debe ser definido en términos de</i> - La población de estudio. - La intervención realizada. - Los resultados ("outcomes") considerados.	☐ SÍ ☐ NO SÉ ☐ NO
2 ¿Buscaron los autores el tipo de artículos adecuado? <i>PISTA: El mejor "tipo de estudio" es el que</i> - Se dirige a la pregunta objeto de la revisión. - Tiene un diseño apropiado para la pregunta.	☐ SÍ ☐ NO SÉ ☐ NO

¿Merece la pena continuar?

Preguntas detalladas

3 ¿Crees que estaban incluidos los estudios importantes y pertinentes?

☐ SÍ

☐ NO SÉ

☐ NO

PISTA: Busca

- *Qué bases de datos bibliográficas se han usado.*
- *Seguimiento de las referencias.*
- *Contacto personal con expertos.*
- *Búsqueda de estudios no publicados.*
- *Búsqueda de estudios en idiomas distintos del inglés.*

4 ¿Crees que los autores de la revisión han hecho suficiente esfuerzo para valorar la calidad de los estudios incluidos?

☐ SÍ

☐ NO SÉ

☐ NO

PISTA: Los autores necesitan considerar el rigor de los estudios que han identificado. La falta de rigor puede afectar al resultado de los estudios ("No es oro todo lo que reluce" El Mercader de Venecia. Acto II)

5 Si los resultados de los diferentes estudios han sido mezclados para obtener un resultado "combinado", ¿era razonable hacer eso?

☐ SÍ

☐ NO SÉ

☐ NO

PISTA: Considera si

- *Los resultados de los estudios eran similares entre sí.*
- *Los resultados de todos los estudios incluidos están claramente presentados.*
- *Están discutidos los motivos de cualquier variación de los resultados.*

B/ ¿Cuáles son los resultados?

6 ¿Cuál es el resultado global de la revisión?

PISTA: Considera

- Si tienes claro los resultados últimos de la revisión.
- ¿Cuáles son? (numéricamente, si es apropiado).
- ¿Cómo están expresados los resultados? (NNT, odds ratio, etc.).

7 ¿Cuál es la precisión del resultado/s?

PISTA:

Busca los intervalos de confianza de los estimadores.

C/¿Son los resultados aplicables en tu medio?

8 ¿Se pueden aplicar los resultados en tu medio? <i>PISTA: Considera si</i> - Los pacientes cubiertos por la revisión pueden ser suficientemente diferentes de los de tu área. - Tu medio parece ser muy diferente al del estudio.	☐ SÍ ☐ NO SÉ ☐ NO
9 ¿Se han considerado todos los resultados importantes para tomar la decisión?	☐ SÍ ☐ NO SÉ ☐ NO
10 ¿Los beneficios merecen la pena frente a los perjuicios y costes? <i>Aunque no esté planteado explícitamente en la revisión, ¿qué opinas?</i>	☐ SÍ ☐ NO

Supplemental Digital Content: JBI critical appraisal tool for analytical cross-sectional studies

RoB assessor:	Date of appraisal:	Record number:
Study author:	Study title:	Study year:

Internal validity	Choice: comments/justification	Yes	No	Unclear	N/A
Bias related to selection and allocation					
1	Were the criteria for inclusion in the sample clearly defined?	☐	☐	☐	☐
2	Were objective, standard criteria used for measurement of the condition?	Yes	No	Unclear	N/A
	Outcome 1	☐	☐	☐	☐
	Outcome 2	☐	☐	☐	☐
	Outcome 3	☐	☐	☐	☐
	Outcome 4	☐	☐	☐	☐
	Outcome 5	☐	☐	☐	☐
	Outcome 6	☐	☐	☐	☐
	Outcome 7	☐	☐	☐	☐
Bias related to classification of the exposure					

Barker TH, Hasanoff S, Aromataris E, Stone JC, Leonardi-Bee J, Sears K, et al. The revised critical appraisal tool for the assessment of risk of bias for analytical cross-sectional studies. JBI Evid Synth. 2025. <https://doi.org/10.11124/JBIES-24-00523>

3	Was the exposure measured in a valid and reliable way?		☐	☐	☐	☐
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Bias related to assessment, detection, and measurement of the outcome

4	Were the outcomes measured in a valid and reliable way?		Yes	No	Unclear	N/A
	Outcome 1		☐	☐	☐	☐
	Outcome 2		☐	☐	☐	☐
	Outcome 3		☐	☐	☐	☐
	Outcome 4		☐	☐	☐	☐
	Outcome 5		☐	☐	☐	☐
	Outcome 6		☐	☐	☐	☐
	Outcome 7		☐	☐	☐	☐

Bias related to confounding factors

5	Were confounding factors identified?		☐	☐	☐	☐
6	Were strategies to deal with confounding factors stated?		Yes	No	Unclear	N/A
	Outcome 1		☐	☐	☐	☐
	Outcome 2		☐	☐	☐	☐
	Outcome 3		☐	☐	☐	☐

Barker TH, Hasanoff S, Aromataris E, Stone JC, Leonardi-Bee J, Sears K, et al. The revised critical appraisal tool for the assessment of risk of bias for analytical cross-sectional studies. JBI Evid Synth. 2025. <https://doi.org/10.11124/JBIES-24-00523>

Outcome 4		☐	☐	☐	☐
Outcome 5		☐	☐	☐	☐
Outcome 6		☐	☐	☐	☐
Outcome 7		☐	☐	☐	☐

Statistical conclusion validity

7

Was appropriate statistical analysis used?					
Outcome 1		Yes	No	Unclear	N/A
Result 1		☐	☐	☐	☐
Result 2		☐	☐	☐	☐
Result 3		☐	☐	☐	☐
Outcome 2		Yes	No	Unclear	N/A
Result 1		☐	☐	☐	☐
Result 2		☐	☐	☐	☐
Result 3		☐	☐	☐	☐
Outcome 3		Yes	No	Unclear	N/A
Result 1		☐	☐	☐	☐
Result 2		☐	☐	☐	☐

Barker TH, Hasanoff S, Aromataris E, Stone JC, Leonardi-Bee J, Sears K, et al. The revised critical appraisal tool for the assessment of risk of bias for analytical cross-sectional studies. JBI Evid Synth. 2025. <https://doi.org/10.11124/JBIES-24-00523>

Result 3		☐	☐	☐	☐
Outcome 4		Yes	No	Unclear	N/A
Result 1		☐	☐	☐	☐
Result 2		☐	☐	☐	☐
Result 3		☐	☐	☐	☐
Outcome 5		Yes	No	Unclear	N/A
Result 1		☐	☐	☐	☐
Result 2		☐	☐	☐	☐
Result 3		☐	☐	☐	☐
Outcome 6		Yes	No	Unclear	N/A
Result 1		☐	☐	☐	☐
Result 2		☐	☐	☐	☐
Result 3		☐	☐	☐	☐
Outcome 7		Yes	No	Unclear	N/A
Result 1		☐	☐	☐	☐
Result 2		☐	☐	☐	☐
Result 3		☐	☐	☐	☐

Barker TH, Hasanoff S, Aromataris E, Stone JC, Leonardi-Bee J, Sears K, et al. The revised critical appraisal tool for the assessment of risk of bias for analytical cross-sectional studies. JBI Evid Synth. 2025. <https://doi.org/10.11124/JBIES-24-00523>

Comprehensiveness of reporting

8	Were the study subjects and the setting described in detail?	Yes	No	Unclear	N/A
		☐	☐	☐	☐

Overall appraisal: Include: ☐ Exclude: ☐ Seek Further Info: ☐

Comments:

Barker TH, Hasanoff S, Aromataris E, Stone JC, Leonardi-Bee J, Sears K, et al. The revised critical appraisal tool for the assessment of risk of bias for analytical cross-sectional studies. JBI Evid Synth. 2025. <https://doi.org/10.11124/JBIES-24-00523>