Evaluation of Hyperhomocysteinemia and *MTHFR* C677T Gene Polymorphism as Independent Risk Factors of Ischemic Stroke among Bangladeshis

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing a degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all main sources of help.

Students' full name and Signature:

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Approval

The thesis/project titled “Evaluation of hyperhomocysteinemia and *MTHFR* C677T Gene Polymorphism as Independent Risk factors of Ischemic stroke among Bangladeshis” Submitted by Mugenyi Raymond of Summer 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology in January, 2025.

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Department Head (Chair)	_____ Dr. Md. Firoze H. Haque, Associate Professor & Chairperson, Department of Mathematics and Natural Sciences BRAC University

Ethics Statement

The study was conducted following the Helsinki Declaration and subsequent revisions. The study was approved by the Institutional Review Board (IRB) of the Department of Mathematics and Natural Sciences, BRAC University.

Abstract

Stroke remains a leading cause of death and disability worldwide. This study investigated the relationship between hyperhomocysteinemia (HHcy) and *MTHFR* C677T gene polymorphism as independent risk factors of Ischemic stroke (IS) in Bangladesh. Blood samples and clinical data were collected from 30 IS patients (>18 years old) and 30 healthy controls. Levels of plasma homocysteine and other biochemical parameters were measured using an automated analyzer, and their relationship with HHcy was determined. DNA was extracted from blood using QIAamp DNA blood kits, and the *MTHFR* C677T gene polymorphism was genotyped using PCR-RFLP. The prevalence of HHcy was higher in IS patients (30%) than in healthy controls (6.67%), and the difference was statistically significant (p-value = 0.02095). The associations between HHcy and IS traditional risk factors were not statistically significant, suggesting that HHcy could influence IS outcomes through mechanisms independent of conventional risk factors. Unique dietary patterns modulating ischemic stroke risk were also identified. Participants eating fish less than three times a week were higher in numbers among IS patients (16.67%) than in controls (0%), and the observed difference in proportions was statistically significant (p-value = 0.0261). The T allele was more prevalent in individuals with HHcy (27.3 %) than in those without HHcy (16.3%), while the C allele dominated in those without HHcy (83.7 %) compared to the hyperhomocysteinemic individuals (72.7%). Similarly, the TT genotype was more prevalent in the HHcy group (9.1%) than in the non-HHcy group (2%). The CT genotype also dominated in the HHcy group (36.4%) compared to the non-HHcy group (28.6%). The high frequencies of the T allele and the TT genotype in individuals with HHcy compared to those without HHcy Indicate that predisposition to the *MTHFR* C677T polymorphism may result in hyperhomocysteinemia. These findings emphasize the need for routine homocysteine screening, maintenance of a healthy diet, and the necessity for genetic testing for the C677T mutation in the *MTHFR* gene for early detection and management of these critical IS risk factors. Future large-scale interventional studies are recommended to fully understand the complex interactions between hyperhomocysteinemia and *MTHFR* polymorphisms in the pathogenesis of ischemic stroke.

Keywords: Ischemic stroke; Homocysteine; Hyperhomocysteinemia; Polymorphism; *MTHFR* C677T; Dietary patterns

Dedication

Dedicated to advancement of stroke research!

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List of Acronyms

LDL	Low-Density Lipoprotein
HDL	High-Density Lipoprotein
RFLP	Restriction Fragment Length Polymorphism
BMI	Body Mass Index
CT	Computed Tomography
MRI	Magnetic Resonance Imaging
ROS	Reactive Oxygen Species
MTHFR	Methylenetetrahydrofolate Reductase
HHcy	Hyperhomocysteinemia
NO	Nitric Oxide
UV	Ultra Violet
BSMMU	Bangabandhu Sheikh Mujib Medical University
EDTA	EthylenediamineTetraacetic Acid
SNP	Single Nucleotide Polymorphisms

Chapter 1

Introduction

Stroke ranks second as a leading cause of death and a major contributor to disability worldwide [1]. It can either be ischemic, in which brain tissues lack blood supply due to blockage of blood vessels, or hemorrhagic, in which blood leaks into the brain after a blood vessel bursts [2]. The chances of having a stroke increase as one grows older, and over the years, it has been known as a disease of the elderly. However, this trend has changed recently, with a rise in stroke incidents in young people from 18 to 44 years of age [3]. This is partly due to the increase in the prevalence of stroke risk factors such as smoking, diabetes, hypertension, and obesity, among others [4]. A deeper understanding of these factors is essential to devising management and treatment strategies beneficial for young and elderly stroke patients.

1.1 Etiology of Ischemic Stroke

Ischemic stroke (IS) occurs following a thrombotic or embolic event that leads to a significant reduction or complete stoppage of blood flow to the brain. In a thrombotic event, clot formation in the arteries (typically due to atherosclerosis) obstructs blood flow to the brain. On the other hand, an embolic event is characterized by debris from other parts of the body and blocks blood flow through the affected vessels [5].

Cardioembolism: This occurs when a clot originating from the heart travels to the brain. On its way, it obstructs or blocks the brain vessel. As a result, blood flow to the brain is prevented, resulting in ischemic stroke. Strokes in this category show magnetic resonance imaging (MRI) or computed tomography (CT) scan findings similar to those for large-artery atherosclerosis. It is, therefore, important that these are ruled out during the diagnosis of cardioembolic strokes. In situations where a patient is found to have a stroke characterized by a medium-risk cardiac source of embolism, with no evidence for any other cause of stroke, then they are considered as one with a probable cardioembolic stroke [5], [6], [7].

Large artery atherosclerosis: This refers to the build-up of atherosclerotic plaques (Figure 1) in the brain arteries. As a result, these brain arteries get blocked, preventing blood flow to the brain and resulting in ischemic stroke. Lesions of sizes larger than 1.5 cm in the cerebellum, cortex, and brainstem, observed on the magnetic resonance imaging (MRI) or computed tomography (CT) scan, are considered to be of large artery atherosclerotic origin. It is essential to have further supportive evidence of a relevant intracranial or extracranial artery that indicates more than fifty percent stenosis. This is usually accomplished through duplex imaging or arteriography [5], [7], [8].

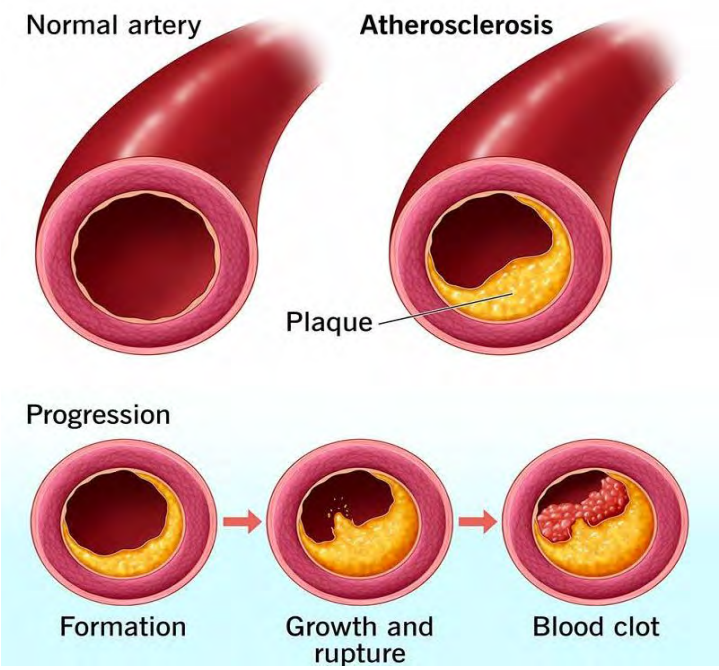

Figure 1: Development of Atherosclerosis [9]

Small vessel occlusion: This refers to the blockage of the small arteries of the brain. It is also known as a lacunar stroke. It is often characterized by high blood pressure and diabetes, which are extensively considered during its diagnosis. Patients suffering from this type of stroke usually have normal MRI and CT scan findings. Some of the clinical manifestations associated with small vessel occlusion include dementia, abnormal gait, and cognitive decline [5], [7], [9].

Stroke of undetermined etiology: This is also referred to as cryptogenic stroke. Strokes falling under this category have no identifiable cause despite undergoing thorough diagnosis or evaluation. A stroke with more than one cause is also found under this category[5], [7], [10].

Stroke of other determined etiology: patients with an uncommon cause of stroke are found in this category. Such causes may include hematologic disorders and nonatherosclerotic vasculopathy. No matter where the lesion is or how big it is, MRI or CT scans for strokes under this category must be suggestive of an acute ischemic stroke. It is also recommended that other studies be conducted to exclude large artery atherosclerosis and cardioembolism [5], [7], [11]

Ischemic stroke occurs when arteries to the brain are blocked, thereby preventing blood flow to specific brain areas. They can be classified into two broad categories: small and large vessel ischemic strokes [12].

1.2 Pathophysiology of Ischemic Stroke

The development of stroke occurs when the ischemic core of the brain fails to receive oxygen and nutrients in about three to four minutes[13], [14]. As a result of this deprivation, a series of events occur, including cellular acidosis, ATP depletion, and ion homeostasis disruption. This deprivation also initiates elevated calcium levels, free-radical mediated toxicity, generation of cytokines and arachidonic acid products, activation of glial cells, disruption of the blood-brain barrier, and leukocyte infiltration. Altogether, these events result in neuronal death [15], [16]. The ischemic core is surrounded by the penumbra. This region consists of cells that are dying and those still receiving some oxygen, just enough for communication[17]. It is this region that most clinical trials are targeting to develop therapies for ischemic stroke [17].

Failure of the brain to receive oxygen and required nutrients results in anaerobic glycolysis. Pyruvate gets converted to lactate, allowing the release of hydrogen (H+) ions. These hydrogen ions end up reducing cellular pH. Simultaneously, the partial pressure of carbon dioxide (pCO_2) levels rise, further contributing to cellular acidosis[18], [19].

At the same time, the reduction in ATP levels disrupts ATP-dependent ion pumps in the ischemic core. This disruption leads to Na^+ and Ca^{2+} ions accumulating, which damage the neuronal cells [15], [20], [21].

The increase in calcium ions allows activation of calpains, caspase and nitric oxide, free radicals, and arachidonic acid metabolite-producing enzymes, which are responsible for not only the activation of pro-apoptotic proteins (specifically BID) but also protein cleavage, rupturing the plasma membrane. The combined accumulation of calcium ions and reactive oxygen species (ROS) triggers mitochondrial permeability transition pore (MPTP), causing mitochondrial swelling, cytochrome C release, and disruption of mitochondrial function. Ultimately, these events activate the apoptotic and necrotic pathways responsible for cellular death[21], [22], [23], [24]. While cells die in the ischemic core and penumbra, they release certain pro-inflammatory signaling molecules such as adhesion molecules (ICAM1, VCAM1), cytokines ((IL-1, IL-6, TNF-α) and chemokines (IL-10, IL-17), which activate post-ischemic inflammation [20], [21], [25]. At the same time, reduction in nitric oxide production allows intravascular clotting to accumulate further, exacerbating ischemic insult [26]. The compound effects of inflammation and oxidative stress allow the blood brain barrier (BBB) to become permeable, allowing cells and protein release from the brain tissue [27], [28]. Simultaneously, mast cells release histamine, proteases, and pro-inflammatory cytokines, further disrupting the BBB and enabling leukocyte infiltration [27], [28], [29]

As long as immediate action is not taken, these processes can extend damage from the core to the penumbra, causing widespread neuronal death.

The pathophysiology of ischemic stroke is further explained in Figure 2

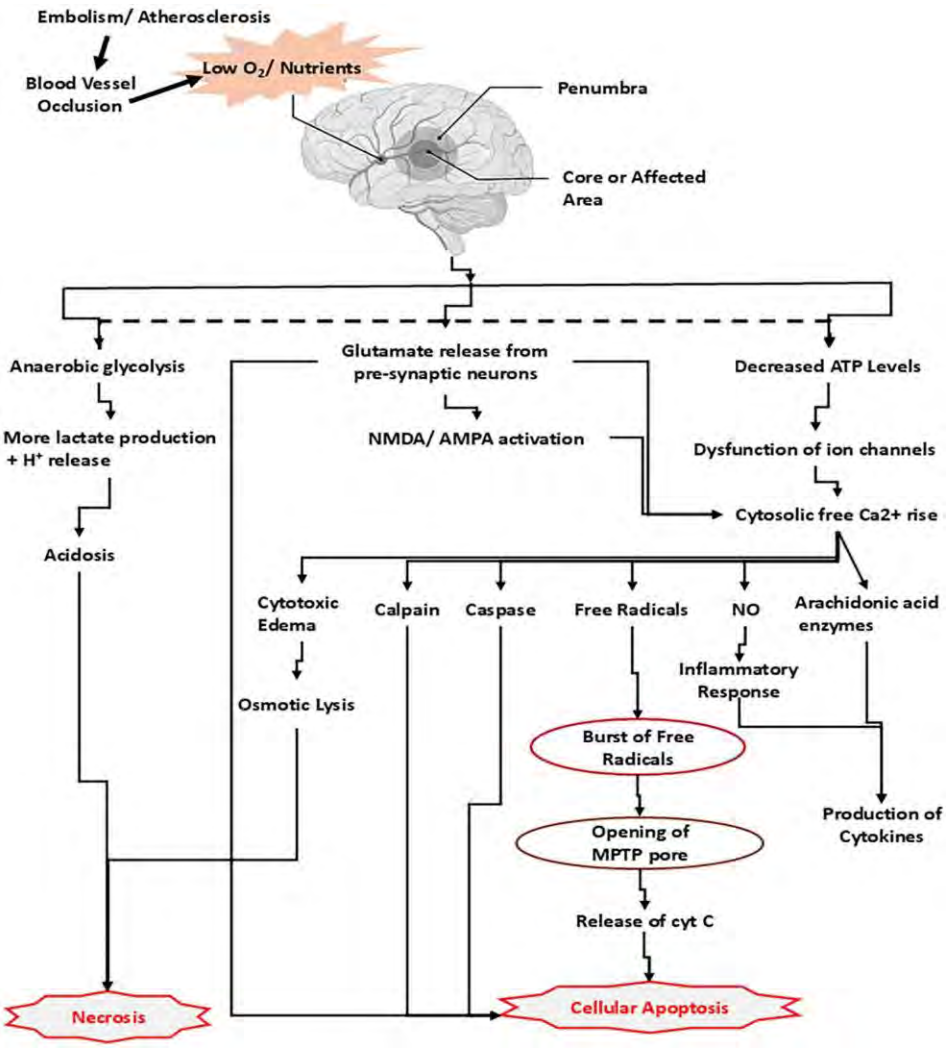

Figure 2: Pathophysiology of ischemic stroke [3]

1.3 Epidemiology of Ischemic stroke

In 2020, the global incidence of stroke was 11.71 million people and, ischemic stroke was approximately 65% of all cases [30]. In the same year, 3.48 million deaths were attributed to Ischemic stroke [30], [31]. In the United States alone, approximately 795,000 individuals experience a new or recurrent stroke each year, with ischemic strokes comprising about 87% of these cases [30]. The mortality of stroke in Asia is more comparable to Eastern Europe than in Western Europe, the Americas, or Australasia [1]. In Bangladesh, stroke is the third leading cause

of death, after coronary heart disease and infectious diseases [32]. According to a recent population-based cross-sectional study, stroke incidence in Bangadesh is about 11.39 per 1,000 population [33]. This value was found to be higher (30.10 per thousand) in the age group of >60 years, but lower (4.60 per thousand) among individuals below 40 years old [33] The same study reported. The same study reported a prevalence rate of 13.62 per thousand compared to 8.68 per thousand for females, indicating the men in Bangladesh are more prone to stoke than women [33]

1.5 Risk Factors of Ischemic Stroke

Different risk factors have been linked to stroke. These include non-modifiable ones, such as race or ethnicity, age, family history, and gender, and modifiable ones, such as diabetes, alcoholism, hypertension, obesity, a lack of physical exercise, smoking, and diet. A better understanding of these factors is essential for better management of stroke[34], [35]. Some of these factors are elaborated below:

Age: Aging is considered the most robust non-modifiable risk factor for ischemic stroke. It is believed to double every 10 years after age 55 years [36]. As people age, arteries become harder and narrower, making them more likely to become blocked, a factor known to increase increase ischemic stroke risk. Furthemore, other medical conditions such as hypertension, diabetes and hypercholesteremia are reported to interact with age, incerasing the risk of acquiring stroke[37].

Diabetes: Diabetes is known to accelerate atherosclerosis, which is the build up of plaques in arteries. It is also known to coexist with other cardiovascular risk factors such as hypertension and dyslipidemia, which further compound the risk of stroke [38]

Obesity: Obesity often leads to metabolic syndrome, which includes hypertension, diabetes, and dyslipidemia, conditions which are independently associated with increased stroke risk [39]

Smoking: Tobacco smoke contains chemicals such as nicotine and carbon monoxide, which can damage blood vessels. This damage leads to inflammation and dysfunction of endothelial cells, promoting atherosclerosis, a condition known to cause ischemic stroke [40]

1.6. Homocysteine Metabolism, hyperhomocysteinemia and *MTHFR* C677T Polymorphism

Homocysteine, an amino acid that contains sulfur, is an intermediate product in the normal biosynthesis of the amino acids methionine and cysteine [41]. Around 1% of it circulates in plasma as free thiol; 70–80% of it is disulphide, bound to plasma proteins, primarily albumin; and 20–30% of it combines with other thiols or with itself to form dimerized homocysteine [42]. Total plasma (or serum) homocysteine (tHcy) is the collective term for all four forms of homocysteine[42]. In case of methionine deficiency, homocysteine can be re-methylated to form methionine and in presence of sufficient methionine, it is instead used to produce cysteine[43]. These pathways are further elaborated in Figure 3

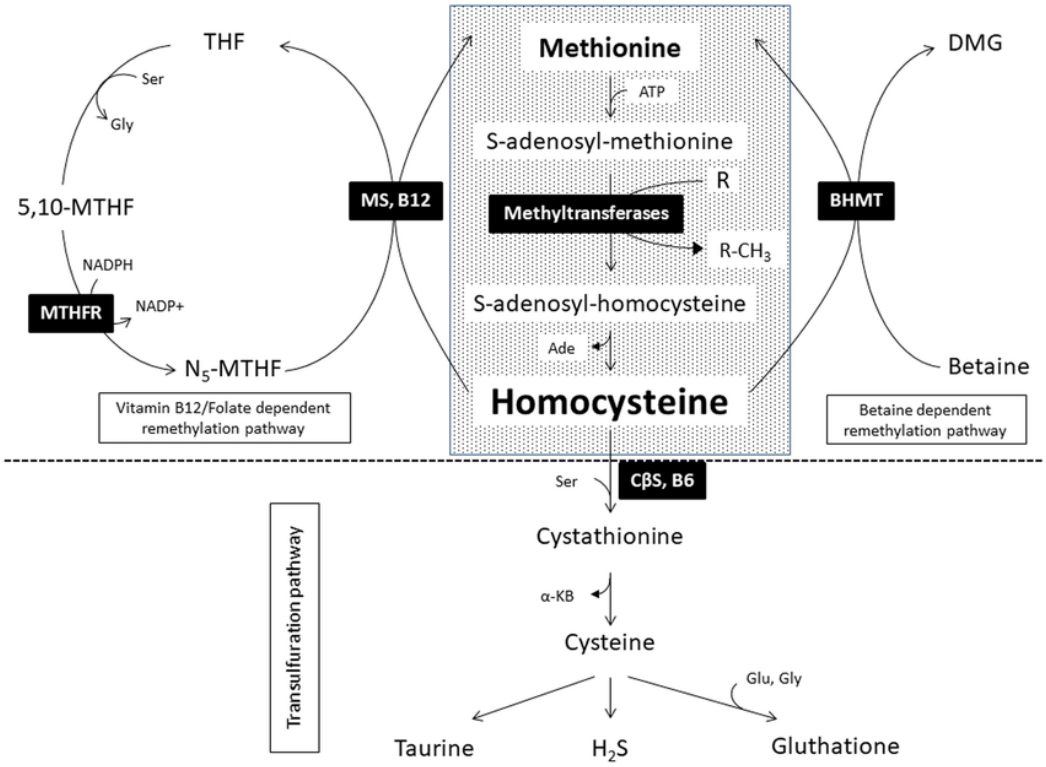

Figure 3: Homocysteine metabolism [44]

[44]SAM—S-adenosylmethionine, SAH: S-adenosylhomocysteine, Glu—glutamate, Gly—glycine,
Ser—serine, R—methylation substrates (DNA, RNA, catecholamine, lipids, guanidinoacetate, etc.), R-CH3—methylated substrates, THF—tetrahydrofolate, MTHF—methyltetrahydrofolate, MTHFR—methylenetetrahydrofolate reductase, MS—methionine synthase, H2S—hydrogen sulfite, DMG—dimethylglycine, α-KG—αketoglutarate

Homocysteine (Hcy) levels can be elevated by a number of factors, including a lack of folate and B vitamins, diabetes, pre-existing atherosclerotic disease, and a number of medications [45]. Other causes include a lack of certain enzymes, resulting in the accumulation of this amino acid and/or its metabolites in the tissue [44]. Hyperhomocysteinemia is an important and independent risk factor for the development of ischemic stroke [46]

In fasting subjects, total homocysteine levels between 5 and 15 μmol/L are regarded as normal [47]. Higher fasting values are divided into three categories: moderate (16–30), intermediate (31–100), and severe (>100 μmol/L) hyperhomocysteinemia [42].

Hyperhomocysteinemia (HHcy) may result from nutritional deficiencies of folic acid and vitamin B12, which are key enzyme cofactors required for normal homocysteine metabolism [48]. By changing homocysteine into methionine, the methylene tetrahydrofolate reductase (MTHFR) enzyme lowers serum homocysteine levels[49]. It has been found that mutations in the gene hinder homocysteine conversion to methionine, thereby contributing to hyperhomocysteinemia[48]. One of the most extensively studied gene variants is MTHFR C677T[50]. Across several population studies, this C677T point mutation has been found to be associated with an increased risk of ischemic stroke [51], [52].

1.7 Research Gap, Hypothesis and Objectives

1.7.1 Research Gap

Last year (2024), we conducted a meta-analysis and systematic review to understand the prevalence of hyperhomocysteinemia as an essential stroke biomarker in the Asia-Pacific region. A total of 136132 articles were initially pooled from PubMed, Google Scholar, and Scopus, and we finally selected 42, per our inclusion and exclusion criteria. Analysis of the data obtained from these articles revealed an overall HHcy prevalence of 45% among stroke patients (5, 393) in the Asia-Pacific region (Figure 4). To our surprise, we could not get more than four studies in Bangladesh as per our eligibility criteria, and therefore, obtaining the pooled HHcy prevalence specifically for Bangladesh was not statistically feasible. Consequently, it became apparent that studies investigating the relationship between HHcy and *MTHFR* gene polymorphisms among stroke patients in Bangladesh were insufficient. Numerous studies conducted previously have found a strong positive correlation between hyperhomocysteinemia and ischemic stroke[53], [54], [55]. However, the strength of this relationship between hyperhomocysteinemia and ischemic stroke is not well established in Bangladesh. A few studies that attempted to do so [46], [56], [57] did not clearly show the reasons behind the relationship. The role of the *MTHFR* gene polymorphism in this association also remains unclear.

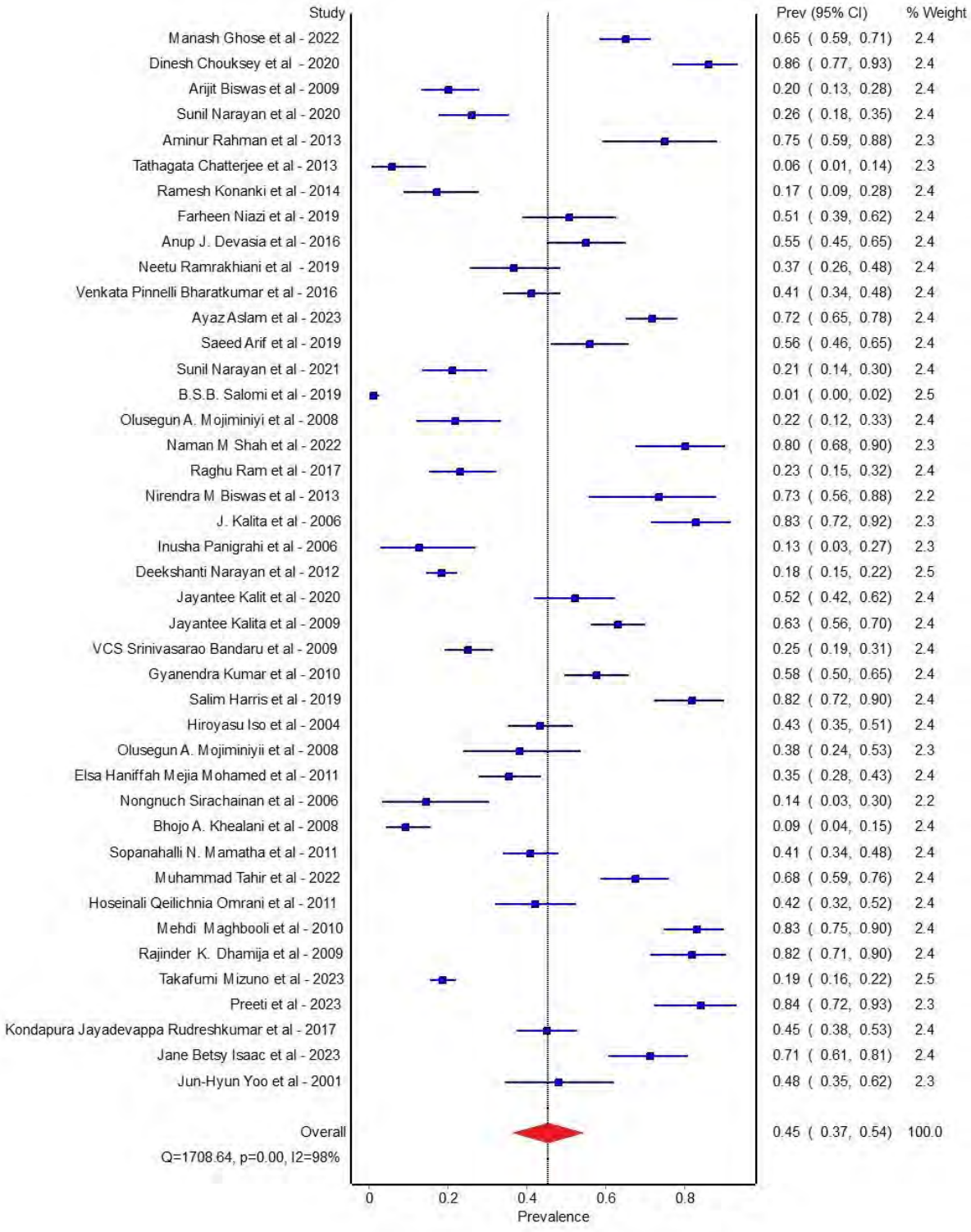

Figure 4: Overall pooled prevalence of HHcy among Asia-Pacific patients with stroke.

1.7.2 Hypothesis

It is hypothesized that HHcy and *MTHFR* C677T gene polymorphisms may contribute to the development of Ischemic stroke in the Bangladeshi population.

1.7.3 Objectives

- To investigate the association between HHcy and *MTHFR* C677T gene polymorphism with IS in the Bangladeshi population.
- To estimate the prevalence of HHcy among ischemic stroke patients (>18 years) in Bangladesh
- To identify specific dietary patterns that might have a link to increased Ischemic stroke risk among Bangladeshi people
- To clarify the role of *MTHFR* gene polymorphism as a potential risk factor for Ischemic stroke in the Bangladeshi population.

Chapter 2

Methodology

2.1 Study design

The study was designed as a hospital-based case-control study (Figure 5).

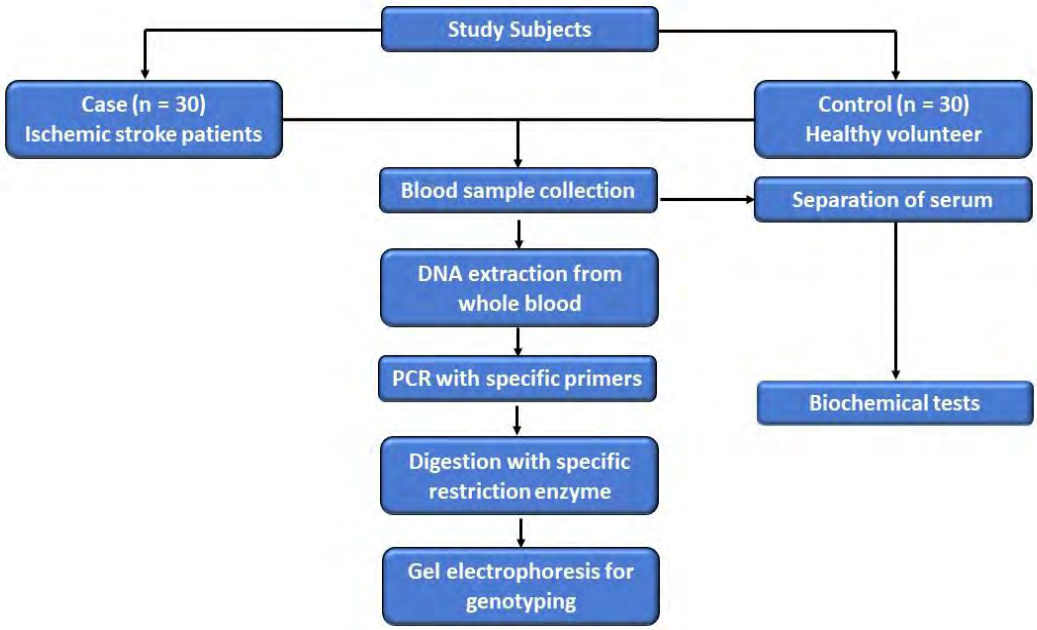

Figure 5: Schematic diagram representing the study design

2.2 Selection of the cases and controls

The case group consisted of 30 patients diagnosed with ischemic stroke from the Neurology Department of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. Ischemic stroke was confirmed by neuroimaging methods (Computed tomography or magnetic resonance imaging). A total of 30 healthy subjects with no previous history of stroke formed the control group. In both groups, participants with chronic illnesses such as myocardial infarction and kidney disease were excluded. Tribal or other ethnic groups were also excluded from the groups.

Inclusion criteria

Case:

I. IS confirmed by either MRI or CT scan (as indicated in the patient's medical reports)
II. Bengali background
III. Age (>18 years old)

Control:

I. Healthy individuals with no previous history of stroke
II. Belonged to the same geographic background and ethnicity as the cases

Exclusion criteria

I. Having a history of any other chronic disease
II. Taking vitamin B supplements
III. Tribal or other ethnic groups

2.3 Questionnaire

A structured questionnaire was created, and details regarding participants' demographic characteristics, lifestyle, pre- and post-admission, and biochemical test results were recorded.

I. **Demographic information:** This section summarized information regarding participants' age, gender, residence, educational qualification, occupation, socio-economic status, and marital status of the respondents.
II. **Clinical and lifestyle-related information:** This section included information regarding height, weight, BMI, weekly food habits, systolic/diastolic blood pressure, heart rate, smoking history, alcohol intake, daily sugar intake, history of physical exercise, diabetes, hypertension, and family history of chronic diseases. Those currently smoking or are no longer smokers but did in the past were considered smokers.

III. **Pre-admission and post-admission clinical information:** This section included information regarding the symptoms experienced before admission, the duration between the first onset of symptom and admission, a list of the initial tests that were prescribed for diagnosis, and the drugs that were suggested right after admission. This section was only applicable to the case group.

IV. **Laboratory findings:** This section summarizes the findings we obtained after the biochemical tests. Total cholesterol, HDL-C, LDL-C triglycerides, Hcy, serum vitamin B12, and serum folate concentrations were recorded in both cases and controls.

2.4 Consent and Ethical Issues

All participants were informed about the study's purpose and the protocol's investigational nature. Each participant's signature was also recorded on the consent form. A structured questionnaire was used to obtain the details from the participants. Participants' clinical data were documented in the presence of the attending physician or recorded from their medical reports. The research adhered to the Helsinki Declaration and its subsequent revisions[58] The study was approved by the Institutional Review Board (IRB) of the Department of Mathematics and Natural Sciences, BRAC University.

2.5 Sample Collection and Storage

About 6.0 mL of venous blood was drawn from each participant, adhering to all aseptic precautions. The drawn blood was immediately transferred to three collection tubes.

I. **Plain Tube/Red Top Blood Tube/Pro-coagulation Tube**: 2.0 mL of blood was taken into one plain tube (Figure 6A), which is used specifically to collect blood samples that require serum [59]After clot formation, the tube was centrifuged, and serum was collected. The serum was then used for biochemical testing in the hospital laboratory.

II. **K2 EDTA tube/ Anticoagulant tube/ Purple Top Blood Tube**: 4.0 mL of blood was transferred into two K2 EDTA tubes (2.0 mL in each tube) (Figure 6B). This tube type contains ethylenediaminetetraacetic acid (EDTA), an anticoagulant that helps prevent blood clotting by binding calcium ions necessary for coagulation. EDTA also serves as a

preservative, maintaining the integrity of cellular components and the morphology of blood cells [60], [61], [62]

Figure 6: Plain and K2EDTA Tubes [60], [61]

For each sample, blood in the plain tube and one EDTA tube were submitted to the BSMMU Laboratory for biochemical testing. The other EDTA tube was kept in an icebox for transportation to the Molecular Laboratory of Brac University and stored at −20°C until DNA extraction.

2.6 DNA Extraction

Genomic DNA was extracted from the whole blood samples using a commercial kit (QIAmp Blood DNA Mini Kit) following the protocol from the manufacturer (Figure 7).

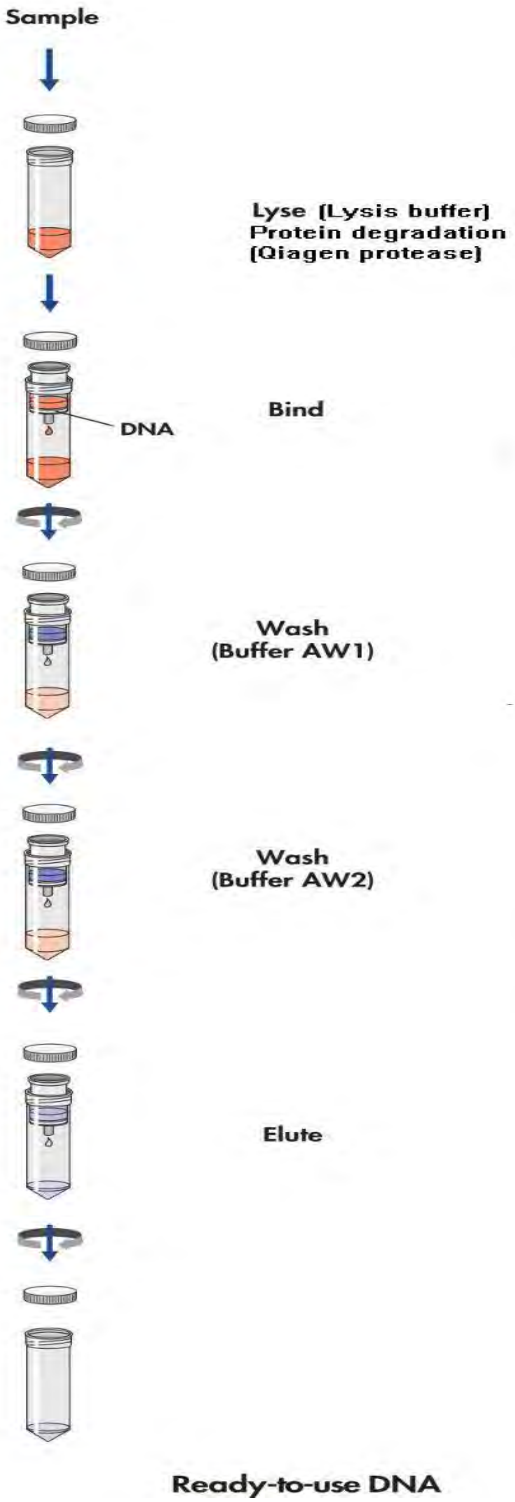

Figure 7: Steps of DNA extraction from whole blood [63]

Cell Lysis: 20 µl of QIAGEN protease were pipetted into a 1.5 microcentrifuge tube. The blood sample and Buffer AL were added (200 µl each), followed by pulse vortexing for 20 seconds to ensure uniform mixing. The mixture was then incubated at 56 ºC for 10 minutes for cell lysis.

Separation of the soluble DNA from cell debris: 200 µl ethanol (96 - 100%) was added to the cell lysate. The sample was mixed thoroughly by pulse vortexing for 20 seconds to ensure uniform mixing. The tubes were briefly centrifuged to remove drops from the lid.

Binding the DNA to a purification matrix: The sample, including any precipitate, was transferred carefully into the QIAmp Mini spin column (in a 2ml collection tube) and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded, and the QIAmp Mini spin column containing the bound DNA was transferred to a new collection tube for washing.

Washing: The QIAmp Mini spin column in the new collection tube was washed with 500 µl AW1 buffer and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded. The column was washed for the second time using a 500 µl AW2 buffer and centrifugation at 14,000 rpm for 3 minutes. The flow-through was discarded. An additional step (drying step) was considered, where the columns were centrifuged at full speed for 1 min to remove any residual liquid. This extra step ensured no chance of possible Buffer AW2 carryover.

Elution: The washed QIAmp Mini spin column was transferred to the elution tube. 200 µl of elution buffer was added to the column membrane and incubated at room temperature (15-25°C) for 5 minutes. Finally, the tubes were centrifuged at 8000 rpm for 1 min to elute the DNA. The extracted DNA was stored at -20 ºC.

2.7 Quantification of DNA

The quantity and purity of the genomic DNA were assessed by taking absorbance values at 260 nm (A260) and 280 nm (A280) using Thermo Scientific™ NanoDrop™. DNA with an A260/A280 ratio of 1.8–2.0 and concentration ranging between 30-45 ng/µl was considered pure.

2.8 PCR-RFLP

The *MTHFR* C677T genotypes were determined using conventional Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

2.8.1 PCR

2.8.1.1 Primer Selection

The primer sequences and PCR conditions used to amplify *MTHFR* C677T were selected based on previously published work [64]. The primer sequences, amplicon size, and digested product sizes of the amplicon are listed in Table 1.

Table 1: MTHFR C677T Primer sequences, amplicon size, and the resultant digested product sizes

***MTHFR* gene polymorphism**	**Primer sequence**	**Amplicon size**	**Restriction Digestion Products**
MTHFR C677T	Forward: TGTGGTCTCTTCATCCCTCGC Reverse: CCTTTTGGTGATGCTTGTTGGC [64]	513 bp	Wildtype (C/C) : 513bp Heterozygous mutant (C/T) : 146 bp, 367 bp, 513bp Homozygous mutant (T/T):146 bp, 367 bp

2.8.1.2 PCR Reagent

The reagents used for PCR-RFLP are listed in Table 2.

Table 2: Reagents and the respective companies used for PCR-RFLP

	Reagents	**Company**
PCR	EmeraldAmp® GT PCR Master Mix	Takara, Japan
	Primer	Macrogen, South Korea
	Nuclease Free Water	Thermo Scientific™, USA
Restriction Digestion	Restriction enzymes (HinfI)	Takara, Japan
Gel Electrophoresis	SeaKem™ LE Agarose	Lonza™, Switzerland
	Quick-Load® 100 bp DNA Ladder	New England Biolabs, USA

2.8.1.3 Composition of PCR mix

The composition of the PCR mix is listed in Table 2.3. Following the manufacturer's protocol, the composition of the PCR reaction mixture was calculated [65]. 0.3 µg DNA concentration was considered, and therefore, adjustments regarding the equivalent volume for each DNA sample and the required nuclease-free water were determined accordingly (Table 3).

Table 3: Composition of the PCR reaction mixture

Components	**Volume (µL)**
Master Mix	10
Forward Primer	0.4
Reverse Primer	0.4
Nuclease-free water	Variable
Genomic DNA	Variable
Total Volume	20

2.8.1.4 PCR Conditions

The PCR conditions used for the amplification of *MTHFR* ***C677T*** are illustrated in Figure 8

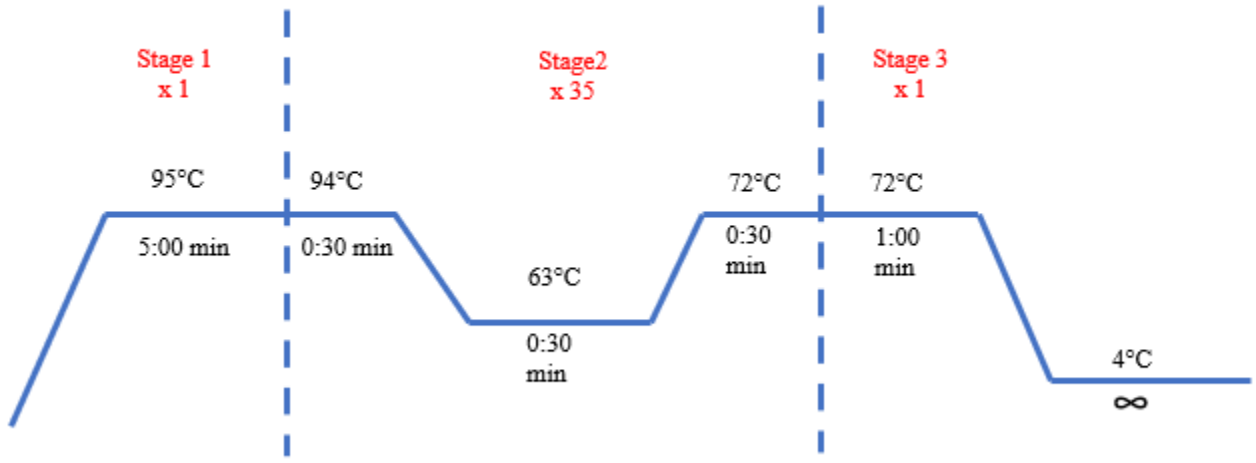

Figure 8: Thermal conditions for amplification of MTHFR C677T

2.8.1.5 Agarose Gel Electrophoresis for the *MTHFR* C677T PCR Products

The *MTHFR* C677T PCR products were analyzed using agarose gel electrophoresis. A 1% gel was used to resolve the PCR products. The gel was stained using Ethidium Bromide (EtBr). After running the gel for 90 minutes at 100 V, the bands were visualized under UV light.

2.8.2 RFLP Analysis

2.8.2.1 Restriction Digestion of *MTHFR* C677T:

An amplicon of size 513 bp in length was selected since it does not contain any restriction sites for the *Hinf*I enzyme in the wild-type version of the *MTHFR* gene, ensuring appropriate detection of the mutation in the gene [64]. 15µl of PCR product, 1µl of *Hinf*I enzyme, 2µl of buffer, and 2µl of nuclease-free water (total volume, 20µl) were placed in a single Eppendorf tube. Mixing was done by flicking the tube, and the drops from the lid were removed by brief centrifugation. The tubes were then incubated at 37 °C for 1 hour, according to the manufacturer's instructions. The digestion of the MTHFR C677T polymorphism by the restriction enzyme HinfI reveals distinct banding patterns depending on the presence or absence of the HinfI recognition site, which is introduced when cytosine (C) at position 677 of the *MTHFR* gene is replaced by thymine (T). In the normal version of the gene (CC genotype), there is no restriction site for the *Hinf*I enzyme; therefore, the 513 bp DNA fragment remains undigested (Figure 9 A). When MTHFR C677T polymorphism is present, the substitution of a C with a T creates a restriction site for *Hinf* I, and the 513 bp DNA fragment is cut into two smaller fragments, one of 146 bp and the other of 367 bp. The CT genotype (heterozygous) yields three fragments: the undigested 513 bp from the normal C allele and two smaller fragments of 367 bp and 146 bp from the mutated T allele (Figure 8 B. The TT genotype (mutant) yields only two small fragments, 367 b,p and 146 bp, indicating the presence of the mutation in both copies of the gene (Figure 9 C)

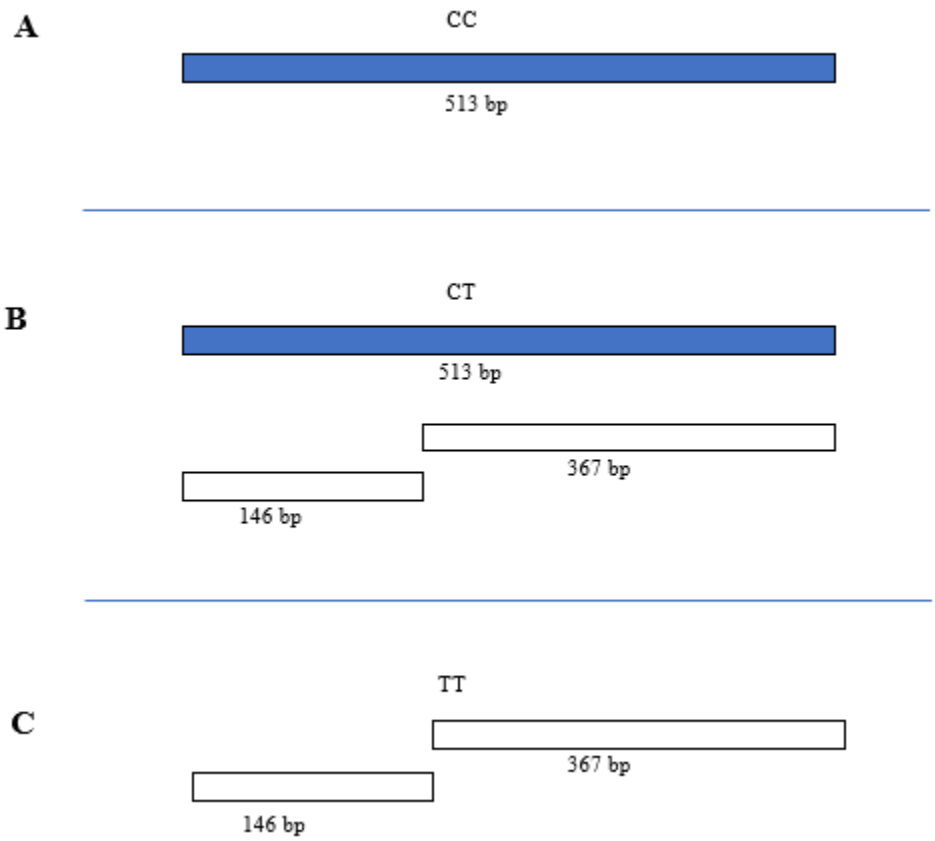

Figure 9: Restriction Digestion of MTHFR C677T Amplicon with HinfI enzyme

2.8.2.2 Composition of Restriction Digestion Mix:

Table 4: Composition of the reaction mixture for HinfI restriction enzyme digestion:

Name of the Component	**Volume (µl)**
Hinf I	1
10X H Buffer	2
Substrate DNA	15
Nuclease-free water	2
Total	20

2.8.2.3 Agarose Gel Electrophoresis for the RFLP Digested Products:

The digested RFLP products were analyzed using agarose gel electrophoresis. A 1.5% gel was used to resolve the products. The gel was stained using Ethidium Bromide (EtBr). After running the gel for 60 minutes at 100V, the bands were visualized under UV light. The band sizes were determined using a 100-base pair DNA ladder.

2.9 Statistical Analysis

Categorical variables are reported as proportions, and continuous variables as median values or means±standard deviations. The Fisher's exact test, t-test, chi-square, Mann–Whitney U, Kruskal-Wallis test and logistic regressions were performed using the R statistical Programming language (Version 4.4.2). Statistical tests were one-sided and two-sided; a $p<0.05$ was taken as the significance level.

Chapter 3

Results

3.1. Demographic characteristics among ischemic stroke patients and healthy controls

The study included 60 participants aged 18 years and above. Thirty were IS patients, and thirty were healthy controls. Table 5 summarizes information regarding their age, BMI, gender, history of diabetes, hypertension, smoking, family history of stroke, and dyslipidemia.

Mean age significantly differed between IS patients and controls (p-value <0.001). Additionally, older age (> 45 years) was found to be significantly associated with ischemic stroke (<0.001). Diabetes was found to be significantly associated with IS (P value = 0.0012), with a higher proportion of diabetic individuals among cases (56.67%) compared to healthy controls (4%). IS patients were more likely to have hypertension compared to healthy controls (80% in patients vs 40% in controls), and this association was statistically significant (p-value = 0.0037). Compared to healthy controls, patients were more likely to have had a stroke in the past (50% in patients vs 1% in controls). This association between previous stroke history and IS was statistically significant (P value <0.001). IS patients were more likely to have had dyslipidemia before compared to healthy controls (53.33% vs 16.67%). This association between previous dyslipidemia and IS was also statistically significant (p-value = 0.0068). BMI, gender, smoking, and family history of stroke were not significantly associated with IS in this study.

Table 5: Demographic characteristics in IS patients and Healthy Controls

Characteristics		Cases (30)		Controls (30)		P-value
		Mean±SD	n (%)	Mean±SD	n (%)	
Age (years)	Overall	56.41±11.96	-	42.43±6.00	-	**<0.001**(s)[a]

	> 45		25(83.33)		8 (26.67)	**<0.001**(s)[b]
	≤ 45		5(16.67)		22 (73.33)	
BMI (Kg/m^2)	Overall	25.81 ± 5.23	-	24.38 ± 3.82	-	0.2331 (ns)[c]
	≥ 25		15 (50)		12 (40)	0.6038 (ns)[b]
	<25		15 (50)		18 (60)	
Gender	Male	-	18 (60)	-	23 (76.67)	0.2670 (ns)[b]
	Female	-	12 (40)	-	7 (23.33)	
Diabetes	Yes	-	17 (56.67)	-	4 (13.33)	**0.0012** (s)[b]
	No	-	13 (43.33)	-	26 (86.67)	
Hypertension	Yes	-	24 (80)	-	12 (40)	**0.0037** (s)[b]
	No	-	6 (20)	-	18 (60)	
Smoking	Yes	-	5 (16.67)	-	0 (0)	0.0617 (ns)[b]
	No	-	25 (83.33)	-	30 (100)	
Family history of stroke	Yes	-	15 (50)	-	9 (30)	0.1876 (ns)[b]
	No	-	15 (50)	-	21 (70)	
Had stroke before	Yes	-	15 (50)	-	1 (3.33)	**<0.001**(s)[b]
	No	-	15 (50)	-	29 (96.67)	
Had	Yes	-	16 (53.33)	-	5 (16.67)	**0.0068** (s)[b]

dyslipidemia/hyperlipidemia before	No	-	14 (46.67)	-	25 (83.33)	

BMI = Body Mass Index; p-value <0.05 was considered as the level of significance; a = p-value computed using Mann-Whitney U test, b = p-value calculated using chi-square test, c = p-value calculated using the two-sample t-test, s = significant; ns = not significant

3.2 Analysis of Biochemical Parameters

3.2.1 Comparison of biochemical parameters between cases and controls

HDL-C levels were significantly lower in the case group than in controls (Median [IQR]: 35.5 [11.75] vs 41 [7.5], $p = 0.03182$). However, the Fisher exact test revealed low HDL-C levels were not significantly associated with ischemic stroke (p-value = 0.1923). On the other hand, elevated homocysteine levels (>15 μmol/L) were significantly associated with ischemic stroke (p-value = 0.0419). Median levels of total cholesterol, LDL-C, triglycerides, serum vitamin B12, homocysteine, and serum folate did not significantly differ between cases and controls (p values> 0.05). Furthermore, high total cholesterol (≥200 mg/dl), low HDL-C (≤40 mg/dl), high LDL-C (≥130 mg/dl), high triglycerides (≥150 mg/dl), and low serum vitamin B12 (<200 pg/ml), were not significantly associated with ischemic stroke. Both patients and controls showed normal serum folate levels (≥3 ng/ml). As a result, no variation exists between the two groups for low serum folate; therefore, no statistical test for association was performed.

Table 6: Comparison of biochemical parameters between cases and controls

Parameter		Study group						P-value
		Cases (30)			Controls (30)			
		Mean ± SD	Median (IQR)	n(%)	Mean ± SD	Median (IQR)	n(%)	
Total cholesterol (mg/dL)	Overall	165.93 ± 47.10	163.5 (48.75)	-	185.93 ± 45.14	182.5 (50.25)	-	0.0977 (ns)[a]
	≥200	-	-	5 (16.67)	-	-	10 (33.33)	0.2326 (ns)[d]
	<200	-	-	25 (83.33)	-	-	20 (66.67)	
HDL-C (mg/dL)	Overall	35.1 ± 10.61	35.5 (11.75)		41.6 ± 8.93	41 (7.5)	-	**0.03182** (s)[a]
	>40	-	-	10 (33.33)	-	-	16 (53.33)	0.1923(ns) [d]
	≤40	-	-	20 (66.67)	-	-	14 (46.67)	

Parameter		Study group						P-value
		Cases (30)			Controls (30)			
		Mean ± SD	Median (IQR)	n(%)	Mean ± SD	Median (IQR)	n(%)	
LDL-C (mg/dL)	Overall	96.33 ± 36.36	94 (51.5)	-	112.56 ± 35.31	111.5 (38.15)	-	0.0584 (ns)[a]
	≥130	-	-	4 (13.33)	-	-	7 (23.33)	0.5062 (ns)[d]
	<130	-	-	26 (86.67)	-	-	23 (76.67)	
Triglycerides (mg/dL)	Overall	172.67 ± 81.32	156(116.25)	-	163.33 ± 79.89	147.5 (107.75)	-	0.5201 (ns)[a]
	≥150	-	-	15(50)	-	-	14 (46.67)	1 (ns)[d]
	<150	-	-	15 (50)	-	-	16 (53.33)	
Serum Vit B12 (pg/mL)	Overall	468.4 ± 196.23	394 (253)		387.67 ± 108.37	356.5 (159.5)		0.1857 (ns)[a]

Parameter		Study group						P-value
		Cases (30)			Controls (30)			
		Mean ± SD	Median (IQR)	n(%)	Mean ± SD	Median (IQR)	n(%)	
	≥200	-	-	29 (96.67)	-	-	29 (96.67)	1 (ns)[d]
	<200	-	-	1 (3.33)	-	-	1 (3.33)	
Homocysteine (µmol/L)	overall	11.47 ± 6.61	9.595 (10.998)		10.01 ± 3.59	10.08 (4.34)		0.7675 (ns)[a]
	> 15	-	-	9 (30)	-	-	2 (6.67)	**0.0419** (s)[d]
	≤ 15	-	-	21 (70)	-	-	28 (93.33)	
Serum Folate (ng/mL)	Overall	7.44 ± 2.81	6.65 (4.525)		7.48 ± 2.65	7.35 (3.65)		0.8766 (ns)[a]
	≥3	-	-	30 (100)	-	-	30 (100)	NA
	<3	-	-	0 (0)	-	-	0 (0)	

HDL-C = High-Density Lipoprotein Cholesterol; LDL-C = Low-Density Lipoprotein Cholesterol, a = p-value calculated using Mann-Whitney U test; d= p-value calculated using Fisher exact test, s = significant, ns = not significant

Box plots were also used to clarify the distribution of biochemical parameters among IS patients and healthy controls in this study.

Total cholesterol (mg/dL): Ranges for total cholesterol levels were 91 - 291 mg/dL in IS patients and 124 - 322 mg/dL in healthy controls. 25% of the patients had cholesterol levels below 138.5 mg/dL. On the other hand, this threshold was higher in controls at 158.25 mg/dL. The median further highlights this disparity, with half of the IS patients having cholesterol levels below 163.5 mg/dL, compared to 182.5 mg/dL in controls. The 75th percentile (Q3) also reflects a similar trend, with 75% of the cases having total cholesterol levels below 187.25 mg/dL, which is lower compared to the 208.5 mg/dL in controls. It can also be observed that the box plot showing total cholesterol distribution in controls is placed slightly higher than that for cases, clarifying how consistently low triglyceride levels are in cases compared to the controls (Figure 10 (A))

HDL-C (mg/dL): HDL-C levels ranged from 12 - 53 mg/dL and 28 - 66 mg/dL in IS patients and healthy controls, respectively. 25% of the patients had cholesterol levels below 30.25 mg/dL. At the same percentile, healthy controls had below 36.25 mg/dL. Median levels were lower in the case group (35.5 mg/dL) than in the control group (41 mg/dL). At the 75th percentile, triglyceride levels in both cases and controls were almost the same; 75% of the participants in each group were below 42 mg/dL and 43.75 mg/dL, respectively. Visually, the box plot showing HDL-C distribution in controls appears to be slightly above that of cases, further clarifying that, in general, HDL-C levels are consistently lower in cases than in controls (Figure 10 (B))

LDL-C (mg/dL): LDL-C levels ranged from 40.4 - 203.6 mg/dL and 53 - 224 mg/dL in IS patients and healthy controls, respectively. 25% of the patients had cholesterol levels below 66.95 mg/dL. At the same percentile, healthy controls had below 90.5 mg/dL. Median levels were lower in the case group (94 mg/dL) than in the control group (115 mg/dL). The same trend was also observed at the 75th percentile; 75% of IS patients had below 118.45 mg/dL, compared to the controls below

128.65 mg/dL. The box plot showing LDL-C levels distribution in controls appears to be slightly above that of cases, making it more evident that, in general, triglyceride levels were lower in cases than controls (Figure 10 (C))

Triglycerides (mg/dL): The ranges for triglyceride levels in cases and contr5ols were almost similar (63 - 353 mg/dL for cases and 58 - 353 mg/dL for controls). The 25th percentile in the two groups was also approximately the same (107.75 mg/dL in cases vs 107.25 mg/dL in controls). Median triglyceride levels were higher in the case group (156 mg/dL) than in the control group (147.5 mg/dL). 75% of IS patients had below 224 mg/dL, a value slightly higher than that for controls (215 mg/dL) (Figure 10 (D))

Serum VitB12(pg/mL): Serum vitamin B12 levels ranged from 190-567.5 pg/mL in cases and 195-619 pg/mL in controls. At the 25th percentile, serum vitamin B12 levels were slightly higher in cases than controls (314.5 pg/mL in cases vs 309 pg/mL in controls). Half of the patients had serum vitamin B12 levels below 394 pg/mL, while controls had below 356.5 pg/ml. At the 75th percentile, IS patients had higher serum vitamin B12 levels compared to controls (567.5 pg/mL in cases vs. 4678.5 pg/mL in controls) (Figure 10 (E)).

Homocysteine (µmol/L): Homocysteine levels ranged from 2.69-25.27 and 2.9-19-16.81 µmol/L in cases and healthy controls, respectively. 25% of the patients had homocysteine levels below 6.4 µmol/L. This value was slightly lower than that for controls (8.35 µmol/L) at the same percentile. The median homocysteine levels in cases and controls were approximately similar (9.59 µmol/L for cases and 10.08 µmol/L for controls). At the 75th percentile, homocysteine levels were higher in cases (17.4 µmol/L) than in controls (12.68 µmol/L) (Figure 10 (F)).

Serum Folate (ng/mL): The ranges for serum folate were 3.5-13.8 and 3.7- 15.1ng/mL in ischemic stroke patients and healthy controls, respectively. 25% of ischemic stroke patients had serum folate levels below 5.25 ng/mL. This value was approximately equal to that for controls (5.45 ng/mL) at the same percentile. The case group had slightly lower serum folate median levels (6.65 ng/mL) than in control subjects (7.35 ng/mL). Finally, 75% of the cases had levels of this

biochemical parameter below 9.78 ng/mL, slightly higher than the value at the same percentile for controls (9.1 ng/mL) (Figure 10 (G)).

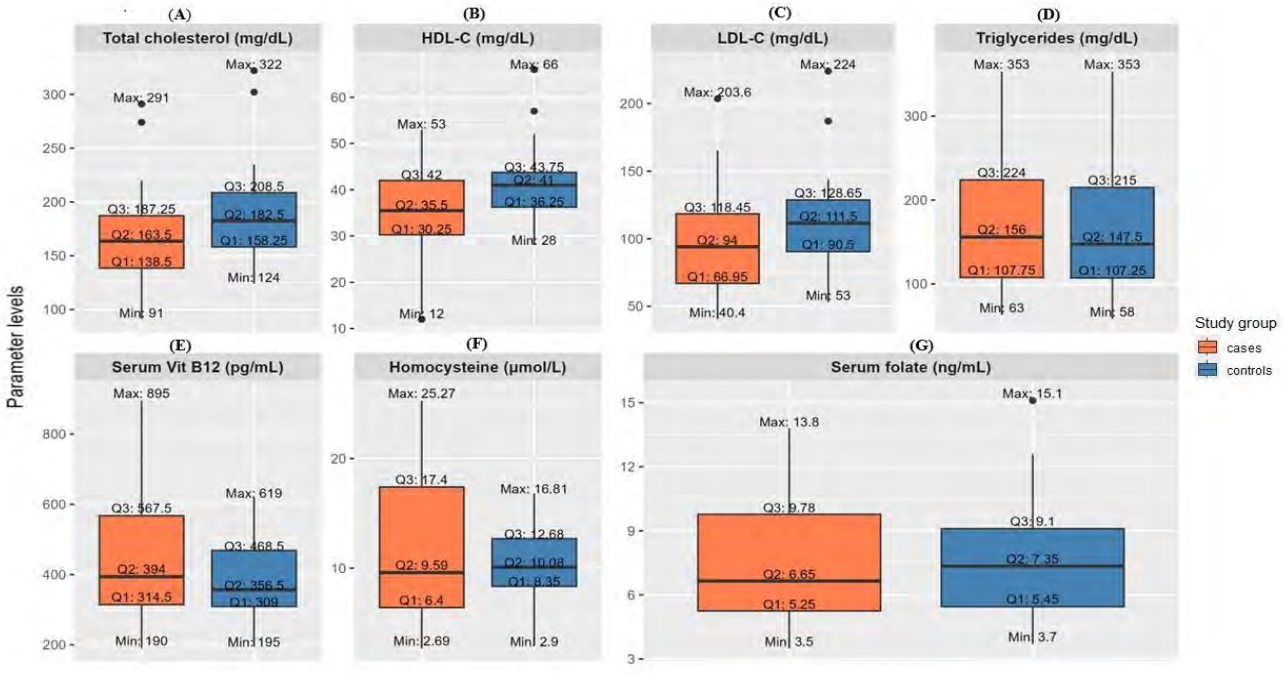

Figure 10: Box plots show biochemical parameter distributions between IS patients and healthy controls.Mas = maximum value, Min = minimum value, Q1 = First quartile/lower quartile/25th percentile, Q2 = 2nd quartile/median//50th percentile, Q3 = third quartile/upp

3.2.2 Correlations between homocysteine and biochemical parameters

After the variables failed the Shapiro-Wilk normality test, Spearman's rank correlation coefficient was used to measure the association between homocysteine and the selected biochemical parameters. A statistically significant weak positive correlation (r = 0.362, p-value = 0.0045) was observed between homocysteine and triglycerides, indicating the association of HHcy with increased triglyceride levels. Conversely, homocysteine was negatively correlated with serum folate (rs = -0.336, p-value = 0.0086). There was no significant correlation between Hcy and total cholesterol, HDL-C, LDL-C, and serum vitamin B12 (Figure 11).

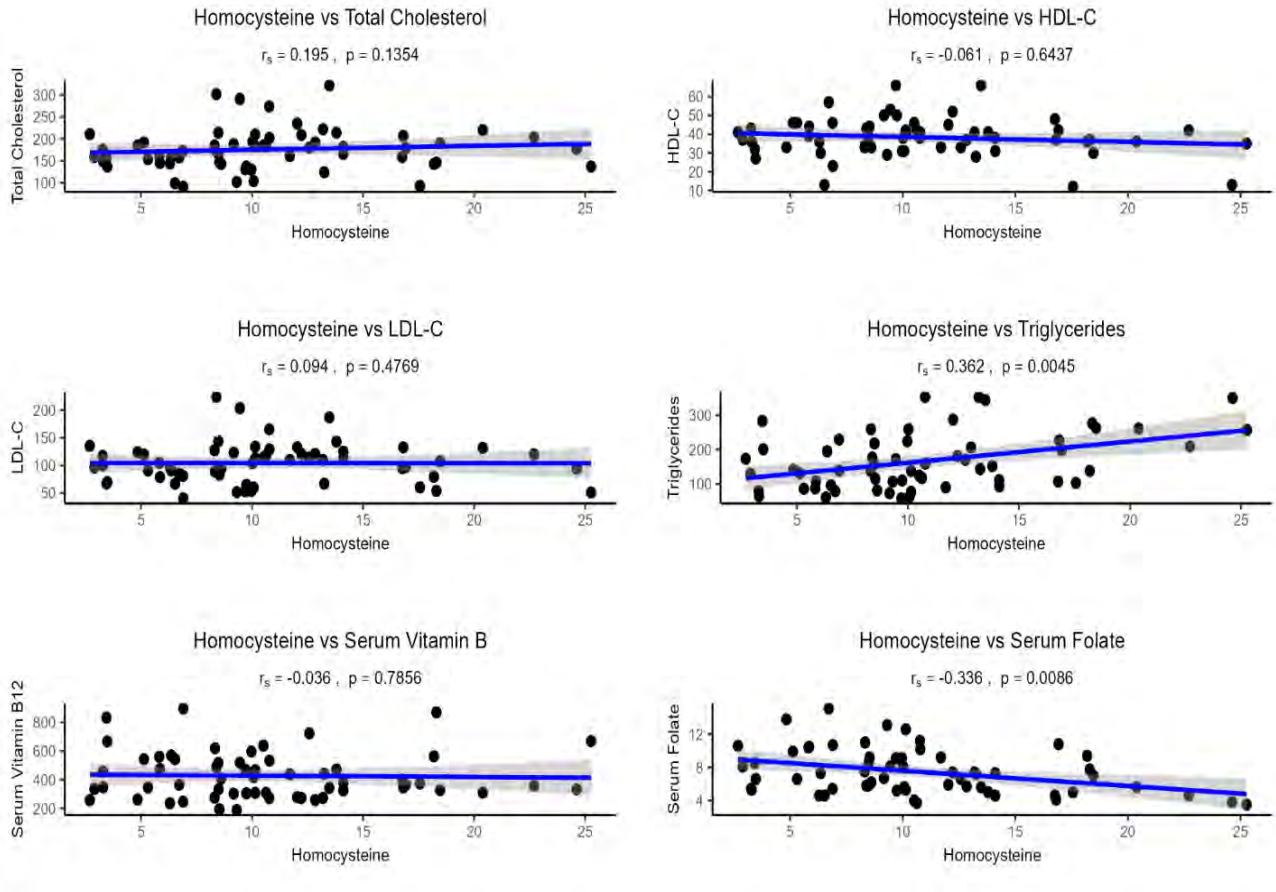

Figure 11: Correlations between homocysteine and biochemical parameters

3.3 Hyperhomocysteinemia

3.3.1 Prevalence of hyperhomocysteinemia

Out of the 60 study participants, 11 (18.33%) had hyperhomocysteinemia (Hcy > 15 µmol/L). This condition was significantly more prevalent in ischemic stroke patients than in controls (9[30%] vs 2[6.67%] in cases and controls, respectively, p-value = 0.02095)

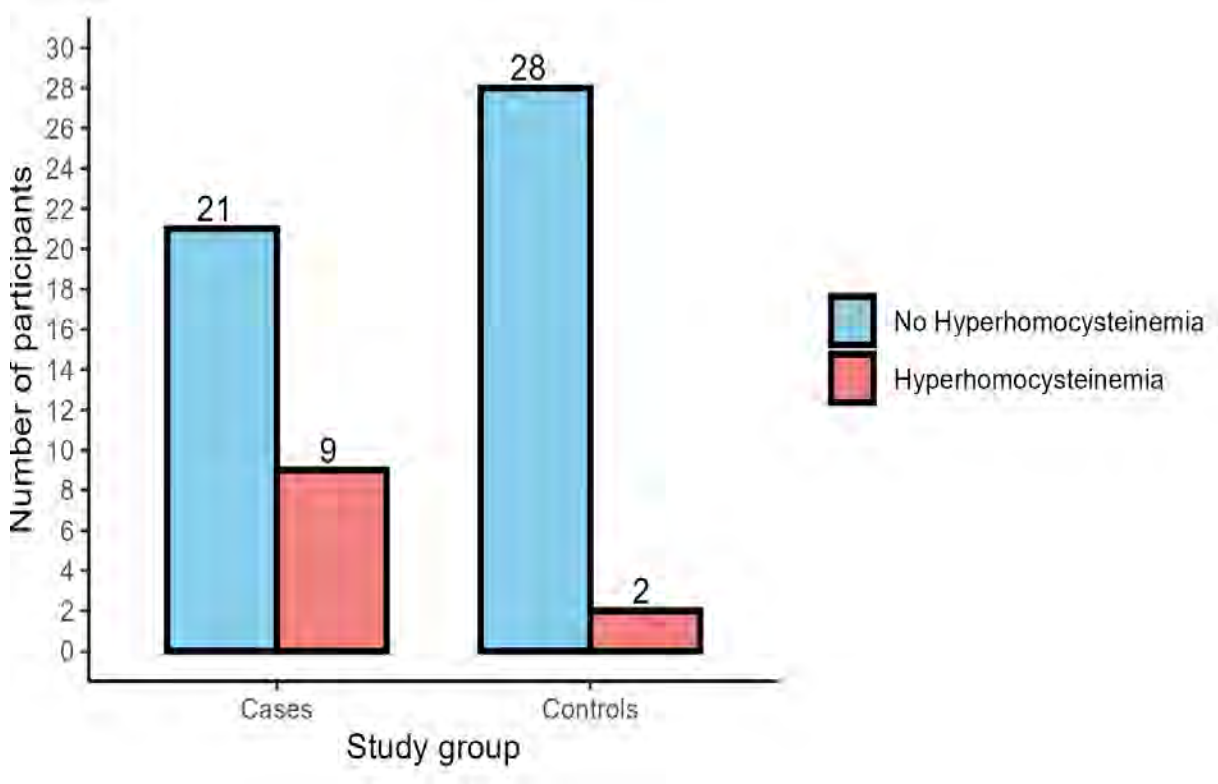

Figure 12: Prevalence of HHcy

3.3.2 Association between Hyperhomocysteinemia and traditional ischemic stroke risk factors among IS patients and healthy Controls

Diabetes, obesity, hypertension, smoking and family history of stroke were not significantly associated with hyperhomocysteinemia in both cases and healthy controls.

Table 7: Association between hyperhomocysteinemia and traditional ischemic stroke risk factors among IS cases and healthy controls

Ischemic stroke risk factors		**IS patients (30)**			**Healthy controls**		
		Hcy > 15 µmol/L (n=9)	**Hcy ≤ 15 µmol/L (n=21)**	**p-value**	**Hcy > 15 µmol/L (n=2)**	**Hcy ≤ 15 µmol/L (n=28)**	**p-value**
		n (%)	**n (%)**		**n (%)**	**n (%)**	
Diabetes	Yes	4(44.44)	13 (61.90)	0.4434	0	4	>0.9999
	No	5 (55.56)	8(38.1)		2	24	
Obesity	Yes	5 (55.56)	10(47.62)	>0.9999	1	11	>0.9999
	No	4(44.44)	11(52.38)		1	17	
Hypertension	Yes	9(100)	15(71.43)	0.1405	0	12	0.5034
	No	0(0)	6(28.57)		2	16	
smoking	Yes	3(33.33)	2(9.52)	0.1432	0	0	>0.9999
	No	6(66.67)	19(90.48)		2	28	
Family history of stroke	Yes	6(66.67)	9(42.86)	0.427	0	9	>0.9999
	No	3(33.33)	12(57.14)		**2**	**19**	

P-values were calculated using Fisher's exact test.

3.4 Dietary patterns

Key findings

Leafy vegetables: Participants consuming leafy vegetables less than three times per week were fewer in cases (33.33%) than in controls (40%). However, the difference was not statistically significant (p-value = 0.3946, Table 8)

Fish: Participants who consumed fish less than three times a week were significantly higher in numbers among IS patients than in controls (16.67% in cases vs 0% in controls, p-value = 0.0261, Table 8)

Milk: Participants consuming milk more than three times a week were more among cases (40%) than in controls (30%). However, the difference was not statistically significant (p-value = 0.3962**,** Table 8**)**

Chicken: Participants consuming chicken less than three times per week were fewer in cases than in controls; however, the difference was not statistically significant (26.67% in cases vs 46.67% in controls, p-value = 0.08993, Table 8**)**

Beef: More cases (10%) than controls (3.33%) consumed beef more than three times a week, but the difference was not statistically significant (p = 0.306; Table 8).

Sugar intake: Consumption of more than 7 teaspoons of sugar per week was slightly less common among the patients (23.33%) than among controls (30%), though no significant difference was observed (p-value = 0.3855, Table 8)

Table 8: Dietary patterns among IS patients and healthy controls

Food item	**Frequency of consumption**		**Cases (30)**	**Controls (30)**	**P-value**
			n (%)	**n (%)**	
Leafy vegetables	<3 times/ week	Yes	10 (33.33)	12 (40)	0.3946
		No	20 (66.67)	18 (60)	-
Fish	<3 times/ week	Yes	5 (16.67)	0 (0)	**0.0261**
		No	25 (83.33)	30 (100)	-
Milk	>3 times/ week	Yes	12 (40)	9 (30)	0.2944
		No	18 (60)	21 (70)	-
Egg	>3 times/ week	Yes	19 (63.33)	17 (56.67)	0.3962
		No	11 (36.67)	13 (43.33)	-
Chicken	>3 times/ week	Yes	8 (26.67)	14 (46.67)	0.08993
		No	22 (73.33)	16 (53.33)	-
Beef	>3 times/ week	Yes	3 (10)	1 (3.33)	0.306
		No	27 (90)	29 (96.67)	-
Sugar intake	>7 tspns/ week	Yes	7 (23.33)	9 (30)	0.3855
		No	23 (76.67)	21(70)	-

The P-values were computed using the Fisher exact test (one-sided)

3.5 PCR and RFLP Results

3.5.1 PCR Results

PCR was done to amplify the *MTHFR* gene segment that contained the SNP C677T. All the 60 DNA samples (30 IS patients and 30 healthy controls) gave the expected band (product size: 513 bp) after agarose gel electrophoresis (Figure 13)

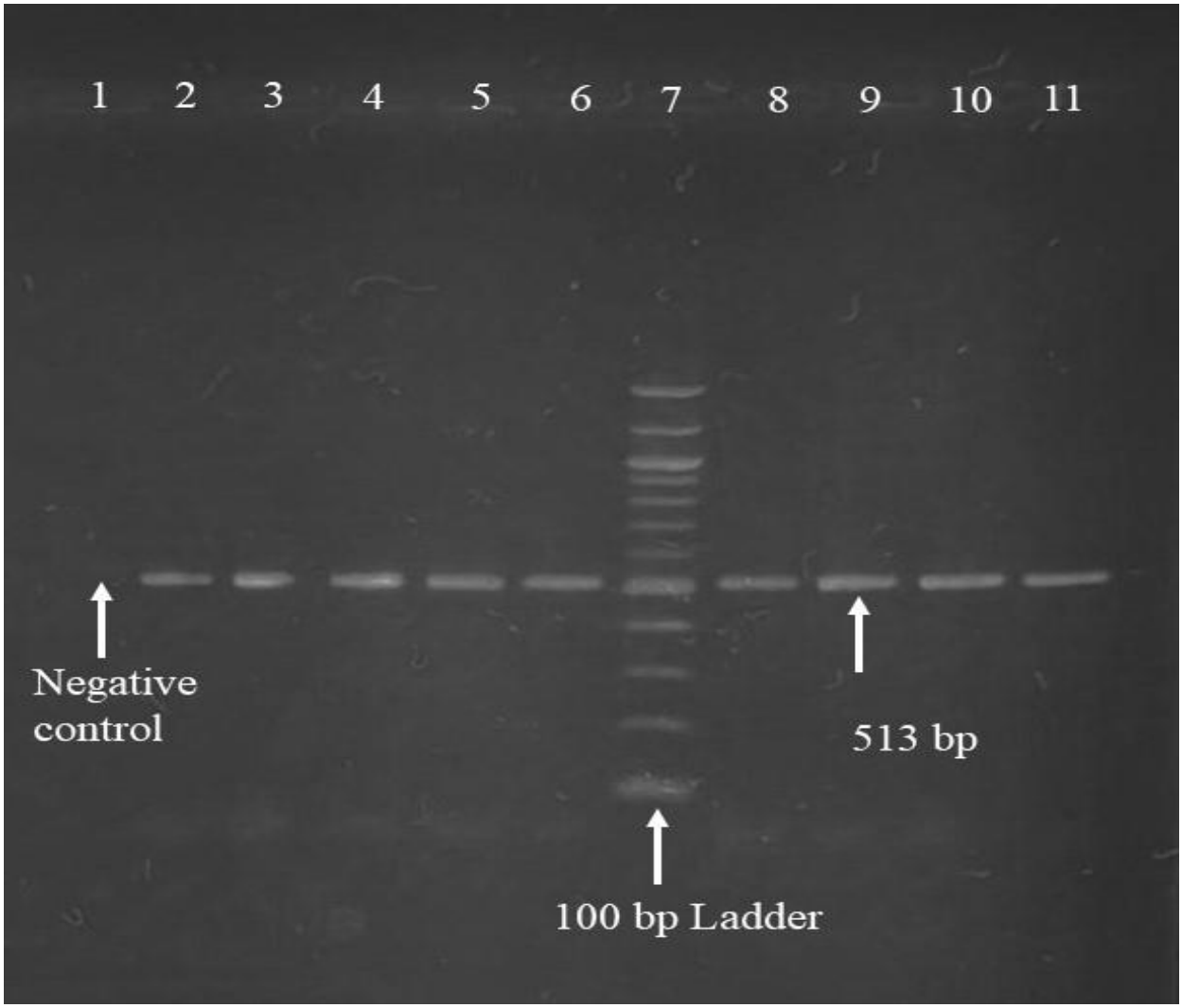

Figure 13: PCR products of MTHFR C677T polymorphism in 1% Agarose gel. Lane 1 contains the negative control. PCR products of MTHFR C677T (513 bp) are in lanes 2 to 6 and 8 to 11. Lane 7 contains the 100 bp DNA ladder.

3.5.2 RFLP Results

All 60 study participants were analyzed for the *MTHFR* C677T mutation, and their genotypes were determined. As expected, if the participant was homozygous normal (CC) for the mutation, one band (513bp) was observed on the gel. If the participant was heterozygous (CT), three bands (513bp, 367bp, and 146 bp) were observed, and if they were homozygous mutants (TT), then two bands (367bp and 146bp) were observed (Figure 14).

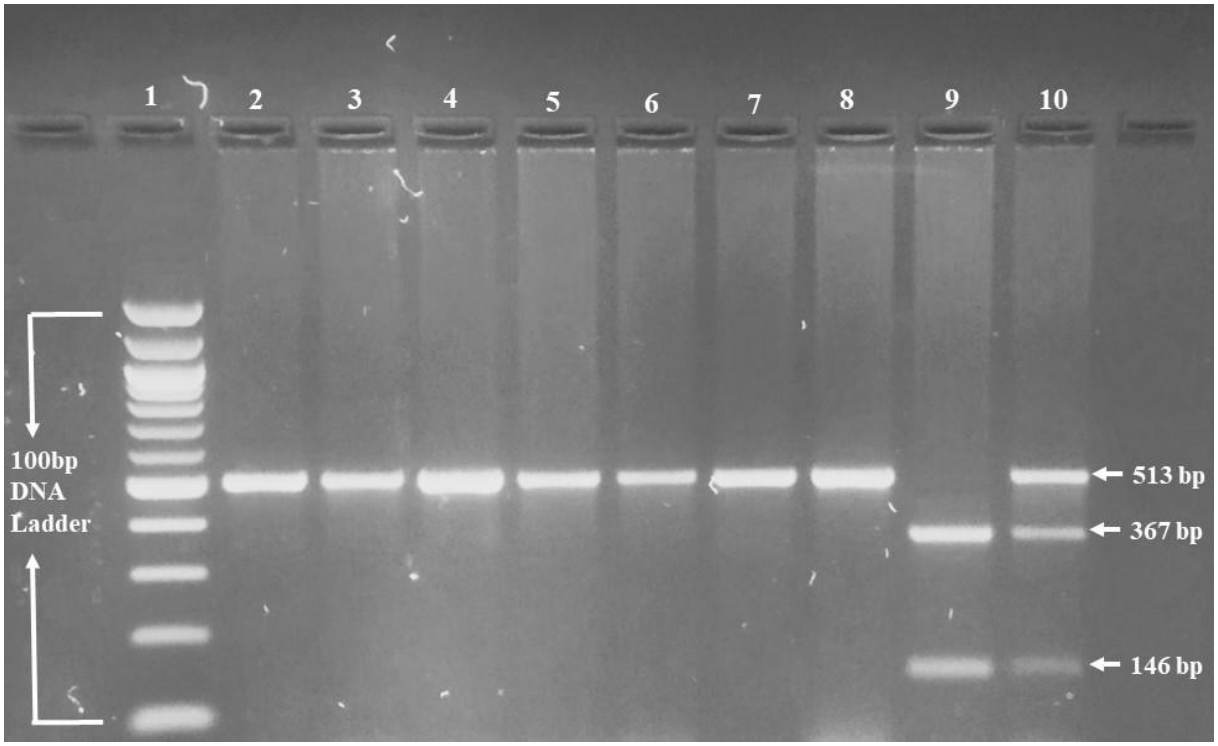

Figure 14: Restricted digested products of MTHFR C667T resolved in 1.5% agarose Gel. Lane 1 contains the 100 bp DNA ladder; lanes 2 to 8 represent the wild-type homozygotes (genotype CC). Lane 9 tontains mutant homozygote (genotype TT). Lane 10 contains heterozygtes (genotype CT).

3.6 Analysis of *MTHFR* C677T genotypes

3.6.1 Distribution of *MTHFR* C677T Polymorphism in IS patients and healthy controls

Among the 60 study participants, 40 (66.67%) were homozygous normal (CC) for the *MTHFR* C677T polymorphism, 18 (30%) were heterozygotes (CT), and 2 (3.33%) were fully mutated (TT). Among the cases, 21 (70%), 7 (23.33%), and 2 (6.67%) had the CC, CT, and TT genotypes, respectively. Of the healthy controls, 19 (63.33%) had the CC genotype, 11 (36.67%) had the CT genotype, and none had the TT genotype.

The distribution of *MTHFR* C677T genotypes and allele frequency show no significant differences between stroke patients and the control group, with the TT genotype observed two times in the cases and neither in the control group. Both the case and the control groups are in HWE for the *MTHFR* C677T variant. The observed genotype frequencies in each group did not significantly differ from the expected values under HWE (P-values> 0.05)

Table 9: Distribution of MTHFR C677T Polymorphism in IS patients and Healthy controls

Genotype	**Ischemic stroke patients (30)**	**Healthy controls (30)**	**P-value**
	n (%)	n (%)	
CC	21 (70)	19 (63.33)	0.2765
CT	7 (23.33)	11 (36.67)	
TT	2 (6.67)	0	
Hardy–Weinberg p-value	0.2247	0.5511	
Allele	n (%)	n (%)	
C	49 (81.67)	49 (81.67)	>0.9999

T	11 (18.33)	11 (18.33)	

3.6.2 Distribution of genotype and allele frequencies among HHcy groups

Genotypes and allele frequency in HHcy and non-HHcy groups were compared. The T allele frequency was 27.3 % in participants with HHcy compared to 16.3% in those without HHcy, while the C allele frequency was 72.7% in the hyperhomocysteinemic participants compared to 83.7 % in those without HHcy (Figure 14(A)). *MTHFR* genotype frequency distribution in the HHcy group was: CC 54.5%, CT 36.4%, and TT 9.1%. In the non-HHcy group, the MTHFR genotypes frequency distribution was: CC 69.4%, CT 28.6%, and TT 2% (Figure 15(B))

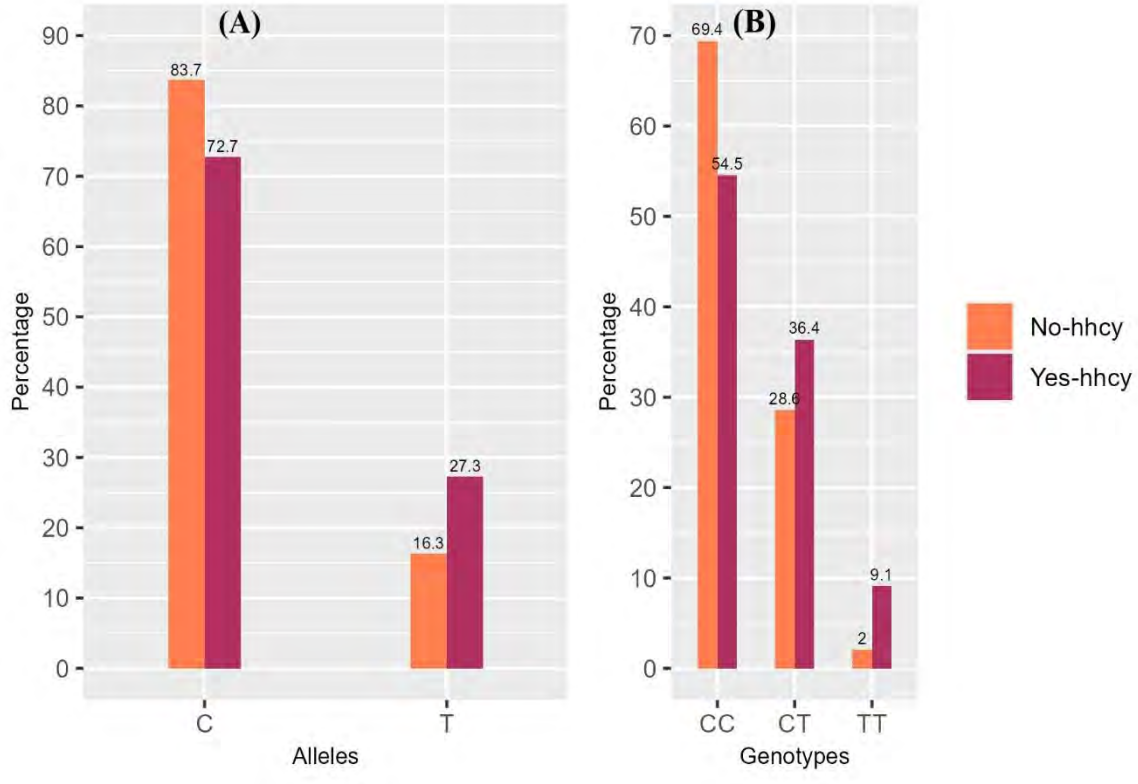

Figure 15: Distribution of genotype and allele frequencies among HHcy groups

3.6.3 Distribution of *MTHFR* C677T Genotypes in Individuals with Hyperhomocysteinemia

Among the 11 hyperhomocysteinemic individuals, 6 (54.55%) had the CC genotype, 4 (36.36%) had the CT genotype, and 1 (9.09%) had the TT genotype (Figure 16 (A)). Among the 9 IS patients with HHcy, 5(55.56%) were either carriers or mutants, and none of the controls had the mutation (Figure 16 (B))

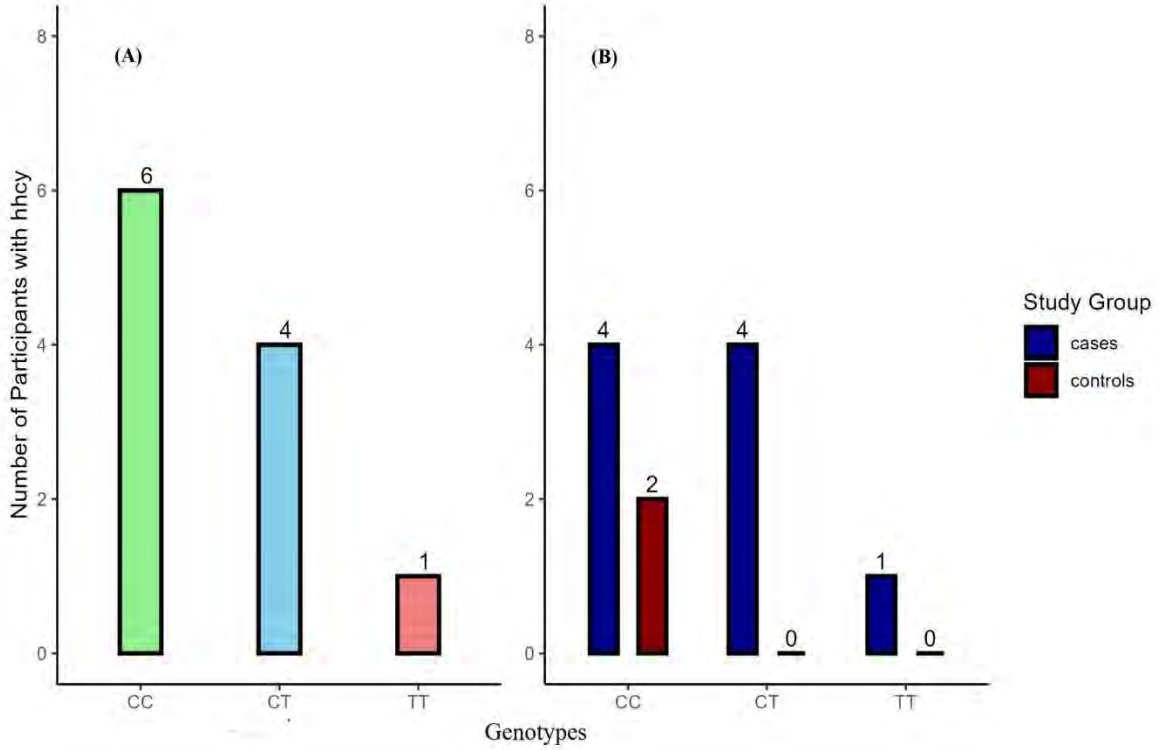

Figure 16: Distribution of MTHFR C677T Genotypes in Individuals with hyperhomocysteinemia. (A) shows the overall distribution of the genotypes among hyperhomocysteinemic participants, and (B) shows the distribution of the genotypes among hyperhomocysteinemic participants in the two study groups: cases and controls.

3.7 Association of biochemical parameters with *MTHFR* C677T genotypes

Median levels of total cholesterol, HDL-C, LDL-C, triglycerides, serum vitamin B12, homocysteine, and serum folate were not significantly different when compared across the three *MTHFR* C677T genotypes (all p values were above 0.05)

Table 10Table 3.7: Association of biochemical parameters with MTHFR C677T genotypes

Parameter	Summary measure	CC (40)	CT (18)	TT (2)	P-value
Total cholesterol (mg/dL)	Mean ± SD	172.18 ± 50.46	186.83 ± 39.31	153.00 ± 9.90	-
	Median (IQR)	169.00 (49.00)	185.00 (47.75)	153.00 (7.00)	0.2393
HDL-C (mg/dL)	Mean ± SD	38.48 ± 11.73	38.61 ± 6.74	33.50 ± 4.95	-
	Median (IQR)	38.00 (12.50)	38.00(8.75)	33.50 (3.50)	0.5732

LDL-C (mg/dL)	Mean ± SD	101.69 ± 35.26	114.12 ± 38.52	72.50 ± 26.16	-
	Median (IQR)	98.50 (44.15)	113.60 (40.00)	72.50 (18.50)	0.1672
Triglycerides (mg/dL)	Mean ± SD	162.40 ± 88.50	167.39 ± 62.90	235.50 ± 57.28	-
	Median (IQR)	138.00 (116.75)	147.00 (94.75)	235.50 (40.50)	0.3012
Serum Vit B12 (pg/mL)	Mean ± SD	416.53 ±159.47	421.28 ±142.00	719.00 ±212.13	-
	Median (IQR)	369.50 (180.50)	372.50 (195.00)	719.00 (150.00)	0.1175
Homocysteine (µmol/L)	Mean ± SD	10.20 ± 4.84	11.78 ± 6.18	12.33 ± 8.44	-

	Median (IQR)	9.88 (6.46)	10.06 (5.69)	12.33 (5.97)	0.9308
Serum Folate (ng/mL)	Mean ± SD	7.37 ± 2.40	7.65 ± 3.49	7.53 ± 0.32	-
	Median (IQR)	6.95 (3.85)	6.40 (4.10)	7.53 (0.23)	0.7349

The Kruskal-Walli's test was used to calculate the p-values

3.8 Distribution of gender, obesity, hypertension, diabetes, family history of stroke, and smoking across the *MTHFR* C677T genotypes in individuals with and without HHcy

3.8.1 Gender-*MTHFR* C677T genotypic patterns in individuals with and without HHcy

Among individuals with HHcy, the CC genotype was more prevalent in males, 6(100%) than in females, 0(0%). Similarly, for the CT genotype, males, 3(75%) outnumbered females, 1(25%). The TT genotype was present in one female and none among males. In contrast, among individuals without HHcy, the CC genotype remained dominated by males, 21 (61.76%), compared to females, 13(38.24%). A similar trend was observed for the CT genotype; males 10(71.43%) outnumbered females 4 (28.57%). The TT genotype was present in one male (100%), and no females were observed. Males were generally more prevalent across the three genotypes in both HHcy and non-HHcy groups (Figure 17).

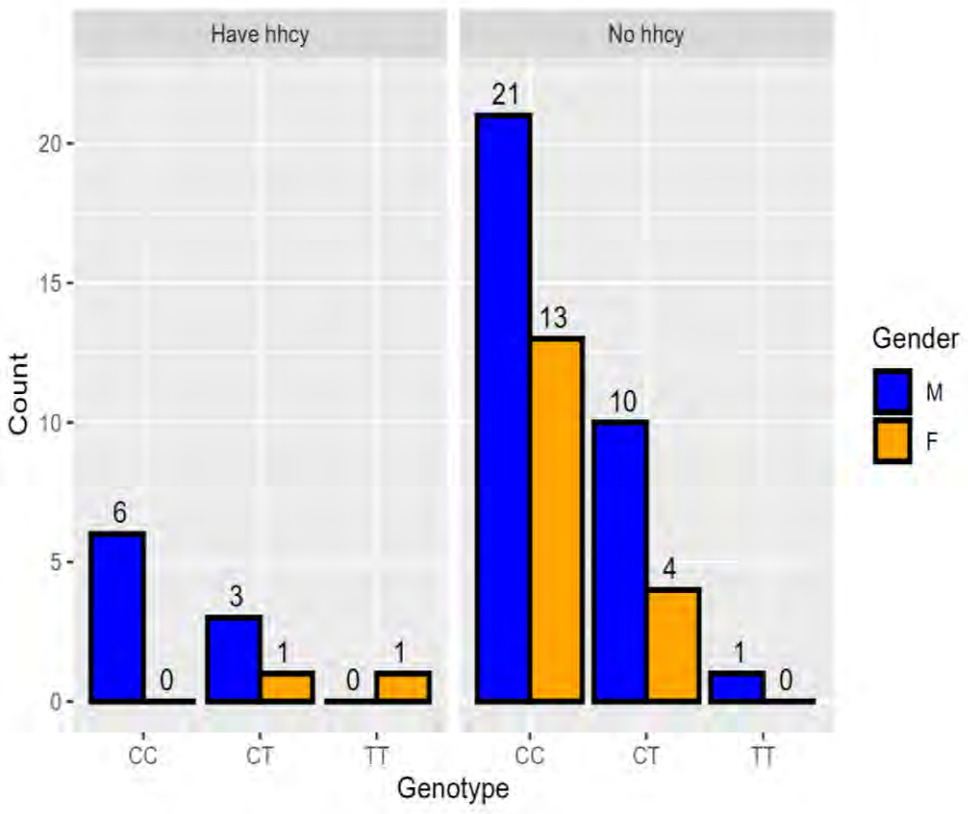

Figure 17: Gender-MTHFR C677T genotypic patterns in individuals with and without HHcy

3.8.2 Obesity-*MTHFR* C677T genotypic patterns in individuals with and without HHcy

Among individuals with hyperhomocysteinemia, the number of obese and non-obese participants with the CC genotype was the same, 3 (50%) in each case. The same scenario was observed in hyperhomocysteinemic individuals with the CT genotype; the number of obese and non-obese individuals was 2 (50%) in each case. The TT genotype was present in one obese participant (100%), and no non-obese participants were observed. In contrast, among individuals without HHcy, non-obese participants, 18 (52.94%) with the CC genotype were more prevalent than those who were obese, 16 (47.06%). A similar trend was observed among non-hyperhomocysteinemic individuals with the CT genotype, where non-obese participants, 10 (71.42%), dominated those who were obese 4 (28.57%). Just like in participants with hyperhomocysteinemia, only one obese participant (100%) with the TT genotype was identified among non-hyperhomocysteinemic individuals, and no none-obese participants were observed (Figure 18)

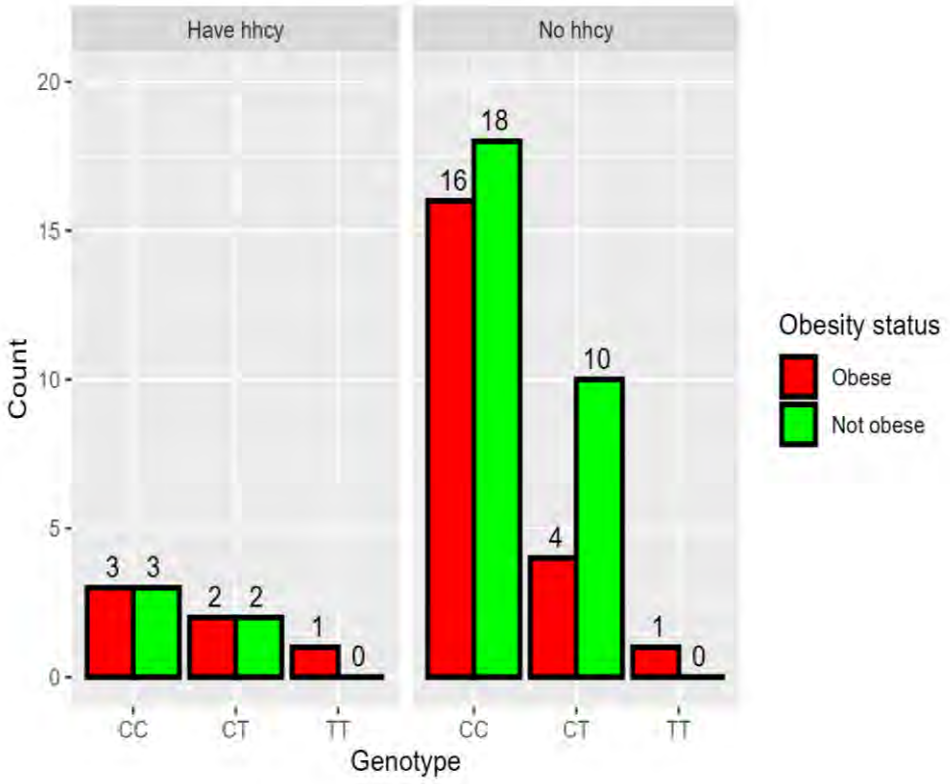

Figure 18: Obesity-MTHFR C677T genotypic patterns in individuals with and without HHcy

3.8.3 Hypertension-*MTHFR* C677T genotypic patterns in individuals with and without HHcy

Among participants with HHcy, the CC genotype was dominated by those with hypertension, 4 (66.67%) than those without, 2(33.33%). Similarly, the CT genotype was more prevalent among those with hypertension, 4 (100%) than in those without, 0(0%). The TT genotype was present in one hypertensive individual (100%), and none without hypertension was identified. On the other hand, among participants with no HHcy, the CC genotype remained more prevalent in individuals with hypertension, 20 (58.82%) compared to those without hypertension, 14 (41.18%). Interestingly, the CT genotype was evenly distributed between those with hypertension, 7(50%), and those without, 7 (50%). The TT genotype was present in one individual without hypertension (100%); no individuals with hypertension were identified (Figure 19).

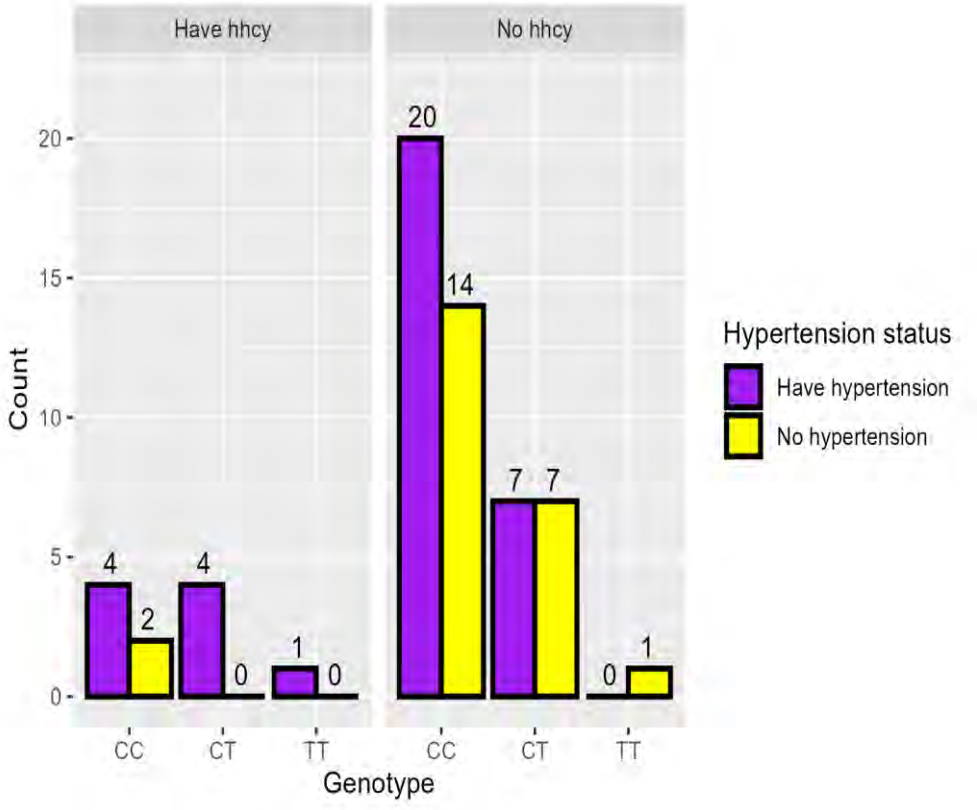

Figure 19: Hypertension-MTHFR C677T genotypic patterns in individuals with and without HHcy

8.4 Diabetes-*MTHFR* C677T genotypic patterns in individuals with and without HHcy

Among participants with HHcy, the CC genotype was evenly distributed between those with diabetes, 3(50%), and those without, 3(50%). The CT genotype was dominated by participants without diabetes 3 (75%), with only one diabetic individual identified (25%). The TT genotype had one diabetic participant (100%), and no non-diabetic individuals were identified. In contrast, among participants without HHcy, the CC genotype was more prevalent in those with no diabetes 20(58.82%) than in diabetic individuals, 14 (41.18%). A similar trend was observed with the CT genotype, which was also dominated by non-diabetic individuals, 12 (85.71%) compared to the diabetic ones, 2(14.29%). The TT genotype was present in one diabetic individual (100%) and none in non-diabetic participants (Figure 20).

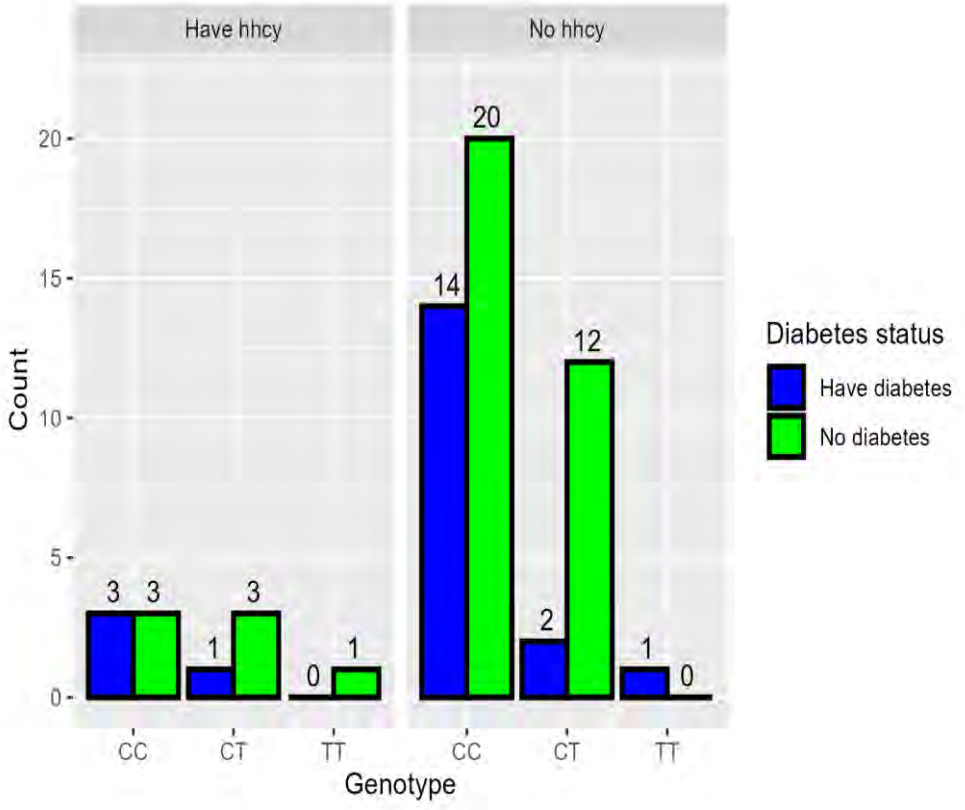

Figure 20: Diabetes-MTHFR C677T genotypic patterns in individuals with and without HHcy

3.8.5 Family history of stroke-*MTHFR* C677T genotypic patterns in individuals with and without HHcy

Among participants with HHcy, the CC genotype was evenly distributed between those with a family history of stroke—3(50%)—and those without, 3(50%). The same scenario was observed for the CT genotype. The TT genotype was present in one participant (100%) with a family history of stroke, and none without a family stroke history was identified. On the other hand, among participants with no HHcy, the CC genotype was more prevalent in participants with no family history of stroke, 22 (64.71%) compared to those with this history 12(35.29%). The same trend was observed regarding the CT genotype, which was more common in individuals without a family stroke history, 9(64.29%) compared to those with this history, 5 (35.71%). The TT genotype was present in one participant with a family history of stroke and in none without this history (Figure 21).

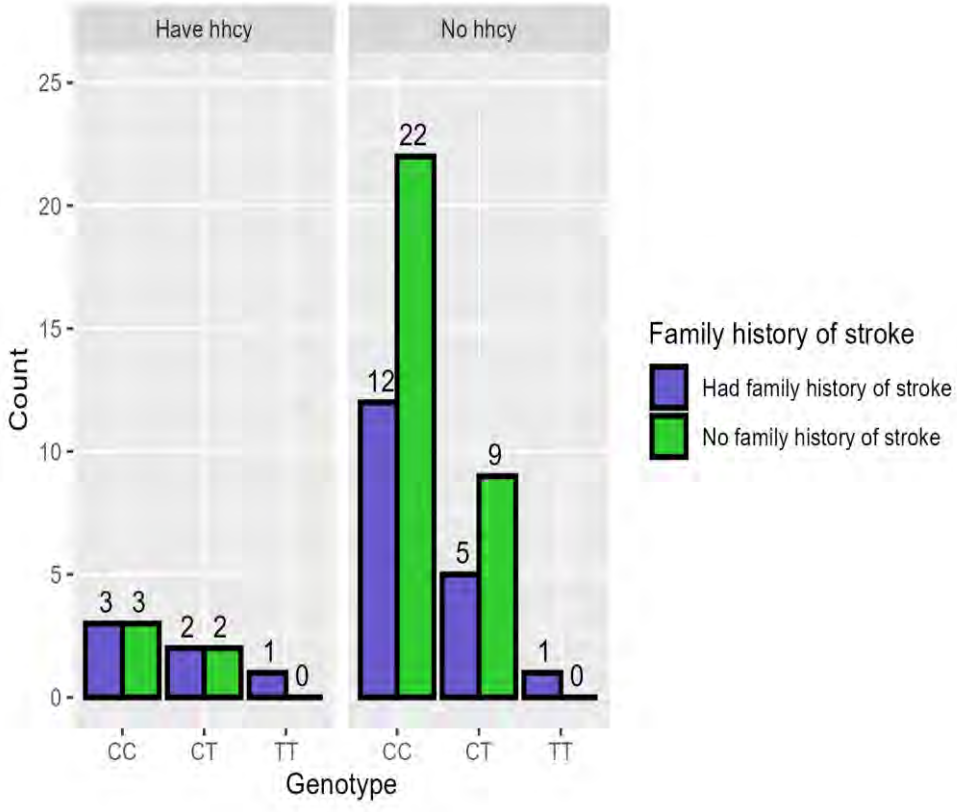

Figure 21: Family history of stroke-MTHFR C677T genotypic patterns in individuals with and without HHcy

3.8.6 Smoking-*MTHFR* C677T genotypic patterns in individuals with and without HHcy

The CC genotype was more prevalent in hyperhomocysteinemic individuals who smoked, 5 (83.33%), compared to the non-smokers, 1 (16.67%). Regarding the CT genotype, it was evenly distributed between hyperhomocysteinemic smokers and non-smokers. The TT genotype was prevalent in one hyperhomocysteinemic non-smoker, and none among smokers was identified. In contrast, among participants with no HHcy, almost all participants with the CC genotype were non-smokers, 33(97.06%). The same trend was observed regarding the CT genotype; all participants were non-smokers, 14(100%). The TT genotype was present in one smoker, and none among non-smokers was identified (Figure 22).

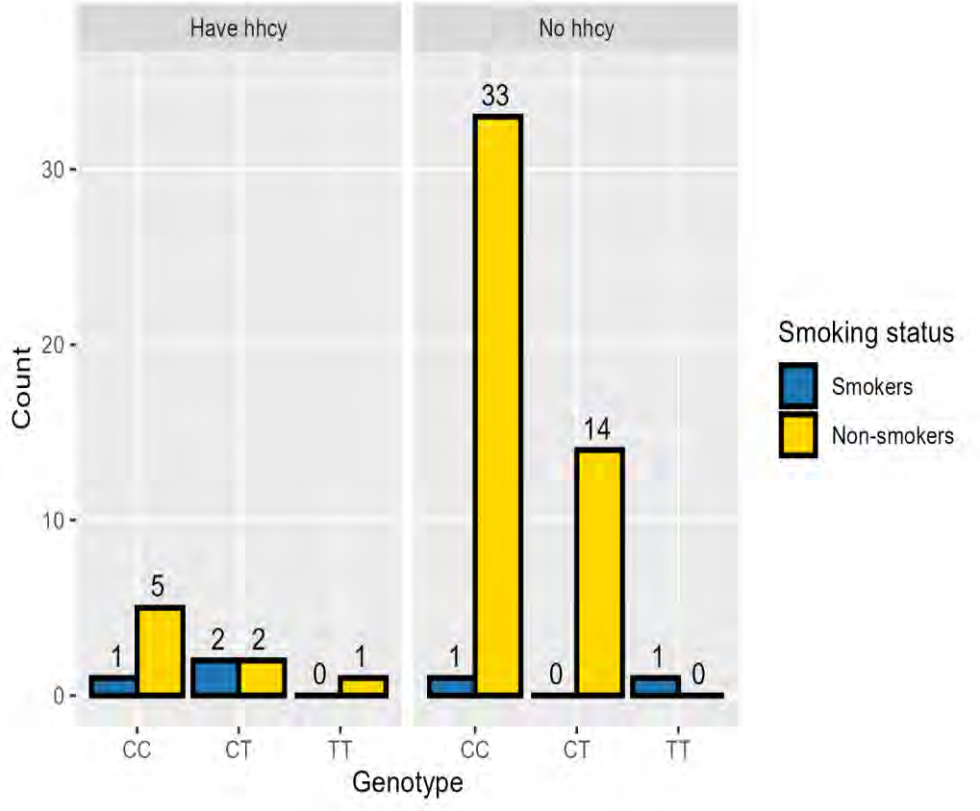

Figure 22: Smoking-MTHFR C677T genotypic patterns in individuals with and without HHcy

3.9 Prediction of HHcy using *MTHFR* C677T genotypes

The unadjusted model suggests that the odds of having HHcy are 1.62 times higher in individuals with the CT genotype than those with the CC genotype. However, this association was not statistically significant (p-value = 0.503). Similarly, those with the TT genotype appeared to have higher odds of having elevated HHcy levels, though this association was also not statistically significant (OR = 5.67, p-value = 0.242). In the adjusted model, after controlling age, BMI, smoking, and diabetes, the association for both genotypes CT and TT remained statistically insignificant (OR = 1.71, p-value = 0.580 and OR = 0.694, P-value = 0.831, respectively)

Table 11: prediction of HHcy using MTHFR C677T genotypes

Genotypes	**Unadjusted values**				**Adjusted values**			
	Odds ratio	95% confidence interval		P-value	Odds ratio	95% confidence interval		P-value
		LCI	HCI			LCI	HCI	
CT	1.62	0.367	6.58	0.503	1.71	0.237	12.0	0.580
TT	5.67	0.206	157	0.242	0.694	0.0157	25.9	0.831

3.10 Prediction of ischemic stroke using HHcy

The unadjusted model suggests that the odds of someone with HHcy having an ischemic stroke are significantly higher than those without HHcy (Unadjusted model; OR = 6.00, 95% CI: 1.37–42.1, P-value = 0.0315). However, after accounting for the confounding variables, age, BMI, smoking, and diabetes, the odds of a person with HHcy developing ischemic stroke turned out to be statistically insignificant (Adjusted model; OR = 2.78, 95% CI: 0.244–31.8, P-value = 0.388), indicating that hyperhomocysteinemia alone may not be responsible for Ischemic stroke.

Table 12: Prediction of ischemic stroke using HHcy

Predictor	**Unadjusted values**				**Adjusted values**			
	Odds ratio	95% confidence interval		P-value	Odds ratio	95% confidence interval		P-value
		LCI	HCI			LCI	HCI	
HHcy	6.00	1.37	42.1	**0.0315**	2.78	0.244	31.8	0.388

Chapter 4

Discussion

4.1 Prevalence of Hyperhomocysteinemia and its association with ischemic stroke.

In this study (60 participants), the overall prevalence of hyperhomocysteinemia (Hcy > 15 μmol/L) was 18.33%. The condition was more prevalent in ischemic stroke patients (30%) than in controls (6.67%), and the difference was statistically significant (p-value = 0.02095), implying that HHcy may potentially be an essential factor in the pathogenesis of ischemic stroke. Our findings are consistent with results from previous studies that reported significant differences in the prevalence of hyperhomocysteinemia between ischemic stroke patients and healthy individuals[66], [67]).

Considering the confounding variables, we conducted logistic regression to evaluate whether hyperhomocysteinemia alone is associated with ischemic stroke. The unadjusted model suggested that having hyperhomocysteinemia is associated with higher odds of developing ischemic stroke compared to those without hyperhomocysteinemia (Unadjusted model; OR = 6.00, 95% CI: 1.37–42.1, P-value = 0.0315). However, after accounting for the confounding variables, in particular, age, BMI, smoking, and diabetes, the association of hyperhomocysteinemia with ischemic stroke turned out to be statistically insignificant (Adjusted model; OR = 2.78, 95% CI: 0.244–31.8, P-value = 0.388), indicating that hyperhomocysteinemia alone may not be responsible for Ischemic stroke. Whereas this is in agreement with some previous studies [68], [69], we cannot ignore the strong evidence that has accumulated over the years that hyperhomocysteinemia is an independent risk factor for ischemic stroke [67], [70], [71]. These differences in study outcomes make it difficult to effectively interpret studies attempting to examine the relationship between hyperhomocysteinemia and ischemic stroke. The underlying reasons might include differences in sample sizes, study designs, study populations, and outcome measures employed in these studies [52]

4.2 Association between hyperhomocysteinemia and traditional ischemic stroke risk factors among IS cases and healthy controls

In this study, the association between HHcy and the traditional risk factors of ischemic stroke was examined to understand the role of homocysteine in the pathogenesis of ischemic stroke. Diabetes, obesity, hypertension, smoking, and family history of stroke were not significantly associated with hyperhomocysteinemia in both cases and healthy controls (p-values>0.05).

As discussed in previous studies, this lack of statistically significant associations indicates that HHcy may influence stroke outcomes through unique mechanisms independent of traditional risk factors [66], [72]. These mechanisms through which HHcy acts to influence stroke need further investigation to aid the development of homocysteine-lowering therapies

4.3 Dietary patterns among IS patients and controls

Diet plays a significant role in managing ischemic stroke. In this study, we aimed to identify specific dietary patterns associated with ischemic stroke among Bangladeshi people. We obtained different percentages for leafy vegetables, fish, milk, chicken, beef, and sugar intake. However, the differences in consumption of these food items between ischemic stroke patients and healthy individuals were not statistically significant except for fish. Participants who consumed fish less than three times a week were significantly more common among IS patients than among controls (16.67% in cases vs 0% in controls, p-value = 0.0261), suggesting that eating less fish might increase the likelihood of one developing ischemic stroke. These findings highlight the importance of maintaining a proper diet to lessen the risk of developing ischemic stroke.

4.4 Analysis of *MTHFR* C677T Genotypes and their Associations with Hyperhomocysteinemia and Ischemic Stroke

All three *MTHFR* C677T genotypes were identified in this study cohort. The CC genotype was observed in 70% of the patients and 63.33% of the controls. The CT genotype was less in patients (23.33%) than in controls 36.67%), and the TT genotype was higher in patients (6.67%) than in

controls (0%). These patterns contradict the results from a recent study that identified the CC genotype in 8% of stroke patients and 46.7% of healthy controls, with the CT genotype being more prevalent in patients (81%) than in controls (47.3%). However, they found the TT genotype higher in patients (11%) than in controls (6%), a pattern similar to the TT genotype prevalence in our study [73]. A study on Turkish stroke patients detected the TT genotype in 6.6% of the patients, a proportion similar to the 6.67% of the same genotype in the patient group in our study. In contrast to our findings, this study detected the TT genotype in 3.3% of the controls [74]. The inconsistencies in the genetic patterns of this polymorphism may be attributed to several factors, including small sample sizes and the differences in the stroke types and subtypes studied [75]

The observed genotype frequencies in the case and control groups did not significantly differ from the expected values under HWE (P-values> 0.05), indicating a stable population with no evolutionary pressures on the *MTHFR* C677T polymorphism. This is in line with what was reported in previous studies [76], [77].

The *MTHFR* C677T polymorphism prevails as one of the most prevalent genetic causes of hyperhomocysteinemia [78], [79]. This study compared genotypes and allele frequencies between participants with HHcy and those without to understand the genetic basis of hyperhomocysteinemia.

The T allele was more prevalent in individuals with HHcy (27.3 %) than in those without HHcy group (16.3%), while the C allele dominated in those without HHcy (83.7 %) compared to the hyperhomocysteinemic individuals (72.7%). Similarly, the TT genotype was more prevalent in the HHcy group (9.1%) than in the non-HHcy group (2%). The CT genotype also dominated in the HHcy group (36.4%) compared to the non-HHcy group (28.6%). Like the C allele, the CC genotype dominated those without HHcy (69.4%) compared to the hyperhomocysteinemic individuals (54.5%). Previous studies have reported similar results, highlighting an increase in the TT genotype and T allele prevalence in individuals with HHcy compared to those without HHcy [80]

The findings from our study underscore a high prevalence of the CT and TT genotypes in people with HHcy compared to those without this condition, further clarifying that the C677T mutation in the *MTHFR* gene predisposes people to hyperhomocysteinemia.

4.5 Strengths and Limitations

The major strength of our study lies in how we selected the control group. It included both hospital-based and non-hospital-based participants, minimizing selection bias. We also excluded tribal or other ethnic groups from participating in the study. This ensured that all the subjects had a uniform genetic background.

The major limitation of our study is the small sample size. It was very challenging to obtain statistical significance with only 60 participants, even with non-parametric tests. Therefore, the interpretation of the findings from this study should be done with caution, and future studies should consider large sample sizes to validate these findings.

Chapter 5

Conclusion

This study focused on understanding the relationship between hyperhomocysteinemia and the *MTHFR* C677T gene polymorphism in ischemic stroke. We found hyperhomocysteinemia to be significantly higher in ischemic stroke patients than in healthy controls. However, when we attempted to run a logistic regression model to understand if hyperhomocysteinemia is an independent risk factor for ischemic stroke, the odds ratio obtained from the adjusted model was not statistically significant. The 95% confidence intervals for the odds ratio were quite large, which indicated that the model was unstable. This could have been because of the small sample size in our study.

The associations between HHcy and the traditional risk factors of ischemic stroke (diabetes, obesity, hypertension, smoking, and family history of stroke) were not statistically significant. This lack of statistically significant associations indicates that HHcy may influence stroke outcomes through unique mechanisms independent of traditional risk factors. Those mechanisms need further investigation.

We also identified dietary patterns that appeared to modulate ischemic stroke among the Bangladeshi people. For example, the analysis revealed that participants who consumed fish less than three times a week were significantly more common among IS patients than controls, suggesting that eating less fish could increase the likelihood of one developing ischemic stroke. These findings underscore the importance of paying attention to our diet, as it is integral in modulating ischemic stroke risk.

Our investigation of the genetic basis of hyperhomocysteinemia revealed a higher prevalence of the T allele in individuals with HHcy than in those without HHcy. Interestingly, the C allele was relatively more common in those without HHcy than in hyperhomocysteinemic individuals. Even the genotypes CT and TT were more prevalent in people with HHcy compared to those without

hyperhomocysteinemia. These findings clarify the critical role the C677T mutation in the *MTHFR* gene plays in the pathogenesis of hyperhomocysteinemia.

Despite the small sample size, our study offers noteworthy contributions to understanding the pathogenesis of ischemic stroke and its associated risk factors. Future large-scale interventional studies are warranted to fully unravel the complex interactions between hyperhomocysteinemia and *MTHFR* polymorphisms in the pathogenesis of ischemic stroke.

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