Genetic Susceptibility to Coronary Artery Disease in Bangladesh: Evaluation of *MTHFR* Gene Polymorphisms (C677T and A1298C) and Hyperhomocysteinemia as independent risk factors of Coronary Artery Disease among young Bangladeshi Adults

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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
B.Sc. in Biotechnology

Mathematics and Natural Sciences
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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. We have acknowledged all main sources of help.

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Approval

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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

Coronary artery disease (CAD) remains a leading cause of morbidity and mortality worldwide. This study investigates the role of elevated plasma homocysteine (Hcy) levels as an independent risk factor for CAD and explores the association of *MTHFR* C677T and A1298C gene polymorphisms with onset of CAD in young Bangladeshi population (≤ 50 years). Blood samples and data from 60 CAD patients and 60 healthy controls were analyzed. Biochemical tests were used to measure plasma Hcy levels and other markers of CAD. PCR-RFLP was used for genotype analysis. Our results showed that 11.7% of CAD patients had elevated Hcy levels compared to 6.7% of controls ($p = 0.53$), indicating a marginally higher prevalence of hyperhomocysteinemia (Hhcy) among CAD patients, though this difference was not statistically significant. The genotype analysis showed 80 individuals with the CC genotype, 35 with CT genotype, and no TT genotype was observed. Genotype analysis identified significant associations between *MTHFR* genotypes and several risk factors: Hhcy and male gender ($p = 0.0004$), family history of cardiovascular disease ($p = 0.0175$), and hypertension ($p = 0.0341$). Furthermore, we found no significant associations between Hhcy and conventional cardiovascular risk factors, suggesting that Hhcy may contribute to CAD through mechanisms independent of traditional risk factors. Our study also uncovered critical dietary factors associated with CAD risk. Higher intake of sugar (OR = 15.129, 95% CI: 4.273–53.565, $p < 0.001$) and chicken (OR = 5.776, 95% CI: 1.287–25.923, $p = 0.022$) were linked to increased CAD risk, whereas frequent fish consumption (OR = 0.227, 95% CI: 0.063–0.816, $p = 0.023$) was associated with a reduced risk. These findings emphasize the importance of dietary modifications in the prevention of CAD. In conclusion, while our study highlights the potential role of Hhcy and specific dietary patterns in CAD risk, the lack of statistical significance in Hhcy's association with CAD suggests a complex interplay between factors.

Keywords: Coronary artery disease, Homocysteine, Hyperhomocysteinemia, *MTHFR* gene polymorphism, C677T polymorphism, A1298C polymorphism, Dietary patterns.

Dedication

Dedicated to the improvement of cardiovascular health and well-being in Bangladesh

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List of Acronyms

CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
IHD	Ischemic Heart Disease
CVD	Cardiovascular Diseases
MI	Myocardial Infarction
CAM	Cell Adhesion Molecules
LDL	Low-density lipoprotein
ACS	Acute Coronary Syndrome
NSTEMI	Non-ST-Elevation Myocardial Infarction
STEMI	ST-Elevation Myocardial Infarction
SIHD	Stable Ischemic Heart Disease
HDL	High-density lipoprotein
HCY	Homocysteine
MET	Methionine
GSH	Glutathione
CBS	Cystathionine β-synthase
CSE	Cystathionine γ-lyase
5-MTHF	5-methyl-tetrahydrofolate
SAH	S-adenosylhomocysteine
HHCY	Hyperhomocysteinemia
MTHFR	Methylenetetrahydrofolate reductase
BMI	Body Mass Index
RFLP	Restriction Fragment Length Polymorphism

PCR	Polymerase Chain Reaction
UV	Ultraviolet
H_2S	Hydrogen Sulfide
5-MTHF	5-methyl-tetrahydrofolate
SAH	S-adenosylhomocysteine
MTR	5-methyltetrahydrofolate-homocysteine-methyltransferase
MTRR	5-methyltetrahydrofolate Homocysteine Methyltransferase Reductase
MMADHC	Metabolism Of Cobalamin Associated D
ST	Smokeless Tobacco
ED	Endothelial Dysfunction
ROS	Reactive Oxygen Species
NO	Nitric Oxide
NF-κB	Nuclear Factor-kappa B
VSMCs	Vascular Smooth Muscle Cells
SNP	Single Nucleotide Polymorphisms
BSMMU	Bangabandhu Sheikh Mujib Medical University
ALP	Alkaline Phosphatase
IRB	Institutional Review Board
EDTA	EthylenediamineTetraacetic Acid

Chapter 1

Introduction

Coronary artery disease (CAD), also known as coronary heart disease (CHD) or ischemic heart disease (IHD), stands as the third leading cause of mortality worldwide [1], posing significant challenges to the global healthcare system. CAD is an umbrella term that encompasses multiple conditions, like stable and unstable angina, myocardial infarction (MI), and sudden cardiac death [2]. In the past, the majority of studies on CAD focused on older individuals, leading to the common belief that CAD primarily affects the elderly. However, there has been a shift in this understanding as current research indicates that CAD is no longer restricted to older adults but is increasingly affecting younger individuals as well. Typically, the younger age bracket for CAD is defined as under 45 years old [3]. CAD before age 55 years for men or 65 years for women is labeled “premature” or “young CAD” [3]. This trend of cardiology has gained importance very recently due to increased prevalence in this age group over the last few decades and is often attributed to the growing prevalence of risk factors like obesity, diabetes, hypertension, and dyslipidemia from an early age, along with genetic predisposition and enhanced detection methods [4].

1.1 Etiology of Coronary Artery Disease

CAD is the most common form of heart disease [2] which is typically characterized by the formation of atherosclerotic plaques that narrows down the vessel lumen [5]. To elaborate, accumulation of fats, cholesterol and other substances on the inner walls of the coronary arteries is called atherosclerosis and the buildup is called plaque. Plaque formation can cause the arteries to narrow down, blocking blood flow (Figure 1.1) [6]. This eventually results in an inadequate supply of blood and oxygen to the myocardium leading to an impaired condition known as myocardial ischemia [5]. Besides, the plaques can also burst/tear, causing a blood clot to form, leading to complete blockage to the arterial blood flow [6]. Apart from high cholesterol, coronary arteries can endure damage due to diabetes or insulin resistance, high blood pressure or hypertension, smoking, obesity, lack of exercise, alcohol intake, excessive stress and so on [6].

Since CAD is a multifactorial phenomenon, the etiological factors of CAD can be broadly classified into non-modifiable and modifiable factors [5]. The factors discussed above are the modifiable factors. Whereas, non-modifiable factors include gender, age, family history, and genetics [5]. Although less often, CAD can also result from coronary artery spasm, known as vasospastic angina [7]. CAD has a tendency to slowly develop over decades. Thus symptoms may go unnoticed until a significant blockage causes problems or until the patient experiences a heart attack [6]. Angina or chest pain is the most common symptom of CAD [6]. Other symptoms indicating CAD include, shortness of breath, pain or discomfort in arms and shoulders, fatigue. With time, CAD weakens the heart muscle to an extent that the heart can not pump blood properly and thus the patient undergoes serious conditions like heart attack or MI [6].

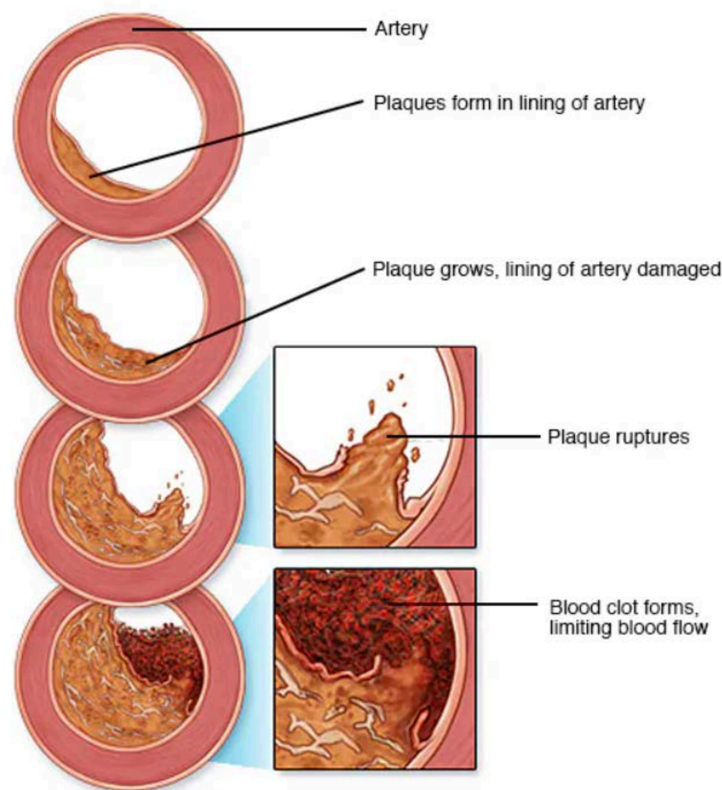

Figure 1.1: Development of atherosclerotic plaque [6]

1.2 Pathophysiology of Coronary Artery Disease

The formation of atherosclerotic plaques marks the hallmark of the pathophysiology of CAD [3]. Plaques are buildup of fatty materials and the formation of these plaques is a gradual process. This process first starts when in response to various risk factors, such as hypertension, dyslipidemia, inflammation, and hyperglycemia, the inner lining of the arteries (endothelium) gets activated and starts to express cell adhesion molecules (CAMs) [8]. CAMs are proteins found on the surface of cells that facilitate cell-cell and cell-matrix interactions [8]. CAMs play a crucial role in the early stages of the disease by promoting the recruitment of inflammatory cells (leukocytes) from the circulation to the arterial wall. Once these cells stick, they can move into the arterial wall (transmigration) with the help of chemoattractant cytokines. In other words, these CAMs, which include VCAM-1, ICAM-1, and E-selectin, facilitate the binding of leukocytes to the endothelium, promoting their transendothelial migration into the arterial intima [8]. Once it reaches the interior of the intima, the recruited leukocytes, mainly monocytes which now convert to macrophages and T cells, interact with smooth muscle cells and endothelial cells, contributing to the formation and progression of atherosclerotic plaques. The macrophages absorb oxidized low-density lipoprotein (LDL) particles, and form foam cells [5]. On the other hand, T cells get activated, releasing cytokines, and contributing to the pathologic process. Additionally, growth factors are also released to activate smooth muscles, which also take up oxidized LDL particles and collagen and get deposited along with activated macrophages, increasing the population of foam cells [5]. All of these collectively contribute to the formation of atherosclerotic plaques [8].

Coronary atherosclerosis is generally distributed in different vessels at variable extents. Over time, as the atheromatous plaque grows in size and becomes stable, the arterial lumen progressively narrows, resulting in ischemia [9]. Once it becomes stable, usually a fibrous cap will form over it, and the lesion will become calcified gradually with time (Figure 1.2) [10]. Eventually, the lesion can undergo ‘hemodynamically significant stenosis’ [5], that enough blood would not reach the myocardial tissue at the time of increased demands (in case of strenuous activity), and angina symptoms might be experienced. However, at rest when the oxygen requirement comes down, these symptoms would subside. For a lesion to cause angina at rest, it must be at least 70% stenosed. Oftentimes, some plaques can rupture and get exposure to tissue

factors, which culminates in thrombosis. This thrombosis could cause subtotal or total occlusion of the lumen and could result in the development of acute coronary syndrome (ACS) in the form of unstable angina, Non-ST-Elevation Myocardial Infarction (NSTEMI), or ST-Elevation Myocardial Infarction (STEMI) [5, 9].

Thus, the classification of CAD is typically done as [5] -

1. Stable ischemic heart disease (SIHD)
2. Acute coronary syndrome (ACS)
    - STEMI
    - NSTEMI
    - Unstable angina

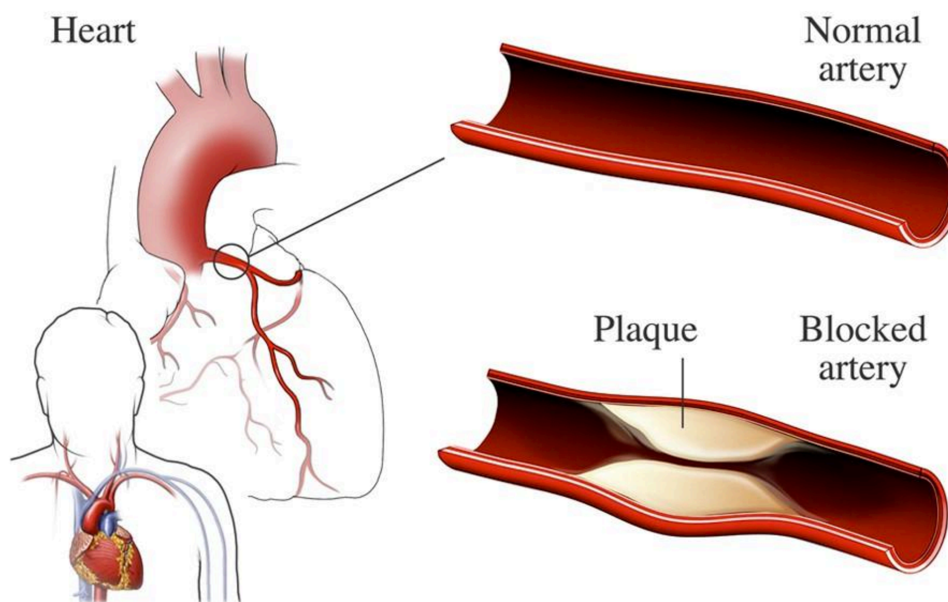

Figure 1.2: Visual representation of a normal vs a blocked artery. [10]

1.3 Epidemiology of Coronary Artery Disease

CAD is the third leading cause of mortality, accounting for one in every six deaths worldwide [13]. Cardiovascular diseases (CVD) contribute to about 17.9 million deaths globally among which CAD accounts for nearly half of it [1,11]. According to the World Health Organization (WHO), CAD contributes to around 16% of the total deaths worldwide [12]. In both 2017 and 2019, CAD alone accounted for around 9 million deaths making it the leading cause of mortality

worldwide [13,14]. Approximately 200 million individuals, 110 million men and 80 million women, are estimated to be living with CAD [14]. The disparity in numbers among the male and female indicate that men are more susceptible to CAD compared to females.

Recent studies indicate that CAD possesses a significant global burden in affecting both developing and developed countries among which 1.2% consist of young CAD cases [15]. Based on regional distribution of the developing countries, CAD is found to be more prevalent in Central and Eastern Europe (Figure 1.3). However, based on ethnicity, South Asians tend to be more at risk of CAD at a younger age with a prevalence ranging from 5% to 10% [15]. In developed nations, CAD was the leading cause of death for both men and women, accounting for one out of every four deaths in the United States [1]. CAD in young adults carries a poor long-term prognosis, and as many as 4% to 10% of AMI events occur in this age group [16].

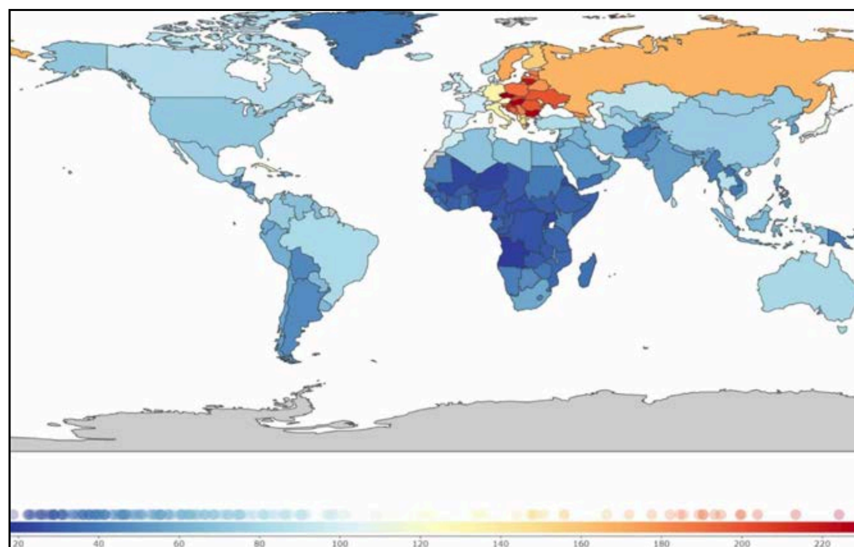

Figure 1.3: Global Distribution of Coronary Artery Disease (CAD): A color-coded representation showing the prevalence of CAD per 100,000 population across different regions worldwide. [12]

1.4 Epidemiology of Coronary Artery Disease in Bangladesh

CAD is a principal contributor to the global mortality rate. In Bangladesh, the prevalence of this disease is escalating in a manner consistent with global trends, thereby evolving from a medical concern to a significant public health issue. CAD has emerged as the leading cause of mortality

in Bangladesh. Comparable to other South Asian populations, individuals in Bangladesh exhibit an elevated predisposition to developing CAD [17]. The detection of CAD in this population is frequently delayed, primarily due to a general lack of awareness and education regarding CAD.

Accurate statistics on the prevalence of CAD in Bangladesh are currently unavailable, as only a limited number of small-scale epidemiological studies have been conducted. The first recorded incidence of ischemic heart disease (IHD) in Bangladesh was documented in 1976 at a rate of 0.33%. Recent data indicates that CAD prevalence ranges from 1.85% to 3.4% in rural populations and reaches 19.6% among urban working professionals. Despite the evident variability in these figures, there is a discernible upward trend in the incidence of CAD within the country.

A study conducted in rural Bangladesh revealed a significant increase in CAD cases between 1986 and 2006. Specifically, the age-adjusted CAD mortality rate among males surged from 16 deaths per 100,000 to 483 deaths per 100,000, an approximate 30-fold increase. For females, the rate escalated from 7 deaths per 100,000 to 330 deaths per 100,000, representing a 47-fold increase. These findings underscore the urgent need for a comprehensive nationwide investigation to accurately characterize the current state of CAD in Bangladesh [17].

1.5 Risk factors of Coronary Artery Disease

CAD arises from a combination of risk factors that can be changed and those that cannot. Modifiable risk factors are behaviors or lifestyle choices that individuals can alter, while non-modifiable risk factors are inherent characteristics or conditions that cannot be changed (Figure 1.4). Unhealthy eating habits, excessive intake of saturated and trans fats, consumption of foods high in salt, and low levels of physical activity are major contributors to the development of CAD.

In addition to these well-known risk factors, several unexpected adverse effects also play a role. For instance, vitamin D deficiency, arsenic contamination in water and food products, and air pollution have all been identified as potential risk factors for CAD [18]. Some of the risk factors are discussed below-

- **Age:** CAD risk increases with age, and men aged 45 or above and women aged 55 or above are at higher risk. Aging, which constitutes one of the main causes of CAD, involves hardening and narrowing of the arteries, thus causing damage and making plaque buildup more possible. Moreover, aging can cause other risk factors to develop such as high blood pressure, high cholesterol and diabetes, which in turn can make this disease worse [19].
- **Gender:** Men have a higher risk of CAD than premenopausal females. Nevertheless, after menopause, the risk for women can naturally be the same as that for men [20]. The components required for estrogen in women are responsible for improving the lipid profile, promoting vasodilation, hindering inflammation, and serving as an antioxidant. As a result, women's risk of heart disease is lower until menopause begins [21].
- **Family History:** Family history indicates a genetic predisposition to CAD beyond the heritability of traditional risk factors. It can also represent both genetic and environmental factors that contribute to CAD risk. Premature family history, defined as CAD occurring before age 55 in males or 65 in females, confers the greatest risk [22].
- **Smoking:** The constituents present in tobacco smoke are the primary agents that impact blood vessels. As a result, there is a rise in atherosclerosis (narrowing of the arteries) that eventually increases the risk of CAD. Smoking or regular tobacco use within the six months preceding the diagnosis is considered a traditional risk factor [22].
- **High Blood Pressure (Hypertension):** High blood pressure (hypertension) puts additional strain in the heart, thereby increasing the chance of CAD [20,22]. The traditional risk factors for hypertension includes positive past history of hypertension and new hypertensives, systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg, based on the average of two or more readings on two or more occasions after initial screening [23].
- **High Cholesterol:** Elevated concentration of low density lipoprotein (LDL) cholesterol (bad cholesterol) along with low level of high density lipoprotein (HDL) cholesterol (good cholesterol) is a cause of atherosclerosis which blocks blood flow in arteries and therefore increases risk of CAD. Hyperlipidemia (total cholesterol> 250 mg/dl, LDL > 160 mg/dl or if the patient was on lipid-lowering therapy) is considered a traditional risk factor [23].

- **Diabetes:** Diabetes is considered a major risk factor for CAD due to the coexistence of multiple cardiovascular risk factors in diabetic individuals, including high blood pressure, abnormal cholesterol levels, obesity, and sedentary lifestyle [22]. The traditional risk factors include diabetes mellitus, positive past history of diabetes and new diabetics, fasting plasma glucose ≥ 120 mg/dl or two hours after glucose load ≥ 200 mg/dl [23].
- **Obesity and Physical Inactivity:** Excess body fat or obesity, along with an inactive lifestyle can often end up with a higher heart disease risk. These factors pose a threat to CAD when they lead to problems such as high cholesterol, high blood pressure, and insulin resistance that is associated with obesity and physical inactivity [17]. In addition to that, physical inactivity also contributes to heart muscle degradation and impaired blood vessel function, giving rise to plaque accumulation and the development of CAD [24].
- **Unhealthy Diet:** Saturated fat, trans fat, cholesterol, and sodium anomalies and also a deficiency in fruits, vegetables, and fiber increased risk of CAD [24]. It can lead to obesity, high blood cholesterol, atherosclerosis, and plaque buildup in the heart's arteries [24].
- **Stress:** Prolonged stress might cause the development of CAD via several mechanisms, including the heightened blood pressure, and some unhealthy defense behaviors [17].
- **Sleep Apnea:** Untreated sleep apnea syndrome, which is a disease characterized by one or more episodes of pauses in breathing during the sleep is also considered as a risk factor of CAD [22].
- **Excessive Alcohol Consumption:** Alcohol abuse raises blood pressure,contributes to obesity and increases triglycerides all of which are common in patients with CAD [17,22].

In Bangladesh, CAD was found to be associated with many risk factors. Many people in Bangladesh are adopting new dietary habits and sedentary lifestyles, increasing their risk due to higher levels of cholesterol, blood pressure, and insulin abnormalities. Besides, genetic predispositions and family history are also considered major risk factors since they can cause inherited conditions and shared lifestyle habits within families can heighten the risk of CAD. In addition, coronary health problems could aggravate from air pollution or secondhand smoke. As

early screening and lifestyle modifications are critical in reducing the risk of CAD, access to healthcare services and awareness of preventive measures are crucial [17].

In most cases, these risk factors operate synergistically. If there are multiple risk factors present, the overall risk to develop CAD may be increased greatly. The risk factors that can be modified like quitting smoking, adopting a healthy diet, regular exercise, and managing stress, play an essential role in the prevention of CAD as well as its complications. The usage of medication to control hypertension and dyslipidemia when necessary is beneficial.

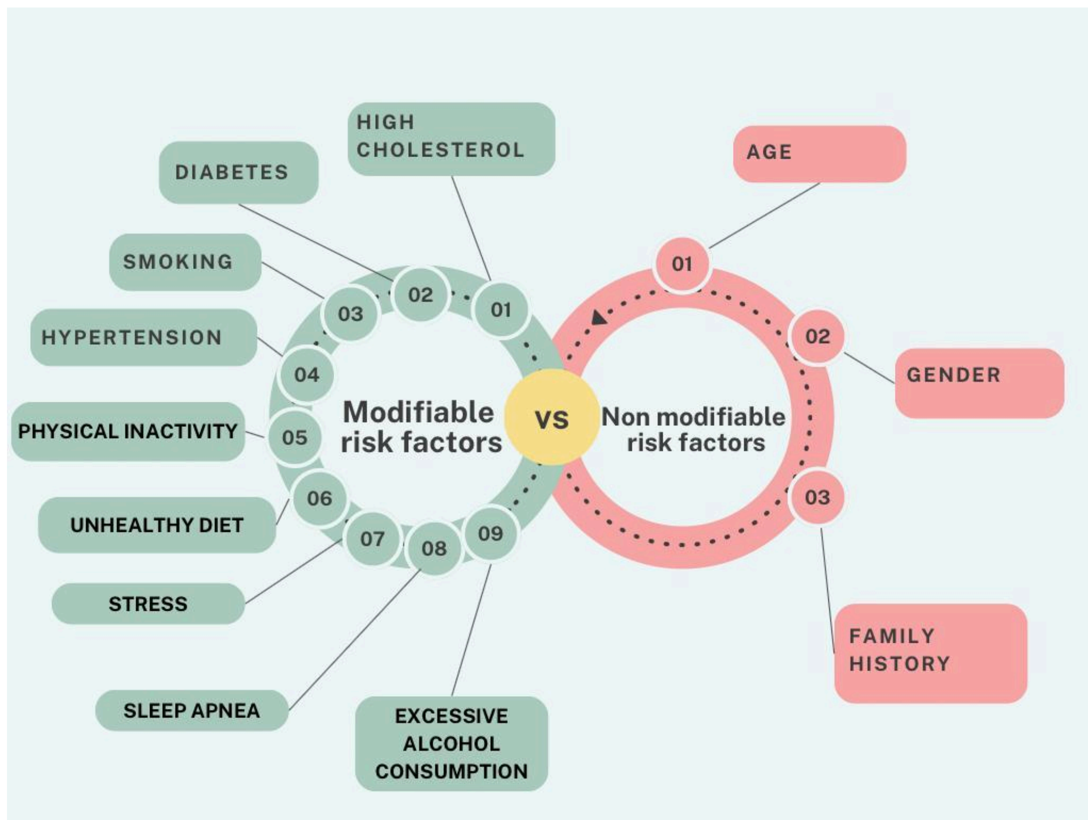

Figure 1.4: Modifiable and non-modifiable risk factors of CAD

1.6 Homocysteine

Homocysteine (Hcy) is a sulfur-containing non-essential amino acid present in the body, synthesized as an intermediate compound during the metabolism of the essential amino acid

methionine (Met) obtained from dietary sources [25]. Animal-based foods such as egg whites, meat, and dairy products, as well as plant-based sources like nuts and seeds, are the primary dietary sources of methionine [26]. With the help of pyridoxine (vitamin B6), folate (vitamin B9), cobalamin (vitamin B12), and riboflavin (vitamin B2), Hcy functions in protein homeostasis, maintaining the methionine level, and DNA methylation, making it essential for post-genomic and epigenetic regulatory processes [27].

Through various biochemical pathways Hcy is converted into other substances required by the body, leaving very little amount left in the bloodstream. Elevated Hcy levels have been associated with a higher risk of CVD, pregnancy complications and congenital abnormalities, neurological and cognitive disorders and various health concerns [28]. Genetics mutation, vitamin B deficiency, medication, diet and environmental factors can influence the level of Hcy in the body [29].

1.6.1 Homocysteine Structure

The structure of Hcy includes a central carbon atom bonded to a hydrogen atom, a carboxyl group (-COOH), an amino group (-NH2), and a sulfur atom (-SH) (Figure 1.5(b)). It also includes two carbon atoms adjacent to the sulfur atom, forming a methylene bridge (-CH2-). Through metabolism of Hcy, it is converted to cysteine and methionine. Hcy is the homologue of the amino acid cysteine where it differs by an extra methylene bridge (-CH2-) (Figure 1.5(b,c)) [30]. Methionine contains an additional methyl group (-CH3) attached to its sulfur atom which is absent in Hcy (Figure 1.5(a)).

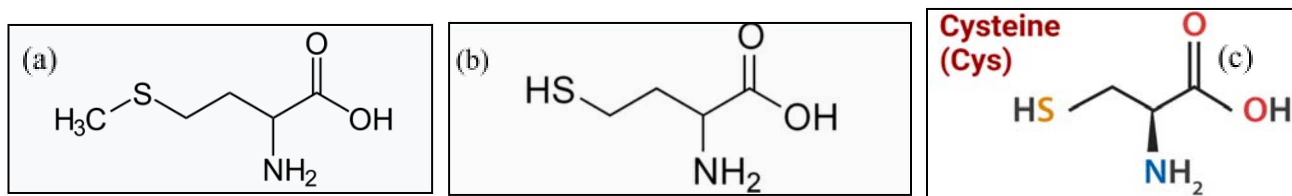

Figure 1.5: Structure of (a) Methionine, (b) Homocysteine and (c) Cysteine [30]

1.6.2 Homocysteine Metabolism

Hcy metabolism is essential for maintaining normal levels of Hcy and methionine in the body which is regulated by the availability of specific cofactors and enzymes. Hcy is transformed into

other substances such as cysteine or is recycled back into methionine. The metabolism of Hcy mainly involves two pathways, transsulfuration and remethylation pathway.

1.6.2.1 Transsulfuration Pathway

Transsulfuration pathway is the metabolic pathway in which sulfur is transferred from Hcy to cysteine (Figure 1.6(b)) [36]. Transsulfuration pathway involves the generation of sulfur metabolites such as cysteine, glutathione (GSH) or the gaseous signaling molecule hydrogen sulfide (H_2S), as well [31]. The transsulfuration pathway, with the help of the key regulators cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzyme enables the transfer of sulfur from Hcy to cysteine through cystathionine, ultimately resulting in the synthesis of cysteine [31].

1.6.2.2 Remethylation Pathway

In the remethylation pathway, Hcy receives a methyl group from 5-methyl-tetrahydrofolate (5-MTHF) to form methionine (Figure 1.6(a)) [36]. This step is catalyzed by the enzyme methionine synthase, which requires vitamin B12 (cobalamin) as a cofactor [31].

SAM donates its methyl group to Hcy in SAM-dependent methylation reactions, leading to the formation of S-adenosylhomocysteine (SAH) as a byproduct. SAH undergoes hydrolysis to yield Hcy and adenosine [28]. Subsequently, Hcy is regenerated to methionine through the transfer of a methyl group from 5-methyltetrahydrofolate by methionine synthases.

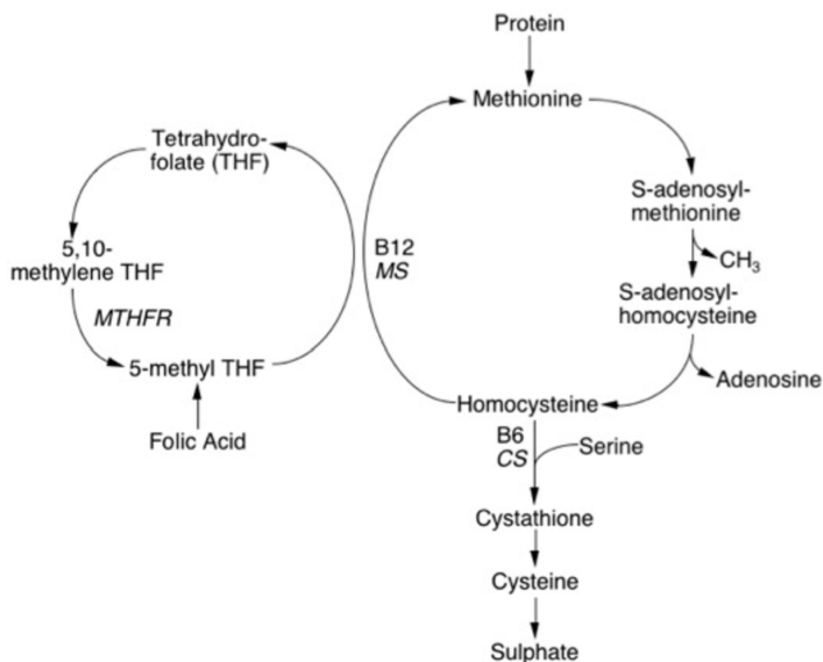

Figure 1.6: Homocysteine metabolism; (a) Remethylation and (b) Transsulfuration [36]

1.7 Hyperhomocysteinemia

Hyperhomocysteinemia (Hhcy) is a medical condition marked by abnormally elevated levels of total Hcy in the bloodstream. Typically, fasting plasma Hcy concentrations between 5 and 15 µmol/L are considered normal [32]. When levels surpass this range, the condition is termed Hhcy. Based on the extent of elevation, Hhcy is classified into three categories: mild (15-30 µmol/l), intermediate (30-100 µmol/l), and severe (>100 µmol/l). It is a recognized risk factor for several diseases (Figure 1.7), including cardiovascular and neurological conditions [33, 34]. Hhcy is linked with inflammation and the development of atherosclerosis. Moreover, it serves as an independent risk factor for CVD, such as stroke and MI [35].

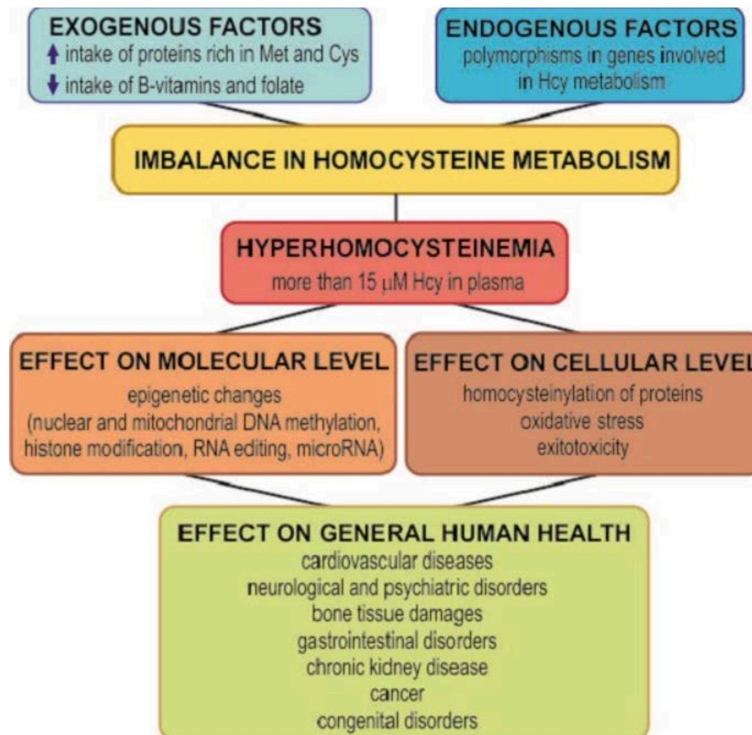

Figure 1.7: Mechanism behind Hyperhomocysteinemia [28]

1.7.1 Causes of Hyperhomocysteinemia

Commonly, high or elevated levels of Hcy in an individual's blood indicates they might have some vitamin deficiencies, particularly of vitamin B6, folic acid (vitamin B9), and vitamin B12 as these are essential for the breakdown of excess Hcy from the blood. However, the causes of Hhcy are diverse and can be categorized into genetic and environmental factors (Figure 1.8):

Genetic factors: Genetic causes involve issues with the metabolism of Hcy, often due to defects/impairments in certain enzymes such as cystathionine β-synthase and Methylenetetrahydrofolate reductase (*MTHFR*). When these enzymes do not function properly, it can cause very high levels of Hcy in the body, leading to severe Hhcy and a genetic condition called homocystinuria. Other genetic factors that can contribute to Hhcy include mutations in the genes Cystathionine Beta-Synthase (CBS), 5-methyltetrahydrofolate-homocysteine-methyltransferase (MTR), 5-Methyltetrahydrofolate Homocysteine Methyltransferase Reductase (MTRR), and Metabolism Of Cobalamin Associated D (MMADHC) [37].

Environmental factors: Environmental factors that can cause Hhcy include -

- **Vitamin Deficiency:** Vitamin B12 and folate are essential for the metabolism of Hcy. Deficiency in either of these vitamins can lead to an accumulation of Hcy in the blood, which can increase the risk of certain health conditions, including atherosclerosis [38].
- **Kidney Disease:** Impaired renal function in kidney disease can contribute to Hhcy due to decreased clearance of Hcy [39].
- **Malabsorption:** Conditions that cause malabsorption, such as celiac disease, can lead to Hhcy due to impaired absorption of vitamins and nutrients [40].
- **Age:** Advanced age, particularly in older adults, can be a risk factor for Hhcy due to decreased renal function and clearance of Hcy [41].
- **Excessive Methionine Intake:** High intake of methionine, an essential amino acid found in high-protein foods, can contribute to elevated Hcy levels [38].
- **Medications:** Drugs such as cholestyramine, metformin, methotrexate, nicotinic acid(niacin), oral contraceptive pills can interfere with Hcy metabolism and contribute to Hhcy [42].
- **Other Diseases:** Conditions like hypothyroidism, psoriasis, malignant tumors and certain medications can also be associated with Hhcy [38].

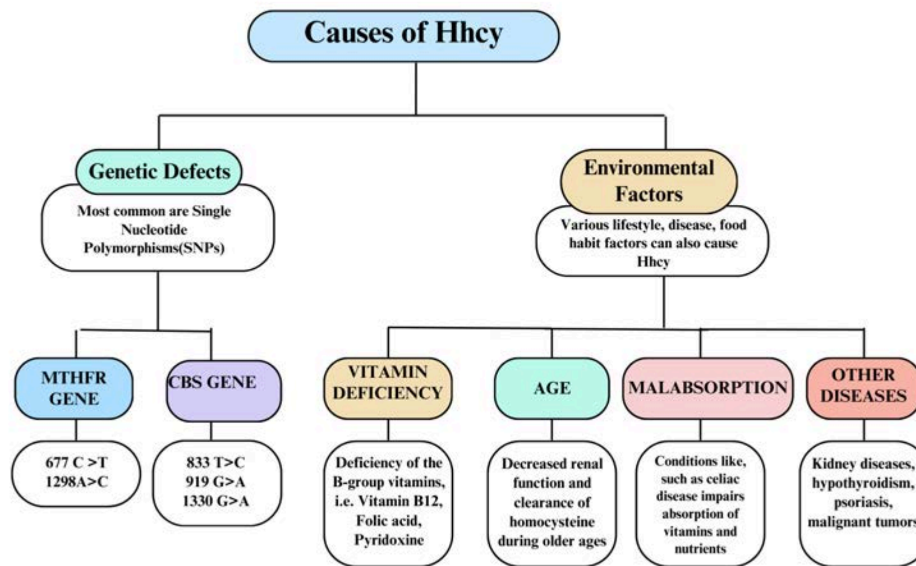

Figure 1.8: Causes of hyperhomocysteinemia

1.7.2 Risk factors for developing Hyperhomocysteinemia in Bangladeshi population

The risk factors for developing Hhcy in Bangladeshi populations include deficiencies in folate (Vitamin B9) and cobalamin (Vitamin B12), which are essential for the metabolism of Hcy, as well as lifestyle factors such as poor dietary habits including low intake of folate-rich foods, and betel nut usage, excess body weight, and smoking behavior [43]. Legumes (beans, peas, lentils), asparagus, leafy greens, beets, brussel, sprouts, eggs, broccoli, nuts and seeds, dairy, fish, clams, sardines, tuna, beef liver, papaya, banana, avocado citrus fruits, and fortified grains are an excellent source of folate and cobalamin [44, 45]. But these food items are not always prioritized in a balanced way in the daily meals of the people of Bangladesh. Additionally, traditional Bangladeshi methods of food preparation involve prolonged cooking, which can result in the oxidation of up to 95% of naturally occurring food folates [43]. As a result, the prevalence of Hhcy among Bangladeshi men is reportedly higher than that among their white European counterparts, and this has been largely attributed to these dietary deficiencies [43]. Apart from dietary habits, smoking behavior has also been identified as an independent risk factor for Hhcy in Bangladeshi populations, with smoking being associated with an increased risk of elevated Hcy levels. A study investigating the association between smokeless tobacco (ST) use and Hhcy

in a low-income urban locality in Karachi, Pakistan found that the occurrence of Hhcy was nearly 15-fold among ST users compared to non-users [46]. Moreover, betel nut usage is another risk factor for Hhcy among Bangladeshi populations. The same study [46] conducted in Karachi, Pakistan, also suggests that ST consumers, especially those who use these products along with betel nuts, are more prone to developing Hhcy. The study also found that Hcy concentrations in the group which consumed ST alone and the group which consumed ST along with betel nut were significantly higher compared to the non-user group [46]. Thus, dietary food habits, smoking behaviour, and betel usage are the key contributors to the prevalence of Hhcy among Bangladeshi population.

1.7.3 Hyperhomocysteinemia and Coronary Artery Disease

Several studies have investigated the link between Hhcy and CAD, with growing evidence indicating that Hhcy is an independent risk factor for CAD, in addition to conventional cardiovascular risk factors [47]. The mechanism behind this link is complex and involves several pathways [35]-

1. **Endothelial dysfunction (ED):** The endothelium is a single layer of cells lining all blood vessels. Endothelial dysfunction (ED) is defined as an impairment of endothelium-dependent relaxation of blood vessels. It is the earliest indicator of atherosclerosis and vascular diseases [48]. Hhcy has adverse effects on the vascular endothelium and smooth muscle cells, which lead to alterations in subclinical arterial structure and function, making Hhcy an independent risk factor for atherosclerosis.

2. **Oxidative stress:** Hhcy also induces endothelial cell dysfunction by decreasing endothelial antioxidant defense to cause oxidative stress and an increase in the intracellular concentration of reactive oxygen species (ROS). ROS, evidently, disturbs lipoprotein metabolism, which contributes to the growth of atherosclerotic vascular lesions.

3. **Impaired Nitric oxide (NO) production:** Hcy acts on vessels by controlling the contractility of vascular smooth muscle cells and the permeability of endothelial cells via

the inhibition of endothelial nitric oxide synthase, which produces nitric oxide (NO). Multiple studies suggested that HHcy actually induces ED via NO inhibition [48].

4. **Inflammation:** Hhcy activates Nuclear Factor-kappa B (NF-κB), which regulates the transcription of various genes involved in inflammatory and immune responses to increase pro-inflammatory cytokines and downregulate anti-inflammatory cytokines. NF-κB activation enhances the expression of genes, including TNF-α, IL-1β, IL-6, MCP-1, and ICAM-1, which initiate and promote atherosclerosis [49].

5. **Vascular smooth muscle cell (VMSC) proliferation:** Hhcy has been shown to promote the proliferation of vascular smooth muscle cells (VSMCs) by inducing cyclin A gene expression, leading to the development of vascular lesions and atherosclerosis [49].

1.8 Methylenetetrahydrofolate reductase gene

The Methylenetetrahydrofolate reductase (*MTHFR*) gene, located at chromosome 1p36.3, is responsible for encoding the enzyme MTHFR, one of the widely studied enzymes in the one-carbon metabolism pathway [50, 51]. MTHFR enzyme plays a role in metabolism of folate and Hcy through reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-methyltetrahydrofolate is an essential circulatory form of folate found in plasma which can be utilized by the cells. It releases a free methyl group for the remethylation of Hcy to methionine which can also be affected by genetic or epigenetic variations of genes associated with the one-carbon metabolism pathway [52].

Polymorphism, or genetic variations in the *MTHFR* gene can lead to reduced enzymatic activity or complete inactivity of the enzyme resulting in higher levels of Hcy [52]. Individuals can have one (heterozygous) or two (homozygous) genetic variations in the *MTHFR*, which can differ from person to person. *MTHFR* C677T and A1298C are two most common single nucleotide polymorphisms (SNP) in the *MTHFR* gene, and are associated with diseases and health conditions like CVD, neural tube defects, neuropathy and pregnancy complications [50, 54].

1.8.1 *MTHFR* C677T Polymorphism

MTHFR C677T polymorphism (NM_005957.4:c.665C>T, rs1801133) is a common *MTHFR* variant involving a missense mutation, where a cytosine (C) is substituted with thymine (T) at the 677 position on exon 4 of *MTHFR* gene [50, 55]. This alteration leads to substitution of the amino acid alanine to valine at codon 222 N-terminal catalytic domain of protein. *MTHFR* C677T polymorphism is responsible for encoding a thermolabile variant with reduced activity at a temperature more than 37℃ [50].

Each individual inherits two copies of the gene, one from each parent. This can result in three possible genotypes (Figure 1.9) [53],

- *MTHFR* 677 CC or Normal/Normal, one C allele inherited from each parent.
- *MTHFR* 677 CT or Normal/Variant, one C allele inherited from one parent and one T allele from the other parent.
- *MTHFR* 677 TT Variant/Variant, two T alleles inherited, one from each parent which is the thermolabile variant which loses its enzymatic activity and becomes denatured as the temperature increases.

The frequency of each of these genotypes vary among different populations. Research shows that the TT genotype is more common among Caucasians in North America present in over 10-15% and in more than 25% of Hispanics 6% of Africans had this genotype [55]. A cross-sectional study among the Japanese population found that the serum folate level among individuals with *MTHFR* 677 TT genotype was lower than those with *MTHFR* 677 CC or CT genotype [56].

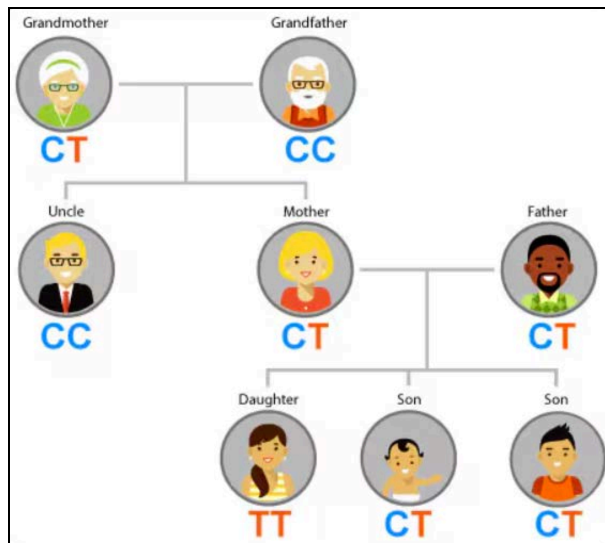

Figure 1.9: Genotypes of *MTHFR* C677T Variant [53]

1.8.2 *MTHFR* A1298C Polymorphism

Another variant of *MTHFR*, A1298C polymorphism (NM_005957.4:c.1286A>C, rs1801131) occurring at position 1298 of the *MTHFR* gene involves replacing adenine (A) with cytosine (C) which leads to substitution of the amino acid glutamic acid to alanine at codon 429 [50, 56]. Though there is insufficient evidence to support the association of *MTHFR* A1298C variant independently affecting the Hcy or folate metabolism. In this case as well three genotypes are possible,

- *MTHFR* 1298 AA or Normal/Normal, one A allele inherited from each parent.
- *MTHFR* 1298 AC or Normal/Variant, one A allele inherited from one parent and one C allele from the other parent.
- *MTHFR* 1298 CC Variant/Variant, two C alleles inherited, one from each parent

1.8.3 *MTHFR* gene polymorphism and Coronary Artery Disease

The *MTHFR* gene polymorphism has been extensively studied in relation to CAD. The available evidence from other South Asian populations, such as India and Pakistan, suggests that *MTHFR*

gene polymorphisms are associated with an increased risk of CVD, including CAD [49]. Research indicates that the *MTHFR* C677T polymorphism is associated with an increased risk of CAD and MI despite normal Hcy levels. Specifically, individuals with the TT genotype of the *MTHFR* gene have a higher risk of CAD severity compared to those with the CC genotype [58]. A study based in Bangladesh found that the prevalence of *MTHFR* gene polymorphisms, such as the C677T variant, is relatively high in the Bangladeshi population [59]. Moreover, the T allele of the rs1801133 polymorphism is linked to an elevated risk of CAD and abnormal lipid levels, such as higher total cholesterol and low-density lipoprotein cholesterol levels [60]. These findings suggest that the *MTHFR* gene polymorphism plays a role in the susceptibility to CAD and may influence disease severity and lipid profiles. However, the search results shed light on a notable scarcity of specific studies examining the correlation between *MTHFR* gene polymorphism and CAD in the Bangladeshi population [61].

1.9 Objective of our study

1.9.1 Research gap

To comprehensively address the gap in understanding the prevalence of Hhcy in the Bangladeshi population and its association with the onset of CAD, we conducted a systematic review and meta-analysis in 2023. The focus of our review paper was particularly on the South Asian population, including Bangladesh, India, and Pakistan. Our results revealed that more than 50% of South Asians patients with heart disease exhibit Hhcy, highlighting the importance of Hhcy in the context of cardiac health. During the evaluation of the finally selected 44 articles meeting our eligibility criteria (out of the 823 articles we found on this topic from Google Scholar, Pubmed and Scopus), which involved 5,613 cardiac patients from these three countries, it became evident that there is a notable scarcity of specific studies examining the correlation between *MTHFR* gene polymorphism and CAD in Bangladeshi population. While a few studies have explored the prevalence of elevated Hcy levels and their relationship with CAD in Bangladesh, a significant research gap persists, especially regarding the role of the *MTHFR* gene polymorphism in this association.

1.9.2 Hypothesis:

It is hypothesized that Hhcy and *MTHFR* C677T and A1298C gene polymorphisms may contribute to the development of CAD among the Bangladeshi population.

1.9.3 Aims of this study

- To investigate the association between Hhcy and *MTHFR* C677T and A1298C gene polymorphisms with CAD in the Bangladeshi population.
- To estimate the prevalence of Hhcy among relatively young (less than or equal to 45 years of age) CAD patients in Bangladesh.
- To identify specific dietary patterns of Bangladeshi people that might have a link to increase CAD risk among young adults.
- To elucidate the role of *MTHFR* gene polymorphism as a potential risk factor for CAD in Bangladeshi population, thereby enhancing our understanding of the genetic basis of cardiovascular health in this population.

Chapter 2

Methodology

2.1 Study Design

The study was designed as a hospital-based case-control study (Figure 2.1).

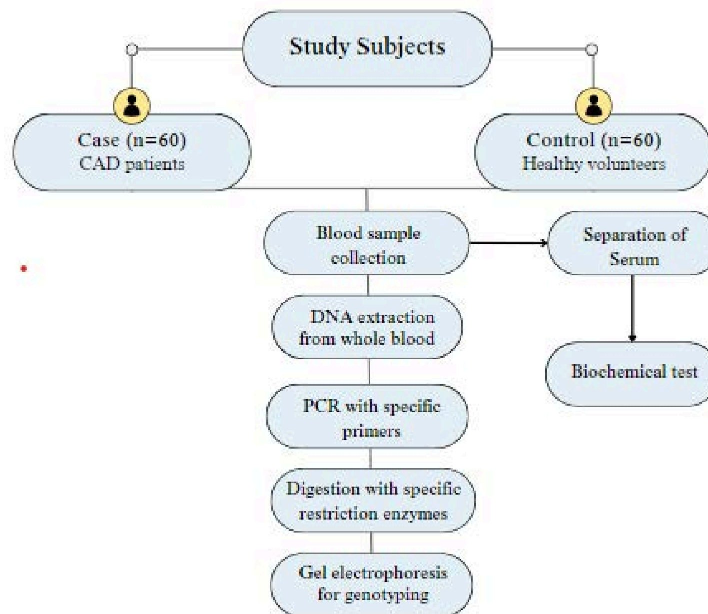

Figure 2.1: Schematic representation of study design

2.2 Selection of the cases and controls

A total of 60 confirmed patients with evidence of CAD, i.e. myocardial infarction or ST-elevated/Non-ST elevated MI or acute coronary syndrome or ischemic heart disease in their prescription were selected as cases in the present study. The cases were selected from the Cardiology Ward/CCU of National Institute of Cardiovascular Diseases (NICVD) and Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. All of them were angiography confirmed CAD patients and <50 yr of age. The control group comprised 60 healthy

volunteers without a CAD background. The two groups were age matched and belonged to the same geographical area. Tribal or ethnic groups were excluded from the study groups.

Inclusion criteria

Case:

I. Admitted patients to the cardiac wards
II. Prescription-confirmed cases of CAD
III. Bengali background
IV. Aged less than or equal to 50 years
V. No previous history of other chronic diseases such as kidney disease, cancer, parkinson's diseases
VI. Not taking any vitamin B or folic acid supplements

Control:

I. Age-matched
II. Healthy people with no previous history of chronic diseases
III. Belonged to the same geographic background and ethnicity as the cases

Exclusion criteria

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

2.3 Questionnaire

A structured and detailed questionnaire was made and divided into four sections-

I. **Demographic information:** This section summarized information like age, gender, residence, educational qualification, occupation, socio-economic status, and marital status of the respondents.

II. **Clinical and lifestyle-related information:** This section includes information regarding height, weight, BMI, Systolic/diastolic blood pressure, heart rate, smoking history, alcohol intake, history of betel nut usage, daily intake of sugar, history of physical exercise, family history of chronic diseases, diabetes, hypertension, and a very detailed history of weekly food habits. Present or ex smokers were considered smokers.

III. **Pre-admission and post-admission clinical information:** This section includes information regarding the symptoms experienced before admission, the duration between the first onset of symptom and admission, list of the initial tests that were prescribed for diagnosis, and the drugs that were suggested right after admission. This section was only exclusive for the selected cases and not the controls.

IV. **Laboratory findings:** This section summarizes the findings we received after the biochemical tests i.e. the concentration of total cholesterol, triglycerides, LDL-C, HDL-C, Fasting blood sugar, HBA1c(%), creatinine, alkaline phosphatase (ALP), Serum Vit B12, Hcy, Serum folate of both the cases and the controls.

2.4 Consent and Ethical issues

Each participant was fully informed of the purpose of the study and the investigational nature of the protocol, and signature on the consent form was received from each participant. Participant details were obtained using a structured questionnaire. Clinical data of the participants were recorded in the presence of an attending physician or directly recorded from the prescription or patients' reports. The study was conducted following the Helsinki Declaration and subsequent revisions [62]. 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 by a trained phlebotomist. The drawn blood was immediately transferred to three blood collection tubes-

I. Red Top Blood tubes/ Plain tube/ Pro-coagulation tube:

2.0 mL of blood was taken into one plain tube (Figure 2.2). The red top tube is used specifically for the collection of blood samples that require serum, which is the liquid portion of blood obtained after the blood has been allowed to clot. These do not contain any additives or anticoagulants. The blood collected in these tubes will naturally clot over time, allowing for the separation of serum from the clotted blood during laboratory processing. After collection of blood samples in this tube, it was left undisturbed for a period of time to allow clot formation. After clotting, the tube is centrifuged to separate the serum from the clot, and the serum was then used for Biochemical testing [63].

Figure 2.2: Red-top blood tube/Plain tube [63]

II. K2 EDTA-tube/ Anticoagulant tube :

4.0 mL of blood was taken and then pushed into two K2 EDTA tubes (2.0 mL in each tube) (Figure 2.3). Inner wall of these tubes is coated with spray-dried K2EDTA which acts as an anticoagulant, binding calcium ions and interrupting the clotting cascade. Calcium is necessary for a wide range of enzyme reactions of the coagulation cascade and its removal irreversibly prevents blood clotting within the collection tube. Ethylenediamine tetraacetic acid (EDTA) has

been recommended as the anticoagulant of choice in these tubes for hematological testing because it allows the best preservation of cellular components and morphology of blood cells [64]. These tubes are used for common hematologic tests and also PCR tests.

Figure 2.3: K2EDTA tube [64]

Blood in a plain tube and one EDTA tube was submitted to the laboratory of BSMMU for biochemical testing. The other EDTA tubes were 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) according to the manufacturer's protocol (Figure 2.4).

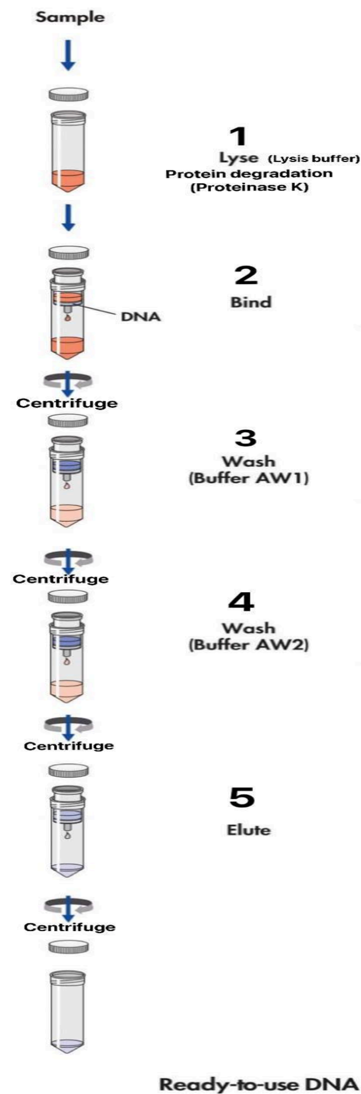

Figure 2.4: Steps of DNA extraction from whole blood [65]

1. **Cell Lysis:** Fresh human blood was collected in anticoagulant-treat collection tubes (EDTA tubes). 200 microliter samples of the whole blood were transferred to a microcentrifuge tube. To that, 20 µl Proteinase K and 200 µl lysis Buffer were added. It was then thoroughly mixed by pulse vortexing for 20 seconds. The mixture was incubated at 56 ºC for 10 minutes for cell lysis.

2. **Separation of the soluble DNA from cell debris:** 200 microliter ethanol (96~100%) was added to the cell lysate. The sample was mixed thoroughly by pulse vortexing for 20 seconds. The tubes were briefly spun to remove drops inside the lid.

3. **Binding the DNA to a purification matrix:** QIAmp Column was placed inside a collection tube. The sample, including any precipitate, was transferred carefully (without wetting the rim) into the QIAmp Column. The tubes were centrifuged at 8000 rpm for 1 minute, and the flow-through was discarded. The QIAmp Column containing the bound DNA is transferred to a new collection tube for washing.

4. **Washing:** The QIAmp 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 second wash uses a 500 µl AW2 buffer. The column was then centrifuged at 14,000 rpm for 3 minutes, and the flowthrough was discarded. For the dry spin, the columns were centrifuged for an additional 1 min to dry the column to remove any residual liquid. The additional step was done to eliminate the chance of possible Buffer AW2 carryover.

5. **Elution:** The washed QIAmp Column was placed inside the Elution Tube. 200 µl of Elution Buffer was added to the membrane of the QIAmp Column. The buffer was allowed to sit in the QIAmp Column for 5 min. Finally, the tubes were centrifuged 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 at 280 nm (A280) using Thermo Scientific™ NanoDrop™. Pure DNA had an A260/A280 ratio of 1.8–2.0 and DNA concentration range between 30-45 ng/µl.

2.8 *MTHFR* Genotyping

The *MTHFR* C677T and A1298C genotypes were determined using conventional Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP).

2.8.1 PCR

2.8.1.1 Primer Selection

To analyze the *MTHFR* C677T and A1298C polymorphisms, primer sequences and PCR conditions were selected based on previously published work [66, 67]. The primer sequences and the resulting digested products of each amplicon are listed in Table 2.1.

Table 2.1: Primer sequences for allele determination, with amplicon size and the resultant digested products size

	Primer Sequence	Amplicon size	Restriction Digestion Products
MTHFR C677T	Forward: TGTGGTCTCTTCATCCCTCGC Reverse: CCTTTTGGTGATGCTTGTTGGC [67]	513 bp	C/C (Wildtype): 513bp C/T (Heterozygous mutant): 146 bp, 367 bp, 513bp T/T (Homozygous mutant):146 bp, 367 bp
MTHFR A1298C	Forward: TGTGGTCTCTTCATCCCTCGC Reverse: CCTTTTGGTGATGCTTGTTGGC [66]	163 bp	A/A(Wildtype): 56bp, 31bp, 30bp, 28bp, 18bp A/C (Heterozygous mutant): 84 bp, 56 bp, 31 bp, 30 bp, 28 bp, 18bp C/C (Homozygous mutant) 84 bp, 31 bp, 30 bp and 18 bp

2.8.1.2 PCR Reagent

The reagents used for PCR-RFLP are listed in Table 2.2.

Table 2.2: 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, MboII)	Takara, Japan
Gel Electrophoresis	SeaKem™ LE Agarose	Lonza™, Switzerland
	Quick-Load® 100 bp DNA Ladder	New England Biolabs, USA
	50 bp DNA ladder	Cleaver Scientific, UK

2.8.1.3 Composition of PCR mix

The composition of the PCR mix is listed in Table 2.3. The composition of the PCR reaction mixture was calculated according to the manufacturer's (EmeraldAmp® GT PCR Master Mi) protocol [68]. Taking 180 ng of DNA, the volume of each DNA sample was determined , and the volume of nuclease-free water was adjusted accordingly.

Table 2.3: 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 µL**

2.8.1.4 PCR Conditions

The PCR conditions used for the amplification of *MTHFR* SNPs 677 and 1298 are illustrated in Figure 2.5 and Figure 2.6, respectively.

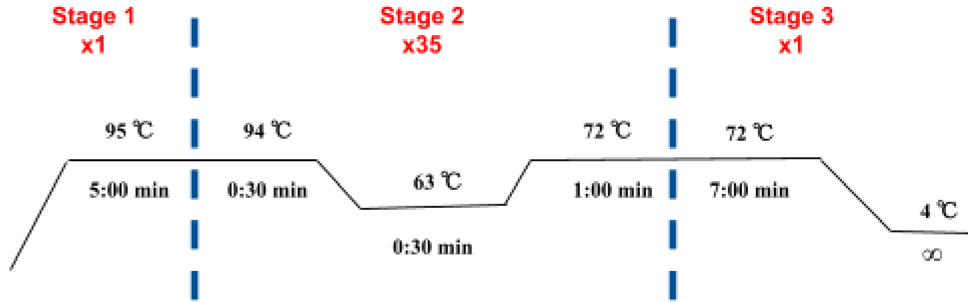

Figure 2.5 : Thermal conditions for amplification of *MTHFR* SNP C677T

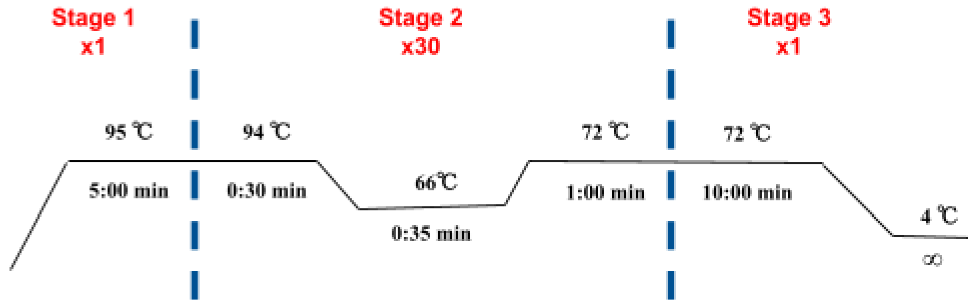

Figure 2.6: Thermal conditions for amplification of *MTHFR* SNP A1298C

2.8.1.5 Evaluation of PCR

The amplification of *MTHFR* PCR products was evaluated through agarose gel electrophoresis, using 1% and 2% gels stained with Ethidium Bromide (EtBr) for the C677T and A1298C polymorphisms, respectively. Subsequently, the amplified products were visualized under UV light.

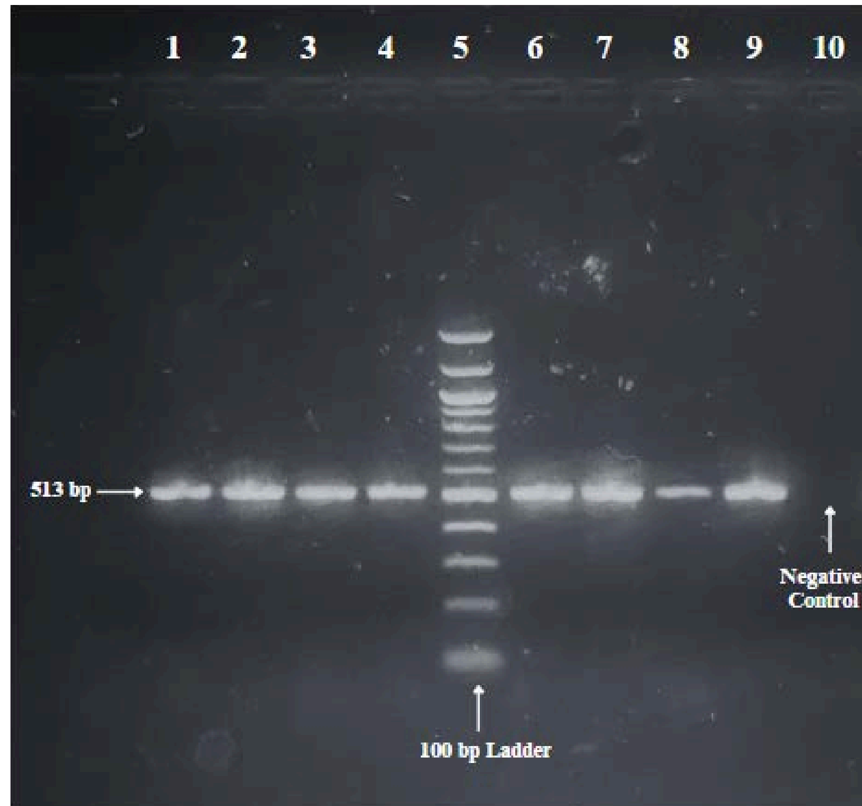

Figure 2.7: PCR products of *MTHFR* gene in 1% Agarose gel for C677T polymorphism analysis. From left to right lanes 1-4, 6-9 PCR products of 513 bp. Lane 5 contains a DNA ladder. Lane 10 is negative control.

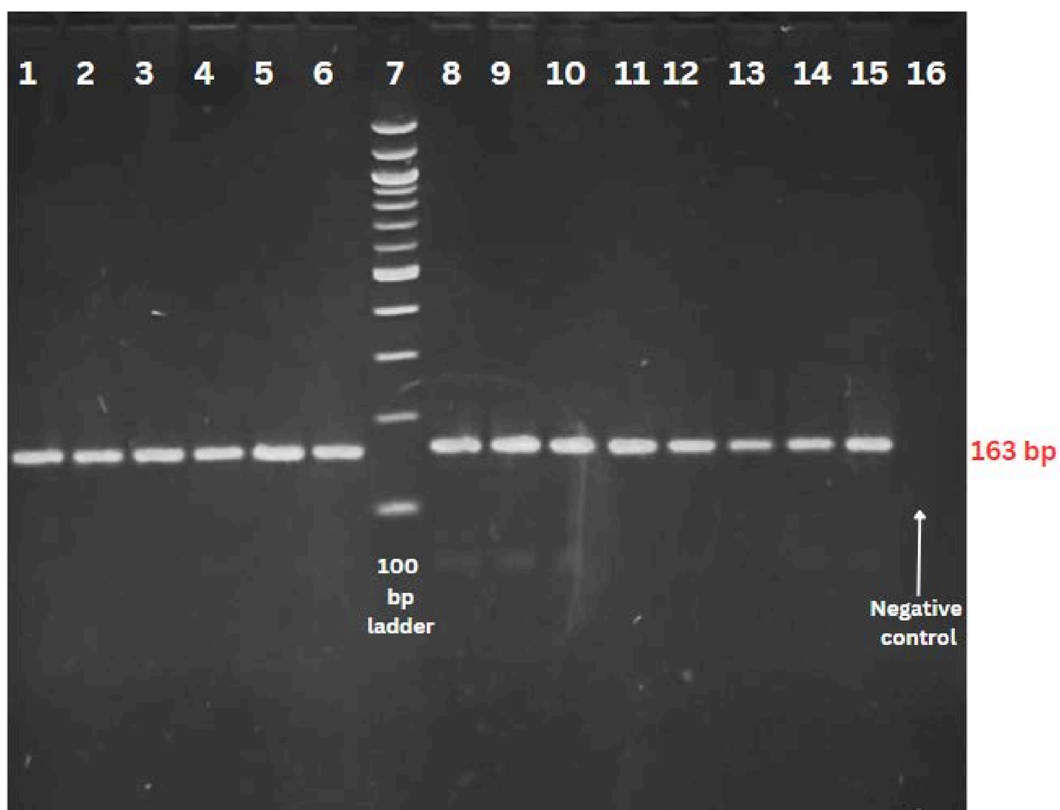

Figure 2.8: PCR products of *MTHFR* gene in 2% Agarose gel for A1298C polymorphism analysis. From left to right lanes 1-6, and 8-15 are PCR products of 163 bp. Lane 7 contains a DNA ladder. Lane 16 is negative control.

2.8.2 RFLP Analysis

2.8.2.1 Restriction Digestion of *MTHFR* C677T:

A 513 base pair (bp) amplicon was carefully considered through analyzing existing relevant articles to specifically avoid any restriction sites for the HinfI enzyme in the wild-type version of the *MTHFR* gene. This selection guarantees the presence of the C677T polymorphism, which is a single nucleotide change from cytosine (C) to thymine (T) at position 677. The PCR product was digested using HinfI (Takara Shuzo Co., Shiga, Japan). This polymorphism modifies the MTHFR enzyme's recognition sequence, allowing for specific cleavage of the DNA.

After doing the PCR, a 15µL PCR product (amplicon) and 1µL HinfI, along with buffer and nuclease-free water, were added to make a total volume of 20µL. The components were mixed all together by flicking and spinning down before incubating them at 37 °C for 1 hour according to the manufacturer's specification. Results obtained from DNA samples containing the C677T polymorphism to enzymatic digestion with HinfI revealed the original amplicon to be accurately divided into two bands in the case of a homozygous mutant: one measuring 146 bp and the other measuring 367 bp, and into three distinct bands, measuring 513 bp, 367 bp, and 146 bp in the case of a heterozygous mutant. The distinct cleavage patterns found in these cases were in opposition to the undigested 513-bp product seen in samples lacking the polymorphism. This allows for a clear distinction between the wild-type and polymorphic alleles.

Restriction Digestion of A1298C PCR Products:

A 163 base pair (bp) amplicon was selected after analyzing existing relevant articles to specifically for our project to avoid any restriction sites for the MboII enzyme in the wild-type version of the *MTHFR* gene. The A1298C mutation, like the C677T mutation, results in a decrease in MTHFR activity that is more pronounced in the homozygous (CC) than in the heterozygous (AC) or normal (AA) states.

After doing the PCR, a 15µL sample and 0.4µL MboII (Takara Shuzo Co., Shiga, Japan) along with buffer and nuclease-free water to make a total of 20µL were mixed all together and incubated at 37°C for 4 hours according to the manufacturer's specification. The RFLP products (15µL) were resolved in 3% agarose gel following 100V and 90 minutes run time. Results obtained from DNA samples containing the A1289C polymorphism to enzymatic digestion with MboII for the wild-type allele 'A', segments of 56 bp, 31 bp, 30 bp, 28 bp, and 18 bp were detected. In contrast, the 'C' allele produced fragments of 84 bp, 31 bp, 30 bp, and 18 bp as a result of the elimination of a restriction site for MboII digestion.

2.8.2.2 Composition of Restriction Digestion Mix:

Table 2.4: Composition of the reaction mixture for HinfI restriction enzyme digestion:

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

Table 2.5: Composition of the reaction mixture for MboII restriction enzyme digestion:

Name of the Component	Volume (µL)
MboII Enzyme	0.4 µL
10X H Buffer	2 µL
Substrate DNA	15 µL
Nuclease-free water	2.6 µL
Total	20 µL

2.8.2.3 RFLP Analysis of Digested Products:

The RFLP products (15µL) were resolved in 1.5% agarose gel following 100V and 60 minutes run time. The gel was then visualized using gel documentation under ultraviolet (UV) light (Figure 2.8). The product size was determined by matching against a 100-base pair DNA ladder.

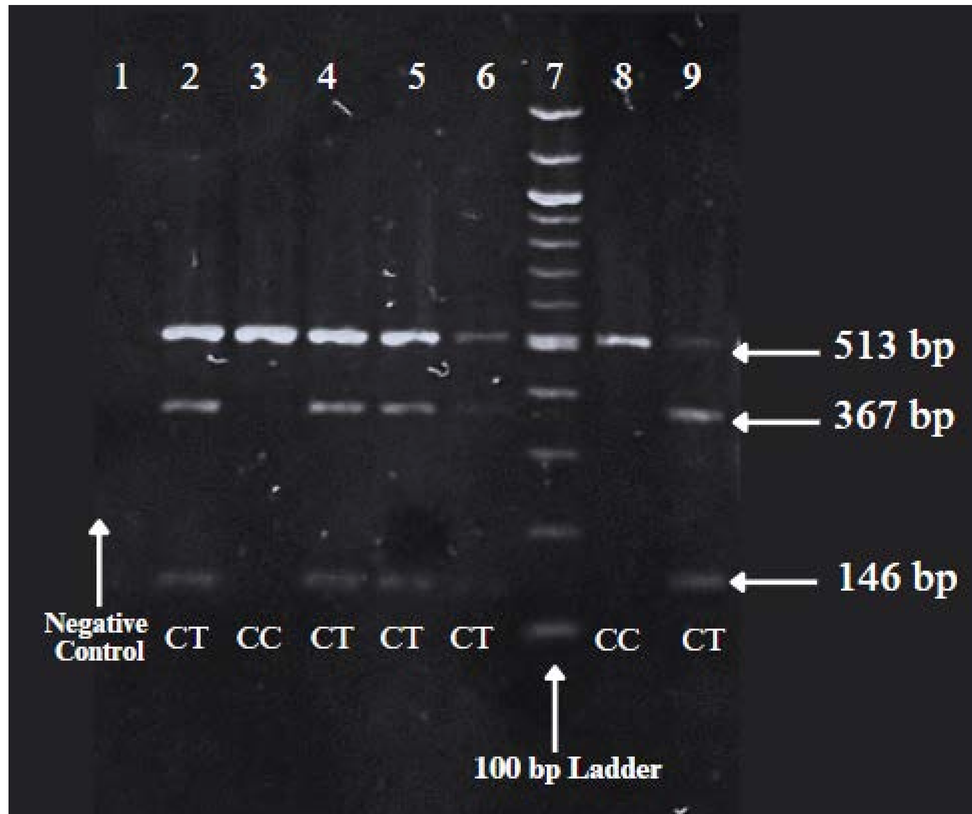

Figure 2.9: Restricted digested products of C667T using HinfI enzyme in 1.5% agarose Gel. CC: wild-type homozygous; TT: homozygous for the polymorphism; CT: heterozygous for the polymorphism. Lane 1 contains negative control; lanes 2,4-6 and 9 contain mutant heterozygous CT (367bp and 146bp).; Lane 7 contains a DNA ladder; Lanes 3 and 8 contain wild-type homozygous CC (513bp).

2.9 Statistical Analysis:

The Mann-Whitney exact tests, Fisher's exact test, chi-square test, and descriptive statistics were performed through GraphPad Prism, version 10. The relative associations with dietary patterns were determined by calculating the odds ratio (OR) with 95 % confidence intervals (CIs) after adjusting for potential confounders and level of significance (p). All statistical tests were two-sided; a $p<0.05$ was taken as the level of significance.

Chapter 3

Result

3.1 Demographic and clinical characteristics

Table 3.1 represents the demographic data of whole participants of our study including 60 patients and 60 healthy controls. All the mean, SD and frequency values were calculated using a descriptive statistics method where gender, smoking habit, diabetes, hypertension and family history of CAD are divided into two sub-groups based on their positive and negative response.

Table 3.1: Demographic characteristics among CAD patients and Healthy Controls

Characteristics	CAD Patients (n=60)			Healthy Controls (n=60)		
	Mean	***SD***	***n (%)***	***Mean***	***SD***	***n (%)***
Age	38.57	5.244	-	38.32	7.354	-
BMI	24.24	3.814	-	24.24	3.642	-
Gender						
Male	-	-	53 (88.33%)	-	-	50 (83.33%)
Female	-	-	7 (11.67%)	-	-	10 (16.67%)
Smoking						
Yes	-	-	33 (55%)	-	-	13 (21.67%)
No	-	-	27 (45%)	-	-	47 (78.33%)
Diabetes						
Yes	-	-	16 (26.67%)	-	-	7 (11.67%)
No	-	-	44 (73.33%)	-	-	53 (88.33%)
Hypertension						
Yes	-	-	25 (41.67%)	-	-	19 (31.67%)
No	-	-	35 (58.33%)	-	-	41 (68.33%)
Family History of CAD						
Yes	-	-	24 (40%)	-	-	11 (18.33%)
No	-	-	36 (60%)	-	-	49 (81.67%)
*CAD- Coronary Artery Disease *BMI- Body Mass Index						

Demographic characteristics of CAD patients and Healthy Controls:

The study included 60 CAD patients and 60 healthy controls, whose gender, age, BMI, history of diabetes and hypertension, smoking status, and family history of CAD are listed in Table 3.2. Patients were 22–47 old with a mean age of 38.57±5.244, while controls were 22–50 years old with a mean age of 38.32±7.354. The main age group focused on for our project was 18-45 years; hence, the majority (45.83%) belongs to the 40-47 age group. Since healthy controls were age-matched with patients, age differences between them were not statistically significant. Compared with control subjects, patients were more likely to be smokers (55% in CAD patients vs 21.67% in healthy control). The family history of CAD was also found in higher numbers in patients than in controls (40% in patients vs 18.33% in controls). With a mean of 24.24, the BMI was found to be the same for patients and controls but other characteristics such as diabetes and hypertension were found more in patients (26.67% and 41.67% in patients vs 11.67% and 31.67% in controls, respectively).

Table 3.2 represents the biochemical data of whole participants of our study including 60 patients and 60 healthy controls. All the mean, SD and frequency values were calculated using a descriptive statistics method.

Table 3.2: Biochemical Parameters in CAD patients and Healthy Controls

Characteristics	**CAD Patients (n=60)**		**Healthy Controls (n=60)**	
	Mean	***SD***	***Mean***	***SD***
Homocysteine	11.05	8.302	10.96	6.347
Triglyceride	178.9	119.3	167.8	88.21
Total Cholesterol	156.5	43.04	182.1	37.17
HDL	35	8.074	39.82	7.710
LDL	87.28	36.85	111.1	30.90
Vitamin B12	411.7	194.7	388.1	115.2
Folate	6.248	3.418	6.604	2.723
*HDL- High Density Lipoprotein *LDL-Low Density Lipoprotein				

Clinical characteristics of CAD patients and Healthy Controls:

The study of total 120 participants including 60 CAD patients and 60 healthy controls where their clinical data were analyzed. In the clinical data report, it can be observed that the mean value of homocysteine is slightly higher among patients than controls (11.05±8.302 in patients vs 10.96±6.347 in controls). The same scenario can be seen in the case of triglyceride and vitamin B12 as well (178.9±119.3 in patients vs 167.8±88.21 in controls and 441.7±194.7 in CAD patients vs 388.1±115.2 in healthy controls, respectively). Although other characteristics such as total cholesterol, HDL, LDL, and folate are found higher in controls than in patients (total cholesterol: 156.5±43.04 in patients vs 182.1±37.17 in controls; HDL: 35±8.074 in patients vs 39.82±7.710 in controls; LDL: 87.28±36.85 in patients vs 111.1±30.9 in controls; folate: 6.248±3.418 in patients vs 6.604±2.723 in controls).

3.2 Prevalence of Hyperhomocysteinemia:

The results showed that 7 out of the 60 CAD patients (11.7%) had Hcy levels higher than the normal range, whereas in the control group, 4 out of the 60 healthy individuals (6.7%) exhibited elevated Hcy levels, p-value = 0.53. (Figure 3.1).

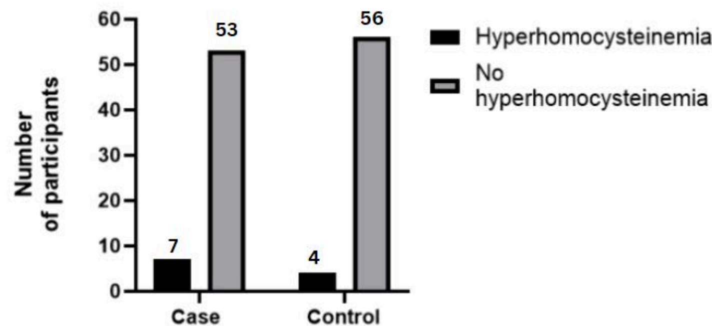

Figure 3.1: Prevalence of hyperhomocysteinemia

3.3 Biochemical Analysis

3.3.1 Association between Hyperhomocysteinemia and Cardiovascular Risk Factors among CAD Patients and Healthy Controls:

Table 3.3 presents the association between Hhcy (Hcy > 15 µmol/L) and various cardiovascular risk factors [69, 70] among CAD patients and healthy controls. The data is stratified into two groups: those with Hhcy and those without (Hcy ≤ 15 µmol/L). The table includes the number and percentage of individuals with each risk factor within these groups, as well as the corresponding p-values from statistical tests, Fisher's exact test and Mann-Whitney test to assess the significance of these associations.

Table 3.3: Association between Hhcy and Cardiovascular Risk Factors among CAD Patients and Healthy Controls:

Cardiovascular risk factors	CAD patients (n=60)			Healthy Controls (n=60)		
	Hcy > 15 µmol/L (n=7)	Hcy ≤ 15 µmol/L (n=53)	p-value	Hcy > 15 µmol/L (n=4)	Hcy ≤ 15 µmol/L (n=56)	p-value
	N (%)	*N (%)*		*N (%)*	*N (%)*	
Serum Folate < 3ng/ml [70]	1 (14.29)	3 (5.66)	0.3995	1 (25)	0 (0)	0.0667
Positive family history of cardiac disease	3 (42.85)	23 (43.39)	>0.9999	2 (50)	12 (21.43)	0.2295
Diabetes	0 (0)	15 (28.30)	0.1760	0 (0)	7 (12.5)	>0.9999
Smoking Habit	5 (71.43)	31 (58.49)	0.6913	1 (25)	13 (23.21)	>0.9999
Hypertension	3 (42.85)	25 (47.17)	0.6994	1 (25)	18 (32.14)	>0.9999
Obesity (≥ 25 BMI score)	2 (28.57)	23 (43.39)	0.6882	2 (50)	25 (44.64)	>0.9999
Note: p-values were calculated using the Fisher's Exact test except for the 'Serum Folate< 3ng/ml' group, which was calculated using the Mann-Whitney test.						

Key Findings:

1. **Serum Folate < 3 ng/ml:**
    - **CAD Patients:** Among CAD patients, 14.29% with Hhcy had serum folate levels <3 ng/ml, compared to 5.66% without hyperhomocysteinemia. The p-value of 0.3995 indicates that this difference is not statistically significant.
    - **Healthy Controls:** In healthy controls, 25% with hyperhomocysteinemia had serum folate levels <3 ng/ml, while none without hyperhomocysteinemia had such low folate levels. The p-value of 0.0667 suggests a trend towards significance but does not reach the conventional threshold for statistical significance.
2. **Positive Family History:**
    - **CAD Patients:** 42.85% of CAD patients with hyperhomocysteinemia had a positive family history of cardiovascular disease, compared to 43.39% without

hyperhomocysteinemia. The p-value of >0.9999 indicates no significant association.

- **Healthy Controls:** 50% of healthy controls with hyperhomocysteinemia had a positive family history, compared to 21.43% without hyperhomocysteinemia. The p-value of 0.2295 indicates no significant association.

3. **Diabetes:**
   - **CAD Patients:** None of the CAD patients with hyperhomocysteinemia had diabetes, whereas 28.30% without hyperhomocysteinemia did. The p-value of 0.1760 suggests no significant association.
   - **Healthy Controls:** None of the healthy controls with hyperhomocysteinemia had diabetes, compared to 12.5% without hyperhomocysteinemia. The p-value of >0.9999 indicates no significant association.
4. **Smoking Habit:**
   - **CAD Patients:** 71.43% of CAD patients with hyperhomocysteinemia were smokers, compared to 58.49% without hyperhomocysteinemia. The p-value of 0.6913 indicates no significant association.
   - **Healthy Controls:** 25% of healthy controls with hyperhomocysteinemia were smokers, compared to 23.21% without hyperhomocysteinemia. The p-value of >0.9999 indicates no significant association.
5. **Hypertension:**
   - **CAD Patients:** 42.85% of CAD patients with hyperhomocysteinemia had hypertension, compared to 47.17% without hyperhomocysteinemia. The p-value of 0.6994 indicates no significant association.
   - **Healthy Controls:** 25% of healthy controls with hyperhomocysteinemia had hypertension, compared to 32.14% without hyperhomocysteinemia. The p-value of >0.9999 indicates no significant association.
6. **Obesity (≥ 25 BMI score):**
   - **CAD Patients:** 28.57% of CAD patients with hyperhomocysteinemia were obese, compared to 43.39% without hyperhomocysteinemia. The p-value of 0.6882 indicates no significant association.

- **Healthy Controls:** 50% of healthy controls with hyperhomocysteinemia were obese, compared to 44.64% without hyperhomocysteinemia. The p-value of >0.9999 indicates no significant association.

Overall, the data in Table 3.3 shows that there are no statistically significant associations between Hhcy and the examined cardiovascular risk factors among both CAD patients and healthy controls. Although some differences in percentages are observed, these differences did not reach statistical significance based on the p-values calculated using Fisher's Exact test (and the Mann-Whitney test for serum folate levels).

3.4 Dietary patterns of Bangladeshi young adults and their association with Coronary Artery Disease:

A logistic regression analysis (Table 3.4) was performed to assess the impact of various dietary factors on the likelihood of developing CAD. The model was adjusted for potential confounders including age, BMI, hypertension, and diabetes. The goodness-of-fit of the model was evaluated using the Hosmer and Lemeshow test, which indicated a good fit ($\chi^2 = 8.305$, df = 8, p = 0.404), suggesting that the model adequately fits the data. A classification table was also generated to indicate the model's accuracy in predicting CAD status. It revealed the model correctly predicted CAD status in 75.8% of cases, with a sensitivity of 75.0% (correctly identifying those with CAD) and a specificity of 76.7% (correctly identifying those without CAD).

Table 3.4: Binary logistic regression analysis of dietary intake pattern with CAD patients and controls

Food group	Consumption frequency	Adjusted values				Unadjusted values			
		Odds ratio	95% C.I		p-value	Odds ratio	95% C.I		p-value
			Lower	Upper			Lower	Upper	
Sugar	>1 tsp/day	15.129	4.273	53.565	**0.000**	8.216	2.840	23.763	0.000
Rice	>1 time/day	0.973	0.227	4.165	0.971	0.275	0.224	3.349	0.834
Leafy vegetables	< 1 time/day	0.516.	0.159	1.675	0.271	0.865	0.227	2.001	0.477
Chicken	>1 times/day	5.776	1.287	25.923	**0.022**	0.674	1.317	19.886	0.018
Beef	>1 time/day	0.785	0.174	3.545	0.753	5.117	0.208	3.012	0.732
Milk	>1 time/day	1.534	0.441	5.335	0.501	0.791	0.503	5.015	0.430
Fish	< 2 times/day	0.227	0.063	0.816	**0.023**	1.588	0.070	0.685	0.009
Egg	>1 time/day	7.849	1.066	57.801	**0.043**	0.219	0.886	32.518	0.067
Junk food	> 1 time/week	0.691	0.225	2.118	0.518	5.369	0.221	1.743	0.366
Milk tea	> 1 cup/day	0.211	0.072	0.624	**0.005**	0.621	0.102	0.744	0.011

Note: The model was adjusted for potential confounders including age, BMI, hypertension, and diabetes.

The logistic regression analysis revealed several dietary factors significantly associated with the likelihood of developing CAD after adjusting for age, BMI, hypertension, and diabetes:

Sugar Intake: Patients consuming more than 1 teaspoon of sugar per day had significantly higher odds of developing CAD (adjusted OR = 15.129, 95% CI: 4.273–53.565, $p < 0.001$). The unadjusted odds ratio also showed a significant association (OR = 8.216, 95% CI: 2.840–23.763, $p < 0.001$).

Chicken Consumption: Consuming chicken more than once per day was associated with higher odds of CAD (adjusted OR = 5.776, 95% CI: 1.287–25.923, p = 0.022), with similar findings in the unadjusted analysis (OR = 0.674, 95% CI: 1.317–19.886, p = 0.018).

Fish Consumption: Patients who consumed fish less than twice per week had lower odds of developing CAD (adjusted OR = 0.227, 95% CI: 0.063–0.816, p = 0.023). The unadjusted analysis also supported this finding (OR = 1.588, 95% CI: 0.070–0.685, p = 0.009).

Egg Consumption: More than one egg per day was associated with higher odds of CAD in the adjusted analysis (adjusted OR = 7.849, 95% CI: 1.066–57.801, p = 0.043), though the unadjusted

analysis did not show a statistically significant result (OR = 0.219, 95% CI: 0.886–32.518, p = 0.067).

Junk Food Consumption: The unadjusted analysis showed that consuming junk food more than once per week was significantly associated with CAD (OR = 5.369, 95% CI: 0.221–1.743, p < 0.001). However, the adjusted analysis did not find a significant association (adjusted OR = 0.691, 95% CI: 0.225–2.118, p = 0.518).

Milk Tea Consumption: Drinking more than one cup of milk tea per day was associated with lower odds of CAD in the adjusted analysis (adjusted OR = 0.211, 95% CI: 0.072–0.624, p = 0.005), supported by the unadjusted analysis (OR = 0.621, 95% CI: 0.102–0.744, p = 0.011).

3.5 Genotype analysis:

3.5.1 *MTHFR* C677T Polymorphism

3.5.1.1 Distribution of *MTHFR* C677T Polymorphism

In Table 3.5, the distribution of *MTHFR* C677T genotypes and allele frequency show no significant differences between CAD patients and the healthy controls group, with the TT genotype not observed in either group. Among the participants including both CAD patients (n=58) and healthy controls (n=57), 80 individuals had the CC genotype, 35 had the CT genotype, and none had the TT genotype. The genotype of 5 samples could not be determined during the PCR-RFLP process. The observed genotype frequencies in both case and control groups were in accordance with the Hardy-Weinberg equilibrium (p= 0.4252 and p= 0.3672, respectively).

Table 3.5: Distribution of *MTHFR* C677T Polymorphism

Polymorphism	Genotype	CAD Patients (n=58)		Healthy Controls (n=57)		p-value
		N	%	N	%	
C677T	CC	42	70.69	39	68.42	0.8411
	CT	17	29.31	18	31.58	
	TT	0	0	0	0	
	Hardy–Weinberg p-value	0.4252		0.3672		
	Allele	N	%	N	%	
	C	99	85.34	96	84.21052632	0.8557
	T	17	14.65	18	15.78947368	

3.5.1.2 Comparison of biochemical parameters according to *MTHFR* genotype

The characteristics of the study population were analyzed on the basis of the *MTHFR* genotype (CC, CT, TT). The distribution of gender showed that 87.5% of individuals with the CC genotype were male, compared to 82.85% in the CT genotype group, with a p-value of 0.5080, indicating no significant difference. Regarding a positive family history of CAD, 25% of the CC group and 37.14% of the CT group reported a positive history, with a p-value of 0.1853. Diabetes prevalence was 21.25% in the CC group and 14.28% in the CT group (p-value: 0.4850). Smoking habits were reported by 15% of the CC group and 34.28% of the CT group, resulting in a p-value of 0.5618. Hypertension was present in 33.75% of the CC genotype group and 40% of the CT genotype group, with a p-value of 0.3823. Obesity, defined as a BMI of 25 or greater, was observed in 42.25% of the CC group and 40% of the CT group, with a p-value of 0.9002.

Overall, no statistically significant differences were found between the CC and CT genotype groups for any of the evaluated characteristics, as all p-values exceeded the conventional threshold of 0.05, indicating no significant association between these characteristics and the *MTHFR* genotype within the study population

Table 3.6: Characteristics of study population

Characteristics	*MTHFR* Genotype			
	CC (n=80)	CT (n=35)	TT (n=0)	p-value
	N (%)	*N (%)*	*N (%)*	
Gender				
Male	70 (87.5)	29 (82.	-	0.5080
Female	10 (12.5)	6	-	
Positive family history of cardiac diseases	20 (25)	13 (37.14)	-	0.1853
Diabetes	17 (21.25)	5 (14.28)	-	
Smoking Habit	32 (15)	12 (34.28)	-	0.5618
Hypertension	27 (33.75)	14 (40)	-	0.3823
Obesity (≥ 25 BMI score)	33 (42.25)	14 (40)	-	0.9002

Values are mean ± S.D.

3.5.1.3 Comparison of Hcy, Folate and B12 level related to *MTHFR* genotype

Table 3.7 shows a comparison of the mean Hcy, serum folate, and serum vitamin B12 levels in individuals with different *MTHFR* genotypes. For the CC genotype, the Hcy levels were 10.06 ± 3.91 µmol/L, folate levels were 6.40 ± 2.95 ng/mL, and B12 levels were 396.87 ± 24.0 pg/mL. Individuals with the CT genotype had Hcy levels of 13.35 ± 10.05 µmol/L, folate levels of 6.31 ± 3.19 ng/mL, and B12 levels of 395.43 ± 15.0 pg/mL. Statistical analysis shows no significant differences in Hcy, folate, and B12 levels with the genotype.

Table 3.7: Comparison of Hcy, Folate and B12 level related to *MTHFR* genotype

Genotype	Hcy	p-value	Folate	p-value	B-12	p-value
CC	10.06±3.91	0.1318	6.40±2.95	0.8786	396.87±.24	0.6596
CT	13.35±10.05		6.31±3.19		395.43±15	
TT	-		-		-	

Values are mean ± S.D. Note: p-values were calculated using the Mann-Whitney test.

3.3.1 Association between Hhcy and *MTHFR* genotype:

Table 3.8 presents an analysis of the association between Hhcy (Hcy > 15 µmol/L) and various cardiovascular risk factors with different *MTHFR* genotypes (CC and CT). The study includes a total of 115 individuals, 80 with the CC genotype and 35 with the CT genotype.

1. **Gender Distribution**

   The analysis reveals that in both the CC and CT genotypes, males with elevated Hcy levels constitute 100% of the group, compared to 86.48% and 24.14%, respectively, of those with lower levels of Hcy, with a significant p-value of **0.0004** which is highly significant ($p<0.001$).

2. **Family History of cardiovascular disease**

   Positive family history shows a significant association with elevated Hcy levels in both genotypes, particularly pronounced in the CT group (66.67% vs. 31.03%, **p = 0.0175**).

3. **Hypertension**

   Hypertension is significantly associated with elevated Hcy levels in the CT genotype (50% vs. 37.93%, **p = 0.0341**).

Other factors such as serum folate levels, smoking habit, obesity did not show significant associations with Hhcy in either genotype. These findings indicate significant interactions between *MTHFR* genotypes, gender, family history, and hypertension concerning Hhcy and cardiovascular risk. Specifically, gender and positive family history show strong associations with elevated Hcy levels, particularly among individuals with the CT genotype.

Table 3.8: Association between Hhcy and *MTHFR* genotype

Cardiovascular risk factors	CC (N=80)		CT (n=35)		p-value
	Hcy > 15 µmol/L (n=6)	Hcy ≤ 15 µmol/L (n=74)	Hcy > 15 µmol/L (n=6)	Hcy ≤ 15 µmol/L (n=29)	
	N (%)	*N (%)*	*N (%)*	*N (%)*	
Serum Folate < 3ng/ml	1 (7.5)	3 (4.05)	1 (16.67)	2 (6.89)	>0.9999
Gender					
Male	6 (100)	64 (86.48)	6 (100)	7 (24.14)	**0.0004**
Female	0 (0)	10 (13.51)	0	2 (6.89)	>0.9999
Positive family history	0 (0)	20 (27.02)	4 (66.67)	9 (31.03)	**0.0175**
Smoking Habit	4 (66.67)	28 (37.83)	2 (33.33)	10 (34.48)	0.6576
Hypertension	0 (0)	27 (36.48)	3 (50)	11 (37.93)	**0.0341**
Obesity (≥ 25 BMI score)	2 (33.33)	31 (41.89)	2 (33.33)	12 (41.38)	0.5718

Note: p-values were calculated using the Fisher's Exact test except for the 'Serum Folate< 3ng/ml' group, which was calculated using the Mann-Whitney test.

3.5.2 *MTHFR* A1298C Polymorphism

The PCR analysis has been completed for the *MTHFR* A1298C polymorphism, though for the RFLP analysis, the optimization is still in process and intended to be completed with the utmost priority.

Chapter 4

Discussion

4.1 Prevalence of Hyperhomocysteinemia

Our findings revealed a marginally higher prevalence (11.7% in CAD compared to 6.7% in Controls) of Hhcy among CAD patients compared to healthy individuals. However, this disparity did not achieve statistical significance (p-value: 0.53), leaving the causal relationship between elevated Hcy levels and CAD unresolved. While the trend observed in our study is congruent with some prior research, which has consistently associated elevated Hcy levels with an augmented risk of CAD [71, 72, 73], we cannot fully reject the notion that there is no significant association between elevated Hcy levels and CAD. This is also consistent with some studies that did not find significant differences in homocysteine levels between CAD patients and controls [74].

The lack of statistical significance in our results may be attributed to several factors, including the relatively small sample size and potential confounding variables not fully addressed in our analysis. This reveals the complexities inherent in homocysteine research, where variations in study design, participant demographics, and methodological approaches can yield divergent outcomes. Despite the preponderance of evidence supporting Hhcy as an independent contributor to CAD and CVD more broadly, the debate still continues due to the lack of conclusive interventional studies establishing a causal link [75].

While our findings provide preliminary insights into the potential association between Hhcy and CAD in our study cohort, the lack of statistical significance warrants cautious interpretation. Moving forward, future investigations should prioritize larger, more diverse sample sizes to capture the full spectrum of Hcy levels and mitigate the influence of confounding variables. Additionally, interventional studies exploring the therapeutic potential of folic acid and vitamin B12 supplementation in reducing homocysteine levels and enhancing endothelial function among CAD patients holds promise and merits further exploration in future research endeavors.

4.2 Association between Hyperhomocysteinemia and Cardiovascular Risk Factors

The association between Hhcy and conventional CVD risk factors was examined to elucidate the role of homocysteine in CAD pathogenesis. Our analysis revealed no significant (p-value > 0.05) associations between Hhcy and various cardiovascular risk factors among both CAD patients and healthy controls. Despite some differences in percentages, none of these associations reached statistical significance based on the calculated p-values.

This lack of significant associations suggests that Hhcy alone may not be a strong independent predictor of the CVD risk factors studied in this cohort. It is possible that Hhcy contributes to CAD development through vascular mechanisms independent of traditional risk factors such as diabetes, hypertension, and smoking. These findings imply that while Hhcy may play a role in CAD pathogenesis, its relationship with traditional cardiovascular risk factors is complex and warrants further investigation.

Our results are consistent with studies that support Hhcy as an independent risk factor for CVD [76, 77], indicating that it contributes to the risk of cardiovascular events even when other risk factors are controlled for. This highlights the potential of Hhcy to influence CAD through mechanisms not fully captured by traditional risk factor assessments. Consequently, future research should aim to explore the underlying pathways through which Hhcy exerts its effects on the vascular system, as well as the potential for targeted interventions to mitigate its impact.

4.3 Dietary Patterns and Their Association with CAD

Dietary factors play a crucial role in modulating CAD risk, and our study sought to identify specific dietary patterns of Bangladeshi population associated with CAD development. The logistic regression analysis identified several dietary factors, including sugar intake, chicken consumption, fish consumption, egg consumption, and milk tea consumption, that were significantly associated with CAD risk after adjusting for potential confounders. These findings revealed higher sugar and chicken intake were particularly associated with increased odds of CAD, while frequent fish consumption was associated with reduced odds. These findings suggest that dietary patterns play a crucial role in the risk of developing CAD, highlighting the importance of dietary modifications in CAD prevention strategies.

4.4 Genotype Analysis

The genotype analysis of the *MTHFR* C677T polymorphism indicates some significant association of genotype with cardiovascular risk factors in individuals with Hhcy. Despite the lack of significant differences in the distribution of the *MTHFR* C677T genotypes between case and control groups, key findings of our analysis highlight the potential impact of this polymorphism on cardiovascular health. For instance, the significant association between Hcy levels and gender is evident, with higher prevalence of Hhcy in males in both the CC and CT genotype groups ($p<0.001$). This suggests that males with these genotypes may be more susceptible to elevated Hcy levels, a known risk factor for CAD. However, this finding is not entirely consistent across available studies. While some studies have reported a higher prevalence of the CT genotype in females and an increased risk of CAD associated with this genotype in females compared to males, other studies have found conflicting results or no significant gender differences [78, 79]. The exact mechanisms by which gender influences Hcy levels in the context of *MTHFR* genotypes warrant further investigation but could involve differences in lifestyle, hormonal influences, or other genetic factors.

Additionally, the significant association between a positive family history of cardiovascular disease and Hcy ($p<0.05$), specifically in the CT genotype group, highlights the hereditary aspect of CAD risk. Individuals with the CT genotype and a family history of cardiovascular diseases may have a compounded risk, highlighting the importance of considering family history in genetic and cardiovascular risk assessments. Furthermore, our study identified a significant association between the CT genotype of the *MTHFR* gene with Hhcy and hypertension ($p<0.05$). The CT genotype is linked to reduced MTHFR enzyme activity, leading to higher Hcy levels, which can cause endothelial dysfunction and increased vascular resistance, contributing to hypertension [80, 81]. This finding underscores the importance of considering genetic factors, such as the CT genotype, in assessing hypertension risk. Targeted interventions to address hyperhomocysteinemia in individuals with the CT genotype may help mitigate hypertension risk. However, some studies have found conflicting results with no association of these risk factors with genotype, while others suggest individuals with the TT genotype have a higher prevalence of family history of cardiovascular diseases and hypertension than those with the CT genotype [82 83, 84].

4.5 Strengths and Limitations

Our control group comprises both hospital-based and non-hospital-based subjects, which minimizes the chance of a selection bias and thus is one of the strengths of our study. We excluded tribal or other ethnic groups from participating in this study, ensuring all subjects had a uniform genetic background.

One of the major limitations of this study is the small sample size, which may have contributed to the insignificant results in most cases. With only 120 individuals in the study, the sample size may not have been sufficient to detect significant associations between the *MTHFR* polymorphisms and cardiovascular risk factors. This is particularly true for the female subgroup, which did not have any individual with any of the mutant genotypes (CT or TT), making it difficult to draw conclusions about the association between the polymorphism and CAD in females. The small sample size may have also led to a lack of power to detect significant associations, resulting in many of the p-values being greater than 0.05. Therefore, the results of this study should be interpreted with caution, and future studies with larger sample sizes are needed to confirm or refute the findings.

Besides, during the study, we encountered a few cases where the genotype could not be determined through the PCR-RFLP process. These instances of missing genotype data were noted and accounted for in our analysis. Consequently, the total number of individuals analyzed for each genotype (CC and CT) may differ slightly from the total number of cases initially included in the study which impacted the overall analysis.

Chapter 5

Conclusion

In this study, we explored the complex relationship between Hhcy, CAD, and associated gene polymorphism analysis within a Bangladeshi cohort. Despite observing a marginally higher prevalence of elevated homocysteine levels among CAD patients compared to healthy controls, the difference did not reach statistical significance, underscoring the need for caution in attributing a direct causal link between Hhcy and CAD. This aligns with the broader scientific discourse, which remains inconclusive due to mixed evidence from various studies and the absence of definitive interventional trials.

Our investigation into the association between Hhcy and conventional cardiovascular risk factors revealed no significant correlations, suggesting that Hhcy may contribute to CAD through mechanisms independent of traditional risk markers like diabetes, hypertension, and smoking. This finding emphasizes the multifaceted nature of CAD pathogenesis and the potential for Hhcy to influence vascular health through unique pathways that merit further exploration.

The dietary analysis within our study highlighted specific consumption patterns that modulate CAD risk in the Bangladeshi population. Elevated intake of sugar and chicken was significantly associated with increased CAD risk, while frequent fish consumption was linked to a reduced risk. These insights underscore the critical role of dietary modifications in CAD prevention strategies and provide a culturally relevant context for public health interventions.

Despite the limitations of sample size and some instances of indeterminate genotyping, our study offers meaningful contributions to understanding CAD risk factors in a specific demographic context. Future research should prioritize larger, well-powered studies to validate these findings and further elucidate the independent and interactive roles of Hhcy, dietary patterns, and genetic factors in CAD pathogenesis. Additionally, interventional studies exploring the efficacy of homocysteine-lowering therapies, such as folic acid and vitamin B12 supplementation, could provide critical insights into potential therapeutic avenues for mitigating CAD risk.

In conclusion, while our findings add to the growing body of evidence on the role of Hhcy in cardiovascular health, they also highlight the intricacies and challenges of CAD research. By addressing these complexities through comprehensive, multidisciplinary approaches, we can advance our understanding and develop more effective prevention and treatment strategies for CAD, ultimately improving cardiovascular health outcomes in Bangladesh.

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