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MPhil Thesis

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Determinants of Hyperhomocysteinemia in Type 2 Diabetic Subjects

Akter, Kuhilika

University of Rajshahi

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Determinants of Hyperhomocysteinemia in Type 2 Diabetic Subjects

MPhil Thesis

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April 2012

DECLARATION

I hereby humbly declare that this Thesis titled as '**Determinants of Hyperhomocysteinemia in Type 2 Diabetic Subjects**' is based on works carried out by me. No part of it has been presented previously for any higher degree.

The research work has been carried out in the Department of Biochemistry and Molecular Biology, Rajshahi University and Biomedical Research Group in BIRDEM under the supervision of Dr Mohammad Amirul Islam, Associate Professor, Department of Biochemistry & Molecular Biology University of Rajshahi and Prof Liaquat Ali, professor of the Dept of Biochemistry & Cell Biology and Director of the Bangladesh Institute of Health Sciences (BIHS), Dhaka, Bangladesh.



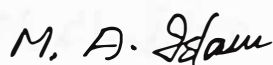
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CERTIFICATE

Certified that **Kuhilika Akter** has done the Thesis titled as **Determinants of Hyperhomocysteinemia in Type 2 Diabetic Subjects'** submitted as partial fulfillment of the requirement for the Degree of MPhil (Dept of Biochemistry and Molecular Biology, Rajshahi University) under the Faculty of Science, Rajshahi University, Rajshahi. This study has been carried out in the Department of Biochemistry and Molecular Biology, Rajshahi University and Biomedical Research Group of BIRDEM during the period of 2007 to 2011. We have gone through the Thesis. To the best of our knowledge no part of the work has been submitted for another degree or qualification in any other Institute.

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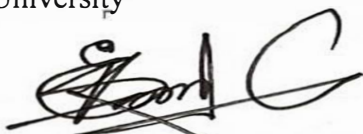
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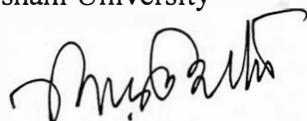
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ABBREVIATIONS

ADA	American Diabetes Association
AG	Blood Glucose After 2 hour oral glucose challenge
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorder
BMI	Body Mass Index
BP	Blood Pressure
Cho	Cholesterol
CHD	Coronary Heart Diseases
CI	Confidence Interval
CRP	C-reactive Protein
CVD	Cardio Vascular Disease
DBP	Diastolic Blood Pressure
DM	Diabetes Mellitus
EASD	European Association for the Study of Diabetes
FBG	Fasting Blood Glucose
GDM	Gestational Diabetes Mellitus
Hcy	Homocysteine
HDL-C	High Density Lipo-protein Cholesterol
IDDM	Insulin Dependent Diabetes Mellitus
LDL-C	Low Density Lipo-protein Cholesterol
NIDDM	Non Insulin Dependent Diabetes Mellitus

OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
SBP	Systolic Blood Pressure
SPSS	Statistical Package for Social Sciences
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TG	Triglyceride
TNF α	Tumour necrosis factor- α
VLDL	Very low density lipoprotein
WHO	World Health Organization
WHR	Waist to Hip Ratio

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease that is independent of major risk factors such as diabetes, hypertension, hypercholesterolemia, and smoking. Although the mechanisms by which homocysteine promotes atherothrombosis are unknown, the epidemiological evidence for the association of hyperhomocysteinemia with atherothrombotic disease is strong (Ueland, Refsum, Brattstrom, 1992; Boushey et al, 1995; Welch, Loscalzo, 1998). Recent retrospective and prospective studies, it is now widely accepted that increased total plasma homocysteine is a risk factor for cardiovascular disease. Impaired enzyme function as a result of genetic mutation or deficiency of the essential B vitamins folic acid B₁₂ and B₆ can lead to hyperhomocysteinemia. Several evidence suggests that it may play a role in atherothrombotic disease. Gradually increased homocysteine causes dysfunction of vascular endothelium.

It was an observational study with a case-control design. Two Groups of subject were included in this study. 108 subjects were diabetic without CVDs in Group I and 101 subjects were selected diabetic with CVDs in group II. A total of 209 subjects included in this study and its association with clinical, socio-economical and biochemical risk factors. Purposively and their economical, and clinical characteristics were noted in a pre-designed case record form. Nutritional intake was assessed by food frequency questionnaire (24 hr dietary recall) method. Each subject went through OGTT following appropriate preparation and DM was diagnosed as per WHO Study Group Criteria. Blood glucose was measured by glucose oxidase method (Randox, UK) using a semi auto analyzer (300 MICROLAB). Lipids were measured by enzymatic methods using the same analyzer in the lab of Rajshahi Diabetic center. Plasma homocysteine concentration was measured by Fluorescence Polarization Immunoassay (FPIA) technology (Abbott Laboratories, USA). Significance of differences will be analyzed by Student's t- test, as appropriate. Pearson's correlation and multivariate regression, as appropriate, were used to analyze the association between the other variables.

Two Groups were age (yrs, M \pm SD) - matched and 55.4% male subjects were in Group II.

BMI (kg/m^2 , $M \pm \text{SD}$); systolic blood pressure (mm of Hg) and diastolic blood pressure (mm of Hg) were significantly higher in Group II [(29 \pm 4.7); (130.5 \pm 12.9) and (88.4 \pm 8.6)] as compare to Group I [(24.1 \pm 3.8); (120.7 \pm 13) and (82.7 \pm 8)]; ($p=0.013$; $p<0.001$ and $p=0.007$) respectively.

Fasting serum glucose level (mmol/l, $M \pm \text{SD}$) were significantly higher in Group II compared to Group I (18.5 \pm 6.01 vs 11.2 \pm 4.2; $P<0.001$). Serum glucose at 2 h after 75 g glucose administration were found to be (18.3 \pm 5.9) in Group I and (17.7 \pm 5.7) in Group II; ($p=0.477$). There was no significant difference in the two Groups. Serum total cholesterol (mg/dl, $M \pm \text{SD}$) were significantly higher in Group II compared to Group I (226 \pm 47 vs 207 \pm 34.2; $P<0.001$). Serum triglyceride (gm/dl, $M \pm \text{SD}$) were significantly higher in Group II compared to Group I (218 \pm 85.5 vs 172 \pm 67.3; $P<0.001$). Serum HDL levels also significantly higher in Group I (47 \pm 8.1) as compare to Group II (39.8 \pm 10); ($p=0.004$).

Serum homocysteine ($\mu\text{mol}/\text{L}$, $M \pm \text{SD}$) were also significantly higher then compare to Group I (7.6 \pm 1.5) and Group II (14.5 \pm 5.8); ($p<0.001$). 37.6% subjects were in Group II their homocysteine level was below cut off value ($<12 \mu\text{mol}/\text{L}$) and 62.4% subjects were above cut off value of homocysteine level.

The daily mean intake of dietary folate (mg/day, $M \pm \text{SD}$) in the study subjects as follows: as Group I (7.7 \pm 1.8) and Group II (1.8 \pm 0.93) respectively ($p<0.001$). Daily vitamin B₆ as follows: as Group I (1.6 \pm 0.38) and Group II (1.0 \pm 0.61) respectively ($p<0.001$). And daily vitamin B₁₂ as follows: as Group I (277 \pm 74.1) and Group II (119.1 \pm 29.8) respectively ($p<0.001$). There was significant difference in dietary intake folate, vitamins B₆ and B₁₂ in between the two Groups.

On the coefficient correlation of homocysteine with other variables. From the data, a highly positive correlation of homocysteine was found with BMI, duration of DM, habit of exercise fasting blood glucose level and serum triglyceride ($r=0.468$, $p=0.050$; $r=0.392$, $p<0.001$; $r=0.181$, $p=0.009$; $r=0.332$, $p<0.001$ and $r=0.146$; $p=0.035$) and

negative correlation was found between dietary folate, vitamin B6 and B12 ($r=-0.563, p<0.001$; $r=-0.379, p<0.001$ and $r=-0.484$) respectively ($p<0.001$) among the Group II.

On further analysis by multiple linear regressions Hyperhomocystenemia was found to have significant negative association only with dietary folate and vitamin B6 ($\beta=-0.408$; $p<0.001$ and $\beta=-0.128$; $p=0.053$) when the effects of Age, BMI and groups were adjusted.

From the above discussion it may be concluded that, Hyperhomocysteneimia is a important risk factor of cardiovascular disease in Bangladeshi diabetic population. Dietary deficiency of folate and vit B₆ are the major determinants of hyperhomocystienemia in type 2 diabetic subjects.

INTRODUCTION

1 INTRODUCTION

Diabetes mellitus (DM) is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (WHO, 1999; ADA, 2005). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (ADA, 2005).

The World Health Organization (WHO) estimated that there were 135 million diabetic individuals in the year 1995 and it has projected that this number would increase to 300 million by the year 2025 (King et al, 1999). It has also declared that diabetes had reached epidemic proportions and predicts that most of the increase will be contributed by developing countries (King et al, 1999). In another review global prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030 (Wild et al, 2004).

Distributive pattern of diabetes shows higher rates among people of developing countries, and in lower socioeconomic groups of more developed countries (Bennett et al, 2000). Incidence of diabetes worldwide is expected to double in the next 10 years affecting over 200 million people. From 2003 to 2025, the worldwide prevalence of diabetes in adult (20-79 years of age) group is expected to increase from 5.0 to 6.2%.

The largest proportional and absolute increase will occur in developing countries, where the prevalence will rise from 4.2 to 5.6%, and in Bangladesh, from 3.9 to 4.8%. Most of the expected population growth between 2000 and 2030 will be concentrated in the urban areas of the world (UNPD, 1999). The ten countries estimated to have the highest numbers of people with diabetes in 2000 and 2030 are India, China, U.S, Indonesia, Japan, Pakistan, Russian federation, Brazil, Italy and Bangladesh.

Globally diabetes prevalence is similar in men and women but it is slightly higher in men <60 years of age and in women at older ages. In developing countries, the majority of people with diabetes are in the 45- to 65-year age range. In contrast the majority of people with diabetes in developed countries are >64 years of age. By 2030, it is estimated that the number of people with diabetes >64 years of age will be >82 million in developing countries

and >48 million in developed countries (Wild et al, 2004). The IDF Diabetes Atlas 2000 used different and less stringent criteria for the inclusion of studies to estimate prevalence of diabetes for 20-to 79-year old individuals in the 172 IDF member countries (~ 90% of the population of the world) (IDF, 2000). It was estimated that there were 151 million people with diabetes in this subpopulation in 2000. The IDF has subsequently released estimates of the numbers of people with diabetes for 2003 and forecast for 2025 of 194 million and 334 million, respectively (IDF, 2003).

Classification of T2DM:

According to World Health Organization (WHO) diabetes is divided into etiologic subclasses, of which type 1 and type 2 are the most prevalent (WHO, 1999). Type 1 or Insulin Dependent Diabetes Mellitus (IDDM) results from an absolute deficiency of insulin due to autoimmune destruction of the insulin producing pancreatic beta cell (Atkinson and Eisenbarth, 2001). Type 2 or Non-insulin Dependent Diabetes Mellitus (NIDDM) is characterized by insulin resistance and/or abnormal insulin secretion, either of which may predominate (Cavaghan et al, 2000). Maturity-Onset Diabetes of the Young (MODY) and Gestational diabetes mellitus (GDM) are less frequently occurring forms of diabetes. It is also worth noting that the current classification of diabetes on the basis of age is becoming increasingly problematic because the age of individuals presenting Type 1 diabetes is getting older and there is an increase of type 2 diabetes in the young.

TYPE- 1: Characterized by beta cell destruction, usually leading to absolute insulin deficiency.

It has two forms:

- **Immune-Mediated Diabetes Mellitus:** Results from a cellular mediated *autoimmune* destruction of the *beta cells* of the pancreas.
- **Idiopathic Diabetes Mellitus:** Refers to forms of the disease that have no known etiologies.

TYPE- 2: Diseases of *insulin resistance* that usually have relative (rather than absolute) insulin deficiency. Can range from predominant insulin resistance with relative insulin deficiency to predominant insulin deficiency with some insulin resistance.

- **Impaired Glucose Homeostasis:** A metabolic stage intermediate between normal glucose homeostasis and diabetes. A risk factor for diabetes and cardiovascular disease. Impaired Fasting plasma glucose higher than normal, and less than impaired

Glucose Tolerance Plasma glucose higher than normal, and less than diagnostic, following administration of a glucose load of 75 g.

Gestational Diabetes Mellitus: Glucose intolerance in pregnancy.

Other Specific Types

- Diabetes caused by other identifiable etiologies.
- Genetic defects of beta cell function
- Genetic defects in insulin action
- Diseases of the exocrine pancreas (eg, cancer of the pancreas, cystic fibrosis, pancreatitis)
- Endocrinopathies (eg, Cushing's)
- Drug or chemical induced (eg, steroids)
- Infection (eg, rubella, Coxsackie, CMV)
- Uncommon forms of immune-related diabetes

Other genetic syndromes

Elevated circulating concentrations of glucose have an autoregulatory effect in enhancing glucose uptake, decreasing hepatic glucose production and increasing insulin production. Hyperglycemia may also cause peripheral insulin resistance as a result of a down-regulation (i.e. decreased numbers) in peripheral tissues. Type 2 DM is characterized by three pathophysiologic abnormalities: peripheral insulin resistance, impaired insulin secretion, and excessive hepatic glucose production (Powers et al, 2001). In type 2 DM, insulin resistance associated with obesity (abdominal or visceral) augments genetically determined insulin resistance. In early-stage disease, pancreatic beta cells compensate for increased insulin resistance by increasing insulin output. The beta cells cannot sustain hyperinsulinemia in the setting of progressively increased insulin resistance (IR). Early beta-cell decline is characterized by postprandial hyperglycemia and impaired glucose tolerance, further decline is characterized by fasting hyperglycemia and overt DM.

IR is associated with a variety of cardiovascular risk factors. Insulin-resistant nondiabetic or prediabetic patients with several of these risk factors have insulin resistance syndrome (IRS, metabolic syndrome, syndrome X). The IRS concept has important implications for prevention, identification, and management of cardiovascular risk (Grundy, 2001).

Cardiovascular diseases (CVD), which include hypertension, stroke, and heart disease, are considered as the leading causes of mortality and morbidity, which accounts for 16.7 million or 29.2% of total global deaths in 2003. In Canada CVD accounts for approximately 40% of all deaths (Heart disease and stroke in Canada, 1997) in the future.

Recently CVDs are showing declining mortality rates in developed countries but the recent World Health Report (WHO, 2002) draws attention to the increasing importance of cardiovascular diseases in developing countries. South Asian populations (from Pakistan, India, Bangladesh and Sri Lanka) represent a quarter of the developing world population and are known to be at a higher risk of CVDs both in native and expatriated settings. In the year 2000, among 16.7 million deaths caused by CVD half of these occurred in developing countries (Nishtar et al, 2002). People of South Asian origin living in the UK have a 1.5-fold greater susceptibility to ischemic heart disease than the general population (Office of Population Census and Surveys, 1993; Wild et al, 1997) and 40-50% higher mortality from coronary heart disease than the population average (McKeigue et al, 1991; Balarajan, 1996).

Cardiovascular diseases are emerging as a serious health problem in Bangladesh. Amongst the heart diseases hypertension, rheumatic fever, rheumatic heart diseases, ischemic heart diseases and congenital heart diseases are common in Bangladesh. The percentage for cardiovascular diseases in Bangladesh is accounted for about 4.5 % (US Library of Congress, 1989). In Bangladesh about 20 per cent adults have got hypertension, 10 per cent adults have ischemic or coronary heart diseases, eight per thousand have got rheumatic heart diseases and three per thousand new born have got congenital heart diseases. Nearly 15 lakh children in Bangladesh suffer from congenital heart disease, according to reports of world organizations (The independent, 2004).

Many factors, such as hyperglycemia, dyslipidemia, and abnormalities in homeostasis, are implicated in the development of diabetic macroangiopathy. Certain factors can increase the risk for cardiovascular diseases. Some of these factors referred to as *nonmodifiable* risk factors, these include: age, gender and family history. And some can change to reduce the risk, referred to as *modifiable* risk factors include: cigarette smoke, high blood cholesterol, high blood pressure, physical inactivity, obesity and overweight and diabetes mellitus. Other factors that contribute to heart disease risk include: homocysteine, C-reactive protein (CRP), lipoprotein (a), stress, and alcohol.

Age plays an important role in type 2 diabetes cardiovascular diseases. Blood can move easily through the supple elastic artery of young people, but as the person grows older the arteries become harder or scleritic, unable to expand properly. As the age advances; with the reduction of flexibility, more resistance is offered to the flow of blood, forcing the heart to work harder causing rise in blood pressure. In most populations the risks of cardiovascular diseases rise steeply with increasing age; advancing age increase in the rise of CVD diseases (Khan, 2008). Adults with diabetes are thought to have a high risk of cardiovascular disease (CVD), irrespective of their age. The age at which people with diabetes develop a high risk of CVD, as defined by an event rate equivalent to a 10-year risk of 20% or more; or an event rate equivalent to that associated with previous myocardial infarction.

Overall, men have a higher risk of heart attack than women. After menopause *sex* hormones appear to play a role in heart disease. Within ages between 40 and 65, women will have a heart attack gradually increasing.

Smoking is considered to be a major factor of risk in heart disease. As many as 30% of all coronary heart disease (CHD) deaths in the United States each year are attributable to cigarette smoking, with the risk being strongly dose-related. Smoking also nearly doubles the risk of ischemic stroke. Smoking acts synergistically with other risk factors, substantially increasing the risk of CHD. Smokers are also at increased risk for peripheral vascular disease, cancer, chronic lung disease, and many other chronic diseases. Cigarette smoking is the single most alterable risk factor contributing to premature morbidity and mortality in the United States, accounting for approximately 430 deaths annually.

Regular *physical activity* reduces the risk of dying prematurely from CVD. It also helps prevent the development of diabetes, helps maintain weight loss, and reduces hypertension, which are all independent risk factors for CVD. Physical inactivity is a significant risk factor for CVD itself. It ranks similarly to cigarette smoking, high blood pressure, and elevated cholesterol. One reason it has such a large affect on mortality is because of its prevalence. Twice as many adults in the United States are physically inactive than smoke cigarettes. Regular physical activity has been shown to help protect against first cardiac episode, help patients' recovery from coronary surgeries, and will reduce the risk of recurrent cardiac events.

It is estimated that approximately 35% of coronary heart disease mortality is due to physical inactivity. The significance of this relationship lies in the fact that coronary heart disease is the leading cause of death in the United States with over 700,000 deaths annually. Approximately 60% of all Americans age 18 and older report that they are physically inactive. Physical inactivity has a major economic impact. It is felt through the loss of income and productivity when disabling diseases result.

Obesity is known to predispose to type 2 diabetes, systemic hypertension, and cardiovascular disease (CVD). Concern therefore exists that the current epidemics of both obesity and diabetes may minimize the gains in life-expectancy that have been observed over the past 20 years.

The association between obesity and CVD has been confirmed in several epidemiological studies, which have reported a direct relationship between body mass index (BMI) and death. BMI and waist circumference (WC) are commonly used to define and quantify obesity and adiposity. In recent years, however, attention has focused also on regional fat depots, which appear to have equal or even greater importance than BMI in the development of metabolic abnormalities and CVD. Abdominal visceral adipose tissue (VAT), for example, the largest visceral fat depot in humans, has been shown to be associated with the metabolic syndrome and the development of CVD.

Hypertension is a chronically increased arterial pressure. It implies the persistent elevation of arterial blood pressure above normal. Blood pressure is the force of blood pushing against blood vessel walls. The heart pumps blood into the arteries (blood vessels), which carry the blood throughout the body. High blood pressure, also called hypertension, is dangerous because it makes the heart work harder to jump blood to the body and it contributes to hardening of the arteries or atherosclerosis and the development of heart failure. Present elevation in systolic and diastolic arterial pressure above normal, which acts a risk factor of increased CVD diseases. The exact causes of high blood pressure are not known. Several factors and conditions may play a role in this situation.

A low level of *HDL cholesterol* is also considered a major risk factor because it independently predicts the incidence of CVD. A final major risk factor is advancing age; chronological age is considered a risk factor because it also independently predicts CVD.

High cholesterol levels considerably increase the risk of cardio-vascular diseases and even heart failure. People with high blood cholesterol levels are exposed to developing heart disease. High cholesterol levels are common to people with ages over 50, people with weight problems, people with gastro-intestinal disorders and people with diabetes. High blood cholesterol levels can be the result of either overproduction of the substance (due to liver dysfunctions) or the inability of the body to eliminate it. However, apart from physiological factors that enable the accumulation of cholesterol inside the organism, there are also many other external factors that contribute to cholesterol build up: inappropriate diet, sedentary lifestyle, smoking and alcohol abuse.

Men with diabetes mellitus (high blood sugar) have 3 or 4 times the likelihood of developing atherosclerosis, resulting in angina, heart attacks, strokes, or peripheral vascular disease. Women with diabetes are at an even higher risk - probably 4 times that of non-diabetic.

To compound the global diabetes epidemic, health professionals are witnessing an alarming increase in inflammatory diseases resulting from adult onset (ie, Type 2) diabetes. This phenomenon is referred to as "metabolic syndrome" where confluences of inflammatory conditions occur along with the diabetes. As a result, growing evidence appears to show that metabolic syndrome makes the diabetic patient susceptible to degenerative health conditions such as cardiovascular disease, stroke and, now believed, Alzheimer's disease. As the diabetes epidemic escalates, a new sense of urgency has taken hold. Proactive strategies for prevention of the disease are being put in place by international health organizations such as the World Health Organization (WHO), as well as by the health departments of industrialized and developing countries, and even at the local level where these food ingredients regulations are being passed. This TriMark Publications report charts the changing landscape of the global diabetic population and explores the added health concerns resulting from the metabolic syndrome phenomenon and one of its major risk factors: cardiovascular disease (CVD). Furthermore, this study evaluates widely-accepted therapeutic approaches to diabetes that are currently in use, while providing an in-depth

analysis of emerging technologies that will be used to treat diabetes and other inflammatory diseases (World Health Report, 2002).

Increased concentrations of the marker of inflammation, C-reactive protein (CRP), are associated with insulin resistance, Type 2 diabetes and the development of cardiovascular disease. In particular, inflammation is closely associated with endothelial dysfunction and is recognized as one of the cardiovascular risk factors clustering in the Insulin Resistance Syndrome. The exact mechanisms linking insulin resistance and inflammation remain unclear. However, the close association between insulin resistance and inflammation in atherogenesis suggests that therapies that address both parameters may have benefits in reducing diabetes-related macrovascular complications.

In recent years fasting serum homocysteine (tHcy) levels has emerged as a strong and an independent risk factor for atherosclerotic vascular diseases (Refsum et al, 1998) and is associated with an increased risk for fatal and nonfatal cardiovascular diseases.

A wealth of epidemiological evidence from more than 100 prospective cohorts, cross sectional and case control studies have confirmed the relationship between homocysteine concentrations and vascular disease (Clarke et al, 2002; Wald et al, 2002). A 1995 meta-analysis by Boushey et al estimated that a reduction of 5mmol/L in plasma homocysteine level would decrease vascular risk by one third and also reported that approximately 10% of the coronary heart disease in the general population could be attributed to hyperhomocysteinemia (Boushey et al, 1995). Since then, many additional observational studies reported an association between hyperhomocysteinemia and atherosclerotic vascular disease (Graham et al, 1997; Markus et al, 1997; Lindgren et al, 1995; Hopkins et al, 1995; Dalery et al, 1995; Robinson et al, 1995; Malinow et al, 1996; Alftan et al, 1997).

Prospective longitudinal studies provide more robust evidence of strong and consistent association between a risk factor and disease. Eight cohort studies reported statistically significant positive associations between elevated homocysteine levels and cardiovascular disease (Arnesen et al, 1995; Stampfer et al, 1992; Wald et al, 1998; Perry et al, 1995; Nygard et al, 1997; Stehouwer et al, 1998; Petri et al, 1996; Bots et al, 1998).

Homocysteine is a sulfhydryl-containing amino acid derived from the metabolic remethylation of dietary methionine. There are 2 pathways in homocysteine metabolism, the remethylation and transulfuration pathways. The transulfuration pathway is mainly limited to cells of the liver and kidneys. The enzymes in this pathway, CBS and γ -cystathionase, are both dependent on pyridoxal-5'-phosphate, a biologically active form of vitamin B₆, as cofactor. Homocysteine can also be remethylated to methionine by the enzyme methionine synthase (MS). This enzyme uses methylcobalamin (a biologically active form of vitamin B₁₂) as cofactor. The methyl group for the latter reaction is donated by 5-methyl-tetrahydrofolate (5-methyl-THF). This form of folate is produced by the enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR). MTHFR in turn uses flavin adenine dinucleotide (a biologically active form of vitamin B₂) as a cofactor (Finkelstein, 1990; Guenther et al, 1999). The intracellular homocysteine concentration is precisely regulated and any excess is transported to plasma.

Disturbances in intracellular homocysteine metabolism lead in most cases to elevated tHcy concentrations. The determinants of total plasma homocysteine are complex and involve demographic, genetic, and acquired factors. Thus, genetic background, nutrition, state-of health, life-style, gender, and age influence the homeostasis of homocysteine.

Physical activity is also associated with high tHcy levels (Nygard et al, 1995; 1997). Coffee consumption is positively associated with the tHcy concentration in both men and women in most (Nygard et al, 1997b; Oshaug et al, 1998; Stolzenberg Solomon et al, 1999; de Bree et al, 2001d; Jacques et al, 2001; Koehler et al, 2001), but not all observational studies (Nieto et al, 1997; Rasmussen et al, 2000; Saw et al, 2001). Smoking is positively associated with the tHcy concentration (Nygard et al, 1995; Giles et al, 1999; Rasmussen et al, 2000; de Bree et al, 2001d; Jacques et al, 2001; Koehler et al, 2001). Alcohol consumption is positively associated with elevated tHcy concentration (Cravo et al, 1996b; de Bree et al, 2001b; Koehler et al, 2001).

Dietary folate intake is identified as the most important dietary determinant of plasma total homocysteine concentration (Selhub, 1993; De Bree, 2001). Dietary intake of vitamin B₆, B₁₂ and folate is inversely correlated to plasma tHcy (Selhub et al, 1993). Individuals with a nutritional deficiency that leads to low blood concentrations of folate, vitamin B₁₂, or

vitamin B₆ are at risk of hyperhomocysteinemia, which lead a risk for cardiovascular disease.

Folate was lower whereas cobalamin was higher ($P < 0.0001$ for both) among men than among women. The study concluded that high prevalence of hyperhomocysteinemia is more closely associated with folate than with cobalamin, although other factors, eg, smoking and betelnut use, may also contribute to its cause. Whereas, in Refsum study 47% had serum cobalamin concentrations <150 pmol/L, 38% of whom were vegetarian, (Refsum *et al*, 2001). This finding contrasts with findings in Bangladesh, where vegetarianism is uncommon, and only 11% of our study participants were found to have cobalamin concentrations <150 pmol/L.

1.1 Rationale

In the recent years CVD is considered as an emerging health hazard causing numerous death in Bangladesh. Therefore, it may be postulated that elevated tHcy could be an etiopathogenic risk factor for CVD in Bangladeshi population because Bangladeshis are more susceptible to elevated tHcy levels. Risk for hyperhomocysteinemia as well as CVD is partly predisposed by genetic, ethnicity, social cultural life style, and dietary factors and also with lack of proper health care facilities. As Bangladesh is a least developing country, dietary intake of certain vitamins, which are necessary for homocysteine metabolism, is not optimal and infrastructure of health care facilities is not satisfactory.

Data on the determinants of homocysteine concentration in Bangladeshi populations are scarce. In the study we will estimate the normal value of plasma total homocysteine concentrations need to be estimated by age and sex among middle aged Bangladeshi type 2 diabetic women and men (taking advantage of having large number of diabetic patients who are prone to CVD) along with normal healthy individuals as Control. Furthermore we established the normal cut of value of plasma total homocysteine concentrations of healthy control need to be done and we also explore the determinants of homocysteine concentration including dietary intake, life style factors, plasma concentration of other conventional factors for CVD. The study will generate valuable data on the etiopathogenesis of CVDs in our population and, thus, help to improve the management and prevention of these groups of disorders.

1.2 Hypothesis: Hyperhomocysteinemia is a risk factor for cardiovascular disease that is independent of major risk factors for type 2 diabetes.

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OBJECTIVES

2.1 General objectives

The general objective of the present study was to explore the determinants of hyperhomocysteinemia in a Bangladeshi type 2 diabetic population with and without CVDs.

2.2 Specific objectives

The specific objective of the present study was:

- 1 To measure serum levels of tHcy in type 2 DM subjects with and without CVDs.
- 2 To assess the already known anthropometric, clinical, biochemical and nutritional determinants of serum hyperhomocysteinemia among type 2 DM subjects with and without CVDs.
- 3 To investigate the association of the known determinants with hyperhomocysteinemia in Bangladeshi type 2 DM (with and without CVDs) subjects taking in to consideration the established confounders of the CVD risk factors.

LITERATURE REVIEW

3 LITERATURE REVIEW

Diabetes mellitus (DM) is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (WHO, 1999; ADA, 2005). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (ADA, 2005).

There are three main types of diabetes:

- **Type 1 DM:** results from the body's failure to produce insulin, and presently requires the person to inject insulin. (Also referred to as insulin-dependent diabetes mellitus, IDDM for short, and juvenile diabetes.)
- **Type 2 DM:** results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency. (Formerly referred to as *non-insulin-dependent* diabetes mellitus, *NIDDM* for short, and *adult-onset* diabetes.)
- **Gestational diabetes:** is when pregnant women, who have never had diabetes before, have a high blood glucose level during pregnancy. It may precede development of type 2 DM.

Other forms of diabetes mellitus include congenital diabetes, which is due to genetic defects of insulin secretion, cystic fibrosis-related diabetes, steroid diabetes induced by high doses of glucocorticoids, and several forms of monogenic diabetes.

T2DM is epidemic in most developed and many developing countries. A large numbers of people from wide range of ethnic groups and all social and economic levels throughout the world. Currently, at least 120 million people suffer from type -2 diabetes, by the year 2010.(Diabetes care volume 23) Type 2 diabetes mellitus is characterized by insulin resistance which may be combined with relatively reduced insulin secretion. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. However, the specific defects are not known. Diabetes mellitus due to a known defect are classified separately. Type 2 diabetes is the most common type.

In the early stage of type 2 diabetes, the predominant abnormality is reduced insulin sensitivity. At this stage hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver.

Type 2 diabetes mellitus is characterized by a high incidence of vascular complications. Cardiovascular complications are the leading causes of death in type 2 diabetes mellitus. Many factors, such as hyperglycemia, dyslipidemia, and abnormalities in hemostasis, are implicated in the development of diabetic macroangiopathy. In recent years fasting serum homocysteine (tHcy) levels have emerged as an independent risk factors for the development of atherosclerosis.

Risk factors of T2DM:

Obesity, excessive fat around the waist (one of the two most likely diabetes risk factors, the other being genetic predisposition).

- Blood relatives with type 2 diabetes
- Being older than 45
- High blood pressure
- Malnutrition of the mother when pregnant
- Low weight at birth
- Lack of activity and high-fat diet
- High levels of triglycerides and cholesterol in the blood
- Experience of polycystic ovary syndrome or gestational diabetes.

Diabetes is a risk factor for cardiovascular disease. When elevated glucose levels below the diabetic range increase cardiovascular risk. Glucose and its metabolites have direct toxic effects on vascular endothelium. Abnormal glucose is absolute or relative insulin deficiency. Which can predispose to cardiovascular disease via endothelial dysfunction, lipid abnormalities, and inflammation. Hypertension, may contribute to the development of both diabetes and cardiovascular disease.

Most cardiovascular complications related to diabetes have to do with the way the blood circulates through the body. Diabetes can change the chemical makeup of the substances found in blood, causing blood vessels to narrow or clog up totally. This is called atherosclerosis, or hardening of the arteries.

The increased risk can be attributed in large part to the high rate of concomitant CVD risk factors such as hypertension, dyslipidemia, and obesity in people with diabetes. However, diabetes is an independent risk factor. When people with diabetes and other risk factors are compared with people who have the same risk factors but not diabetes, people with diabetes still have higher rates of death and disability from CVD. Even though death rates from cardiovascular causes have declined for the population as a whole over the past 30 years, the decline has been much smaller in people with diabetes. In fact, the death rate from CVD in adult women with diabetes has increased over that period. Cardiovascular diseases the major causes of mortality and morbidity in modern societies. In developing world as the most common causes of death. The increasing prevalence of major and emerging cardiovascular risk factor accounts for the growing burden of cardiovascular disease in the world. Diabetes in all forms is one of the main cardiovascular risk factors. Two of diabetic patients will die as a result of cardiovascular complications, and approximately 30% of patients treated in cardiovascular intensive care units have diabetics (Circulation).

Certain factors can increase the risk for cardiovascular diseases. Some of these factors referred to as non-modifiable risk factors, these include: age, gender and family history. And some can change to reduce the risk, referred to as modifiable risk factors, includes: cigarette smoke, high blood cholesterol, high blood pressure, physical inactivity, obesity and overweight and diabetes mellitus. Other factors contribute to heart disease risk; these are homocysteine, C-reactive protein (CRP), lipoprotein (a), stress, and alcohol.

Recently hyperhomocysteinemia has emerged as a strong and an independent risk factor for atherosclerotic vascular diseases (Refsum *et al*, 1998) and is associated with an increased risk for fatal and nonfatal cardiovascular disease. A wealth of epidemiological

evidence from more than 100 prospective cohorts, cross sectional and case control studies have confined the relationship between homocysteine concentrations and vascular disease (Clarke *et al*, 2002; Wald *et al*, 2002). A 1995 meta-analysis by Boushey *et al* estimated that a reduction of 5 mmol/L in plasma homocysteine the coronary heart disease in the general population could be attributed to hyperhomocysteinemia (Boushey *et al*, 1995). Since then, many additional observational studies reported an association between hyperhomocysteinemia and atherosclerotic vascular disease (Graham *et al*, 1997; Markus *et al*, 1997; Lindgren *et al*, 1995; Hopkins *et al*, 1995; Dalery *et al*, 1995; Robinson *et al*, 1995; Malinow *et al*, 1996; Alfthan *et al*, 1997). Prospective longitudinal studies provide more robust evidence of strong and consistent association between a risk factor and disease. Eight cohort studies reported statistically significant positive associations between elevated homocysteine levels and cardiovascular level would decrease vascular risk by one third and also reported that approximately 10% of disease (Arnesen *et al*, 1995; Stampfer *et al*, 1992; Wald *et al*, 1998; Perry *et al*, 1995; Nygard *et al*,

Other risk factors, in addition to the aforementioned major risk factors, may further contribute to total risk. They are underlying risk factors and emerging risk factors. The underlying risk factors are overweight/obesity, physical inactivity, atherogenic diet, socioeconomic and psychosocial stress, family history of premature CVD, and various genetic and racial factors. To some extent, the underlying risk factors affect risk by acting through the major risk factors, and they also appear to influence risk in ways unrelated to the major risk factors. Although these underlying risk factors likely add an independent component to total risk, their contribution has been difficult to distinguish in prospective studies from their effects on major risk factors; for this reason, they generally are not included in clinical predictive equations.

Emerging risk factors are factors that are correlated with CVD risk in prospective or case-control studies, but the strength of their correlation and/or their prevalence in the population is less than that for the major risk factors. For this reason, the emerging risk factors generally are not included in risk-prediction equations. Among the emerging risk

factors are various lipid factors [triglycerides, apolipoproteins, lipoprotein(a), and lipoprotein subfractions] and nonlipid factors (insulin resistance, prothrombotic markers, and proinflammatory markers). Similarly, subclinical atherosclerosis may also be useful in predicting the risk of CVD events. Because the emerging risk factors are not incorporated into risk predictions, their use in clinical practice must be individualized and based on clinical judgment. Most importantly, they should not be given more priority in risk assessment than that given to the major risk factors.

Several risk factors that are only moderate often incur a greater total risk in the short term than does a single, severe risk factor. Risk assessment in both individuals and populations must take this fact into account. The greatest efficacy of treatment occurs in patients who are at highest risk. Thus, persons who are at higher total risk will attain greater reductions in absolute risk with any given lowering of risk factors. Giving priority in risk-reduction therapies to patients at higher total risk will produce a substantial reduction in total CVD events. Furthermore, more high-risk individuals will benefit; in other words, the number needed to treat over a given period of time to achieve prevention of 1 CVD event will be fewer in higher-risk persons than in lower-risk persons.

The principle of assessing the total or global risk associated with multiple risk factors was first introduced in New Zealand in 1993, in relation to the management of blood pressure. The following year, the European Society of Cardiology, European Atherosclerosis Society, and European Society of Hypertension proposed total multifactorial risk as the primary determinant of drug treatment for both blood pressure and blood lipids in preventing the development of coronary heart disease (CHD). The US National Cholesterol Education Program (Adult Treatment Panel II) published in the same year, also recommended, for the first time, assessing and managing lipids in the context of other cardiovascular risk factors. The principle of global risk was also supported by conclusions of the 27th Bethesda Conference (Matching the Intensity of Risk Factor Management with the Hazard of Coronary Disease Events), followed by the Sixth report of the Joint National Committee on High Blood Pressure, the AHA Prevention V Conference, the International Task Force on Coronary Heart Disease, the

WHO/International Society of Hypertension Guidelines for Management of Hypertension, the National Cholesterol Education Program (Adult Treatment Panel III), and, most recently, the Third Joint European Societies' Task Force on CVD Prevention in Clinical Practice and the Seventh report of the Joint National Committee on High Blood Pressure. All of these guidelines since 1993 have embraced, to different extents, the principle of multifactorial or global risk assessment as a basis for deciding whom to treat with drugs, although patients with hypertension and end-organ damage such as renal failure or younger patients with hypertension and dyslipidemias whose short-term risk may be low also can benefit from medical therapy directed toward a single risk factor. Because physicians deal with the whole patient and therefore every aspect of their risk of CVD, the principle of total risk assessment and management is consonant with the practice of medicine.

Coronary or CVD risk prediction should be based on a prospective population cohort study undertaken in the population to which the risk score is to be applied. This is because total risk of CVD may differ from one country to another, and the contribution of individual risk factors may also differ to some extent from one part of the world to the other. The published examples of coronary or CVD predictions include the Systematic Coronary Risk Evaluation. Project in liaison with the Third Joint European Societies' risk charts the New Zealand cardiovascular risk assessment and management chart which provides estimates of both CVD risk and the likely benefit of therapy to lower blood pressure or lipids; the Sheffield; the Joint British Societies' coronary risk prediction chart and associated software program; and the ATP III 10-year Risk Estimates for men and women using Framingham Point Scores, which are also available as a computer program. Most of these risk tools are based on the Framingham function. In addition, computer software programs are available based on the PROCAM study of men in Germany, PRECARD from a prospective cohort study of Danish men and women, and the European Society's HeartScore.

First, when we use the European Society of Cardiology's SCORE charts as an example, an individual's short-term risk of developing a CVD event (myocardial infarction or

stroke) over the next 10 years is found by locating the appropriate box in the chart based on the knowledge of age, gender, smoking status, systolic blood pressure, and total cholesterol level. The New Zealand chart estimates CVD rather than CHD, but the risk is over a shorter period, 5 years rather than 10. Systolic and diastolic pressure are both used, as well as the ratio of total to HDL cholesterol. The total cholesterol/HDL cholesterol ratio improves coronary risk prediction, particularly for women and for those in the middle range of cholesterol. The Framingham tables produce a numerical score that also corresponds to a short-term 10-year CHD risk (myocardial infarction and CHD death). Although all of these charts, tables, and computer programs estimate CHD or CVD risk for an individual, it must be emphasized that some individuals will be at higher risk than is evident from these calculations. Patients with clinically established CHD, other atherosclerotic disease, and diabetes; patients with hypertension associated with end-organ damage or familial dyslipidemias; patients with a family history of premature CVD; and those with low HDL cholesterol or raised triglyceride levels also may be at higher risk than indicated by the charts. Use of the Framingham risk function has certain limitations. Although it depends on the population, it or any other algorithm that is derived from a different region may not accurately predict total risk in another population. Nevertheless, coronary risk charts or computer programs can have several useful functions: An individual's total risk of developing a CHD or CVD event Second, relative risk can readily be estimated by comparing the risk in one cell Finally, the chart can be used to illustrate the effect of changing from one risk category to another. with any other in the same age group or with a table of average or low risk. over a defined time period can be read from a chart without any calculations.

Although young people are generally at lower risk, this will rise steadily as age increases. In the European recommendations, short-term risk estimates for clinical decisions in young adults and subjects in early middle age are made to project risk to age 60 years. For example, if the projected risk to age 60 years places a person in the high-risk category, this person can be treated accordingly with more intensive monitoring and earlier intervention. In this way, individuals with low CVD risk today, but who will become high

risk in the long term unless there is lifestyle and, where appropriate, therapeutic intervention, can also be identified and treated earlier.

The concept of total CVD risk also challenges the traditional classification of prevention into tertiary, secondary, and primary. Most patients with established CVD have developed symptomatic disease because they are at high risk, and the management of these multiple risk factors will over the longer term determine their risk of recurrent disease. Healthy individuals at high risk are usually no different (and many will already have asymptomatic atherosclerosis) from those who have declared their disease; all are at high risk of developing and dying from a CVD event. Thus, prevention of CVD at a population and individual level should be considered as a continuum from low to high risk: those at highest risk are patients with clinically manifest CVD, followed by individuals without known CVD at different levels of risk from high to low. The risk for an individual within a population is not just a function of their absolute ranking in relation to others but on the overall risk of the population in which they live. A "low-risk" individual in a high-risk population may actually be at higher total CVD risk than a "high-risk" individual in a low-risk population. The risk of an individual should always be judged in the context of the CVD risk of the population as a whole. An assessment of the determinants of total CVD risk should be a major determinant of priority setting for CVD prevention and management policy at both the clinical and population level, and guideline recommendations should emphasize interventions on all CVD risk factors rather than on single risk factors.

People who present with symptoms or history of atherosclerotic vascular disease (AVD), ie, CHD, stroke, or peripheral arterial disease, are at high risk of recurrent nonfatal and fatal cardiovascular events. Although the initial prognosis of these patients is determined by the extent of tissue damage to heart or brain, the longer-term prospects are strongly influenced by the extent of their atherosclerotic process, lifestyle, and other risk factors responsible for expression of atherosclerotic disease. CHD dominates the clinical presentation of atherosclerosis and accounts for a large majority of CVD patients. Of those with other manifestations of atherosclerosis in the form of stroke or peripheral

arterial disease, many will also have CHD, which is a frequent cause of death. Population-based autopsy studies have shown a strong correlation between the severity of atherosclerosis in one arterial territory and involvement of other arterial beds. Therefore, the prevention of atherosclerosis and its complications is the same regardless of which arterial territory becomes symptomatic. For practical purposes, no distinction needs to be made between those presenting with CHD and other forms of atherosclerosis in terms of lifestyle intervention and risk factor management for blood pressure, lipids, and hyperglycemia. However, specific drug therapies may differ according to the clinical expression of atherosclerotic disease and its complications (eg, preference for β -blockers or angiotensin-converting enzyme [ACE] inhibitors for blood pressure control in CHD patients).

CHD is the most common clinical manifestation of atherosclerosis. Sudden cardiac death in the community is often the first manifestation of CHD and is the terminal event in more than half of CHD patients. Acute myocardial infarction and unstable angina account for approximately one third of all cases, whereas exertional angina is the most common clinical manifestation of this disease, accounting for more than one half of all cases presenting in the community. Because the majority of individuals with CHD survive their first symptomatic presentation, the potential to reduce the risk of recurrent events and death is considerable. Surveys of contemporary clinical practice around the world, however, show that lifestyle and risk factor management, including the use of prophylactic medical therapies, falls far short of evidence-based national guidelines on CVD prevention. To further reduce the risk of recurrent CHD events and death, the standards of preventive care must be raised.

Patients with atherosclerosis of the carotid, vertebral, and cerebral arterial circulations can present with transient episodes of cerebral ischemia (transient ischemic attack) or a full stroke (either thrombotic or embolic), which can leave them temporarily or permanently disabled. Prevention of hemorrhagic stroke is not included here because the pathology is not usually atherosclerosis; however, because of its association with hypertension as a modifiable risk factor, it must be considered to benefit from medical treatment. The risk

of recurrent cerebrovascular disease is determined by multiple risk factors, particularly hypertension. In addition, patients with cerebrovascular disease due to thrombosis usually have CHD as well. Therefore, their risk factors should be managed on the assumption that they have CHD in order to broadly reduce their risk of CVD events. Although it is beyond the scope of this discussion, embolic stroke associated with atrial fibrillation deserves attention as a major preventable entity.

Atherosclerosis of the peripheral arteries usually presents clinically with aneurysmal dilatation of the aorta, aortic dissection, and, most commonly, progressive ischemia ("intermittent claudication") of the lower limbs. Although an aortic aneurysm or dissection can be life threatening, atherosclerosis of the lower limb arteries is usually not, although patients can develop critical ischemia of the foot requiring amputation. However, almost all patients with atherosclerosis of the peripheral arteries also have CHD and therefore are at increased risk of a nonfatal coronary event or coronary death. The risk factors for atherosclerosis of the peripheral arteries are the same as those for the coronary circulation, although smoking is a particularly powerful risk factor for atherosclerosis of the aorta and lower limbs. Therefore, patients with peripheral atherosclerotic disease should also have their risk factors managed in the same way as those with CHD to reduce their risk of CVD complications. In addition, peripheral arterial disease is a powerful predictor of major coronary events. Therefore, the presence of peripheral atherosclerotic disease places a person in a high-risk category. Patients with CHD or other atherosclerotic disease are considered to be at high risk. There is no practical utility in further quantifying their total risk of a future CVD event because risk stratification will not alter recommendations for target goals of risk factor therapy.

The medical technology to detect asymptomatic atherosclerotic disease is already available for coronary atherosclerosis, carotid/vertebral atherosclerosis, and peripheral arterial disease. This technology has revealed the ubiquity of AVD, as understood by pathologists many years ago. Emerging methodologies can aid in the detection of AVD before clinical symptoms. The cost of this technology emphasizes the benefits and importance of primordial preventive strategies, as discussed elsewhere in this article. The

objective of detecting asymptomatic AVD in apparently healthy individuals is to intervene in order to slow disease progression, if possible to induce regression, and to reduce the risk of thrombotic complications, thereby reducing the risk of a first nonfatal or fatal coronary or other atherosclerotic disease event.

The rising prevalence of CVD worldwide is in part a reflection of a rising prevalence of CVD risk factors in many nations. Among these are increasing prevalence rates of cigarette smoking, hypertension, lipid disorders, diabetes, and older people. Changing life habits across broad populations is responsible for the emergence of most of these risk factors. Cultural changes are such that multiple risk factors in individuals are common. To stem the rising tide of CVD worldwide, it will be necessary to attack the causes of CVD risk factors. These underlying causes include increasing obesity, decreasing physical activity, and changes in the composition of the diet. To modify these underlying risk factors, CVD specialists must team with primary healthcare providers, epidemiologists, and public health officials to modify behavioral characteristics of individuals. CVD specialists can assist in the identification of problem areas and serve as a catalyst for change. It is logical that preventive efforts in whole populations should be broad based and directed toward reducing all the risk factors simultaneously.

Nutrition, Population Sector Programme (HNPS) has identified three NCDs-cancer, cardiovascular diseases and diabetes mellitus-as major public health problems. Therefore surveillance of these diseases should be started to assist in formulating country policies and programmes. They have a few common risk factors for which Bangladesh does not have representative data to be addressed for primary prevention

National Institute of Cancer Research and Hospital (NIC), Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), and National Heart Foundation Hospital and Research Institute (NHF) are playing important role in providing services to the patients and education of professionals on cardiovascular diseases, cancer and diabetes mellitus. Recently, Zia Heart Foundation Hospital and Research Center (ZHF), Dinajpur, started providing services to

cardiovascular patients of northern outlying districts of Bangladesh where such services had been lacking. Their capacity needs to be strengthened to ensure quality management, and surveillance (Non-communicable Disease & Mental Health).

Non modifiable Risk factors

- Age
- Sex
- Family History

Modifiable factors

- Cigarette smoking
- High blood pressure
- High blood cholesterol
- Physical inactivity
- Obesity
- Overweight
- Diabetes mellitus.
- Cholesterol Relationship (Cholesterol Abnormalities & Diabetes)

Cardiovascular Risk Factors

Both cross-sectional and prospective studies have linked obesity to hypertension. Recent estimates suggest that, after adjustment for other risk factors (such as age, BMI, degree of weight cycling, physical activity, smoking, and alcohol consumption), each kilogram increase in body weight increases the risk for developing hypertension by 4.4%. In a nationally representative sample the prevalence of elevated blood pressure dramatically increased with increasing weight, particularly among individuals aged less than 55 years. Similarly, among postmenopausal women, the risk of developing high blood pressure doubled with either a high BMI or high WHR , suggesting that both general and abdominal obesity are important risk factors. These observational studies are corroborated by clinical intervention trials, which have consistently found that weight loss effectively lowers blood pressure. In the Framingham study , a weight loss of 6.8 kg

or more led to a 28% reduction in the risk of hypertension (RR = 0.72; 95% CI: 0.49 – 1.05) for middle-aged adults and a 37% reduction for older adults (RR = 0.63; 95% CI: 0.42 – 0.95). The study also reported that sustained weight loss over 4 years resulted in a 22% reduction in hypertension risk among middle-aged adults (RR = 0.78; 95% CI: 0.60 – 1.03) and a 26% reduction (RR = 0.74; 95% CI: 0.56 – 0.97) in older adults. Overall, it appears that the prevalence of hypertension increases even with relatively small increases in body weight. Furthermore, in hypertensive subjects, overweight is associated with cardiovascular abnormalities such as increased progression of left ventricular hypertrophy. Another study showed that in addition to a reduction in blood pressure, weight loss and exercise may induce favorable changes in left ventricular structure related to cardiovascular events. Given that high blood pressure represents one of the most common modifiable risk factors for CVD risk, obesity-related hypertension could be reversed with weight loss, thus reducing CVD risk at the population level.

b. Dyslipidemia

Dyslipidemia is characterized by elevated total cholesterol and triglyceride levels, normal to elevated LDL cholesterol, reduced HDL cholesterol and raised low-density lipoprotein apo B. Several observational studies have observed associations between body weight and plasma lipoproteins. In the Framingham Heart Study, weight gain over a 26-year follow-up period was associated with adverse lipid profiles and weight loss was associated with improvements in cholesterol. Other studies have found that changes in body weight are associated with changes in lipid concentrations. Findings from the Framingham Offspring Cohort provide further evidence that BMI is significantly and linearly associated with total cholesterol, LDL cholesterol and triglyceride concentrations, and is inversely associated with HDL cholesterol in nonsmoking men and woman; the latter observation is consistent with other studies. In contrast to weight gain, weight loss and exercise may result in lower LDL cholesterol and triglyceride levels, decreases in the total cholesterol to HDL cholesterol ratio, and increases in HDL cholesterol levels. Furthermore, cohort, case-control and intervention studies have found that a high LDL to HDL cholesterol ratio, as well as high triglyceride to HDL cholesterol ratio and small LDL-size in the presence of hypertriglyceridemia, are

associated with the highest CVD risk. This unfavorable lipid profile is commonly found in obese adults. Higher fat intake of saturated and trans fats were associated with increased risk of coronary heart disease (CHD), Elevated cholesterol in the blood is due to abnormalities in the levels of lipoproteins,

c. Hyperinsulinemia

Insulin resistance is a condition characterized by increased insulin production and impaired glucose tolerance, and is probably the most frequent abnormality seen in association with central or visceral abdominal adiposity. Insulin resistance may underlie a number of other metabolic disorders including hypertension, hyperglycemia and impaired glucose tolerance, hypertriglyceridemia, and hypercholesterolemia. This clustering of risk factors has been termed insulin resistance syndrome, syndrome X or metabolic syndrome. It is worth noting, however, that although each individual risk factor conveys only a small increase in CVD risk, the overall impact on CVD risk is substantial due to the coincidence of these risk factors.

Increasing central obesity has been independently associated with insulin resistance, hyperinsulinemia and a progressive increase in insulin and glucose concentration in response to an oral glucose tolerance test. Some have proposed that central obesity promotes insulin resistance through increased levels of free fatty acids which causes the muscle tissue to utilize more fat fuel thereby impairing the insulin-mediated uptake and utilization of glucose. The accumulation of free fatty acids is also associated with oxidative stress and the impairment of micro vascular functions. Central obesity may also induce insulin resistance through release of inflammatory cytokines such as IL-6, which in turn impair insulin action in diverse tissues. Alternatively, insulin resistance in obesity may be attributed to both a decrease in insulin receptors and intracellular post-receptor defects in insulin action. Furthermore, abnormal secretion of several adipocyte hormones such as leptin, adiponectin and ghrelin, which are primarily regulated by insulin-induced changes of adiposity metabolism, may be potential targets for managing obesity and insulin resistance.

Insulin resistance has been associated with weight gain in some, but not all observational studies . In young adults, weight gain over a 7-year follow-up period was positively associated with concentrations of fasting glucose and insulin . Wilson and colleagues found that weight gain over 16 years predicted development of features of the insulin resistance syndrome. However, it has been proposed that insulin resistance is an adaptation for maintaining stable weight, such that the oxidation of fat tends to be favored over its storage and over the oxidation of glucose. Several observational studies of different ethnic groups support this hypothesis that higher fasting insulin is associated with lower weight gain . Interestingly, conflicting data have been found in children indicating that hyperinsulinemia and insulin resistance may favor weight gain

In women, the body fat distribution pattern often changes with progression through menopause. Greater increases in waist circumferences and WHR in postmenopausal women compared to women who remain premenopausal may contribute to increase risk of chronic diseases, such as type 2 DM. Van Pelt et al in a large cohort of healthy postmenopausal women found that waist circumference was significantly associated with hyperinsulinemia and elevated triglyceride concentrations among women with a normal range of BMI (24-28 kg/m²). Furthermore, the combination of insulin resistance and the accumulation of visceral adipose tissue in the abdominal compartment contribute to the most unfavorable metabolic risk profiles in post-menopausal women.

Several studies have found a strong association between obesity and CHD risk . again, obesity is strongly linked to several cardiovascular risk factors including diabetes, hypertension, and dyslipidemia. These risk factors could represent intermediate steps in the causal pathway between obesity and CHD risk; therefore, considerable debate exists over whether adjustment for these risk factors is desirable or represents “overcontrol” and introduces, rather than controls, bias. Most observational studies that did not control for risk factors reported associations between BMI and CHD. In the Nurses’ Health Study, the relative risk (RR) of CHD was over three times higher among women with BMI’s of ≥ 29 kg/m² compared to those with BMI’s of less than 21 kg/m², after adjustment for age, smoking, menopausal status, hormonal use and parental history of CHD. After excluding

women with self-reported diabetes or hypertension, the magnitude of the risk of CHD between the same extreme categories of BMI was attenuated, but remained moderate (RR 3.6 versus 2.6). A recent study from an English cohort found that almost 60% of the 10-year coronary risk in this population was attributable to BMI >25 kg/m². Although this study did not control for rtension and dyslipidemia, they observed that systolic blood pressure and total cholesterol increased sharply with increasing BMI among men with WHR less than 0.95 and was high at all levels of BMI among men with WHR exceeding 0.95. Similarly, observation studies that controlled for one or more coronary risk factors in the analyses found that while BMI remained independently associated with CHD risk, associations tended to be attenuated. In 1998, the American Heart Association (AHA) concluded that obesity was a independent coronary heart disease risk factor. Other studies support the AHA's statement that obesity increases the risk of CHD events, although other CHD risk factors such as hypertension, dyslipidemia and diabetes might explain some, but not all of the association between obesity and CHD . Furthermore, a large international, case-control study reported that in all regions of the world, for both men and women, abdominal obesity increased the population attributable risk of myocardial infarction, (one of the most common CHD events) to 80.2%, from 75.8% attributed from hypertension, diabetes and dyslipidemia. At the population level, obesity appears to be a well-defined and consistent hazard for CHD. contrast to the findings of studies of young and middle-aged adults a direct relation between BMI and CHD risk has not consistently been found among older age groups (>60 years) Rimm and colleagues found that among men <65 years of age, the risk of CHD increased threefold (RR 3.44; 1.67-7.09) for men with a BMI greater than 33 compared with lean men (BMI <23.0), yet in older men the risk was substantially lower (1.26; 95% CI 0.37- between the same extreme categories. Other prospective studies have reported a lack of association between BMI and coronary disease among older populations. These age-related differences in obesity and CHD risk may reflect early onset coronary artery disease incidence among overweight persons, changes in the relative proportion of fat free and lean body mass with age or weight loss due to a sub-clinical disease.

A growing body of evidence indicates that abdominal visceral adiposity may have more significant health consequences than BMI on CVD incidence and mortality. While the exact mechanism is unknown, it is postulated that excess abdominal adiposity may be more predictive of CVD risk than BMI because of its stronger association with other cardiovascular risk factors. Furthermore, a positive association between abdominal visceral fat and pathological changes of the coronary arteries indicate that the process of coronary atherosclerosis would have occurred even before individuals are clinically diagnosed for CHD. Rexrode and colleagues found that after adjustment for BMI and other cardiac risk factors, women with a WHR greater than 0.88 were more than 3 times as likely to develop CHD during an 8-year follow-up compared to women with a WHR of less than 0.72. In this same cohort, waist circumference was also significantly associated with increased risk of CHD, even after controlling for BMI. Another large population-based cohort found that WHR was positively associated with the incidence of CHD in both younger and older women while BMI was related to CHD only among women aged 55 years or under. Several other longitudinal studies also observed associations between abdominal obesity and CHD among middle-aged and older women. Using a similar methodology in a large prospective study of men, Rimm et al found that in men <65 years of age, BMI was strong predictor of CHD, whereas after age 65, WHR was a better predictor of risk among men. In contrast, another prospective study from a different cohort found that abdominal obesity was an independent risk factor for CHD in middle-aged men. Interestingly, Rexrode et al found a modest relationship between abdominal obesity, as measured by either WHR or waist circumference, and risk of CHD both in middle-aged and older men. These associations were reduced substantially when BMI was accounted for. In a case-control study, Sonmez et al did not observe WHR to be statistically different between the age groups of male-CHD cases. As also shown in the WHO MONICA study the sensitivity and specificity of detecting abdominal adiposity may be population-specific. Furthermore, measures of abdominal obesity such as waist circumference may complement BMI assessment in cardiovascular risk assessment. Specific thresholds of waist circumference within BMI categories may be required to identify those at increased risk of CHD.

Weight gain, even in modest amounts (5-7.9 kg), has been associated with increased risk of CVD. In fact, Willet and colleagues estimated that for every kilogram increase in body weight, the risk of developing CHD among women increased 3.1% (122). Furthermore, fluctuations in body weight have also been associated with CHD risk. In the Framingham Heart Study, individuals who fluctuated in body weight, as reflected in large relative standard error of the regression coefficient, experienced more CHD events than individuals who maintained a normal weight. In the Nurses Health Study, weight gain from age 18 to age 55 was significantly associated with future risk of CHD after adjustment for BMI. Rapid weight gain in childhood has been shown to be associated with CHD later in life. Individuals with a low weight at birth who gain weight rapidly after 1 year of age are at an elevated risk for developing CHD in later in life.

d. Obesity, body fat distribution and risk of stroke

In contrast to the epidemiological studies linking obesity and CHD risk, fewer studies have examined the association between obesity and stroke incidence and mortality. Current evidence for an association between general obesity and risk of stroke is conflicting, with some studies suggesting that a higher BMI is associated with stroke incidence while others report no association. In the Nurses' Health Study, the risk of ischemic stroke was directly related to BMI, with women whose BMI was greater than 32 kg/m² at 2.4 (RR 2.4; 95% CI greater risk of ischemic stroke compared to those with a BMI of <21 kg/m² (15). In contrast, the risk of hemorrhagic stroke was inversely related to obesity in the same cohort, with highest risk among the leanest subjects. Using a similar methodology in a large prospective study of men, Walker et al. reported no association between BMI and incidence of total stroke, of which approximately 70% were ischemic strokes. Interestingly, recent studies demonstrate a significant association between BMI and risk of stroke among men especially for total stroke and ischemic stroke. It has been suggested that abdominal obesity, as measured by WHR, is a better predictor of stroke than BMI. Men with a WHR of >0.98 were twice as likely to suffer a stroke compared to men with a WHR ≤0.89. In women, these associations seem less consistent. Significant associations between WHR and stroke incidence have been reported in other observational studies as well.

Prospective studies indicate that all of the major cardiovascular risk factors—cigarette smoking, hypertension, and high serum cholesterol—continue to act as independent contributors to CVD in patients with diabetes. As already mentioned, clustering of metabolic risk factors, called the metabolic syndrome, occurs commonly in type 2 diabetes. The onset of hyperglycemia in patients with the metabolic syndrome appears to accelerate atherogenesis, possibly by enhanced formation of glycosylated proteins and advanced glycation products and/or by increasing endothelial dysfunction. These direct consequences of hyperglycemia probably contribute to the microvascular disease underlying nephropathy and retinopathy, and they may promote macrovascular disease as well.

Predisposing Risk Factors

Diabetes mellitus

Several predisposing factors simultaneously affect the development of CVD and diabetes mellitus. Among these concomitant factors are obesity, physical inactivity, heredity, sex, and advancing age. The mechanisms whereby they predispose to chronic diseases are complex and often overlapping. To some extent, these predisposing factors exacerbate the major risk factors: dyslipidemia, hypertension, and glucose tolerance; and they may cause CVD and diabetes mellitus through other pathways as well. To a large extent, both CVD and diabetes must be prevented through control of the predisposing risk factors. Modification of life habits is at the heart of the public health strategy for prevention of CVD and diabetes mellitus. High priorities are the prevention (or treatment) of obesity and promotion of physical activity. Drug therapy nonetheless may be required to control the metabolic risk factors, particularly when they arise from genetic aberration and aging. Effective drugs are currently available for treatment of hypertension and dyslipidemia. Hypoglycemic agents also are available for treatment of type 2 diabetes, but new pharmacological strategies are under investigation for more effective treatment and prevention.

Insulin Resistance and the Metabolic Syndrome

Most patients with type 2 diabetes have insulin resistance. Indeed, insulin resistance seems to predispose to both CVD and diabetes.⁶⁰ Research suggests that insulin resistance is a multisystem disorder that induces multiple metabolic alterations. Factors that contribute to insulin resistance are genetics obesity-physical inactivity-and advancing age. Patients with insulin resistance often have abdominal obesity. Metabolic risk factors that occur commonly in patients with insulin resistance are atherogenic dyslipidemia, hypertension, glucose intolerance, and a prothrombotic state. Each of these risk factors can be reviewed briefly.

Atherogenic Dyslipidemia

Atherogenic dyslipidemia is characterized by 3 lipoprotein abnormalities: elevated very-low-density lipoproteins (VLDL), small LDL particles, and low high-density-lipoprotein (HDL) cholesterol (the lipid triad). The lipid triad occurs frequently in patients with premature CHD and appears to be an atherogenic lipoprotein phenotype independent of elevated LDL cholesterol. Most patients with atherogenic dyslipidemia are insulin resistant.^{69 70 71} Atherogenic dyslipidemia in diabetic patients often is called diabetic dyslipidemia. Many patients with atherogenic dyslipidemia also have an elevated serum total apolipoprotein B. Growing evidence suggests that all of the components of the lipid triad are independently atherogenic. Together they represent a set of lipoprotein abnormalities besides elevated LDL cholesterol that promote atherosclerosis.

Hypertension

Hypertension, also known as high blood pressure, is a persistent elevation in blood pressure that taxes the heart and can, over time, cause damage to organs such as the kidneys, brain, eyes, and heart. Blood pressure (BP) is the amount of force blood exerts on the walls of the arteries and veins. BP depends on the force and rate of the contraction of the heart as it pumps oxygenated blood from the left ventricle (compartment) of the heart into the arteries and the resistance to that flow. The amount of resistance depends on the elasticity and diameter of the blood vessels and how much blood is flowing through them.

Blood pressure is dynamic; it rises and falls depending on a person's level of activity, time of day, and physical and emotional stresses. In healthy people, it is largely controlled by the autonomic nervous system but is also regulated by hormones, including:

- Angiotensin II — produced by the kidneys, it causes increased resistance in blood vessels.
- Aldosterone — produced by the adrenal glands in response to angiotensin II, it affects the amount of sodium, potassium, and fluids excreted by the kidneys.
- Catecholamines — such as epinephrine, also called adrenaline, produced by the adrenal glands in response to stress and increases heart rate and resistance in blood vessels.

When one or more of the regulating factors is not able to respond appropriately to the demands of the body, then the pressure of the blood may become persistently increased.

Elevated Plasma Glucose

For several years after onset of insulin resistance, fasting and postprandial glucose levels typically are normal. During this period, pancreatic β -cells are able to increase insulin secretion in response to insulin resistance and thereby maintain normal plasma glucose levels. In some people, however, insulin secretion declines with aging, and elevated glucose concentrations appear. The first abnormality in plasma glucose in patients with insulin resistance is IFG (or impaired glucose tolerance). The presence of IFG usually accompanies long-standing insulin resistance. It is currently estimated that 13.4 million adults, 7.0% of the US population, have IFG. Many prospective studies show that IFG (or impaired glucose tolerance) is a risk factor for CVD; the degree of independence as a risk factor, however, is uncertain, because IFG commonly coexists with other components of the metabolic syndrome. A patient with IFG nonetheless must be considered at risk for both CVD and type 2 diabetes. As already indicated, once categorical hyperglycemia develops, it counts as an independent risk factor for CVD.

Other Risk factors

- Homocysteine
- C-RP

- Lipoprotein
- Stress
- Alcohol
- Hyper-Homocysteinaemia

Homocysteine (Hcy), a sulfurcontaining amino acid, is emerging as an important risk factor for atherosclerosis in patients with end stage renal disease, we examined the significance of serum Hcy levels in diabetic patients on HD (1999)

Total plasma homocysteine levels. Homocysteine is an amino acid and is used as a building block for proteins. Homocysteine is naturally occurring molecule in the body and it is required in several reactions that occur within the cells, that comprised the human body. Formation of cysteine and methionine, which can be further used by the body. Recent studies have provided evidence that increased levels of homocysteine in blood plasma can be related to increased risk in coronary heart disease, stroke, and peripheral vascular disease. here is also information available that suggests homocysteine may not be a risk factor for CVD. he previously cited studies indicate that homocysteine levels may be lowered with folic acid, vitamin B12, and/or vitamin B6. The studies also indicate that clinical trials have not been completed to show whether adjusting homocysteine levels with these common vitamins can reduce the risk of cardiovascular disease. Applying the American Heart Association deaths figures and costs, a one percent reduction in deaths from CVD may save approximately 9600 lives annually and reduce the annual costs from CVD by as much as \$2.59 billion. Although a clinical trial to study the effects on incidence of CVD mortality and morbidity by reducing the total plasma homocysteine levels with vitamin supplements would be costly, there is potential for large savings if clarification is provided to the outstanding questions that now exist.

- Cardiovascular disease is the major cause of death in diabetes, accounting for some 50% of all diabetes fatalities, and much disability.
- On average, people with type 2 diabetes will die 5-10 years before people without diabetes and most of this excess mortality is due to CVD diseases.

- Stroke occurs twice as often in people with diabetes and hypertension as in those with hypertension.

High plasma homocysteine level has been associated with increased risk for coronary heart disease (CHD) events in nondiabetic individuals, especially in those with previously diagnosed CHD. In persons with type 2 diabetes mellitus, the association between homocysteine level and cardiovascular disease may be stronger than that in nondiabetic individuals, but no large prospective studies have examined the relationship between homocysteine level and CHD mortality in persons with type 2 diabetes.

Vascular disease is a major cause of morbidity and mortality in end stage renal failure patients and cannot be explained entirely by the prevalence of traditional risk factors for atherosclerosis. A high plasma homocysteine concentration, which is a risk factor for vascular disease is found in patients with end stage renal disease. The exact cause for the hyperhomocysteinaemia seen in these patients is unknown, although metabolism of homocysteine. High homocysteine concentrations may also be attributable to a deficiency of folate, vitamin B6 or vitamin B12 although, because of supplementation, these vitamins may be present in high concentrations in renal patients. The occurrence of hyperhomocysteinaemia despite high plasma vitamin concentration could be due to altered metabolism or inhibition of intracellular vitamin activity. A number of studies have now established hyperhomocysteinaemia to be an independent risk factor for atherosclerosis in patients with vitamin B12 or vitamin B6 with end-stage renal disease. Plasma homocysteine concentrations can be reduced by administration of folic acid either alone or combined.

At elevated levels, homocysteine can block production of nitric oxide in the cells of the blood vessel walls, making the vessels less pliable and allowing plaque to build up. Several different mechanisms have been proposed to explain the apparent association between homocysteine and atherosclerotic vascular disease.

Possible Mechanisms of Homocysteine-Mediated Atherogenesis:

1. Endothelial dysfunction
2. Endothelial cell injury
3. Promotion of smooth muscle cell proliferation
4. Enhanced platelet aggregation
5. Increased binding of lipoprotein(a) to fibrin
6. Generation of free radical species
7. Stimulation of low-density lipoprotein oxidation
8. Procoagulant effects

Homocysteine is a naturally occurring amino acid, derived from methionine and produced in small amounts by the human body. It is metabolized by transsulfuration (which depends on vitamin B6) and remethylation (which relies on folate [folic acid] and vitamin B12). According to a 1999 science advisory from the American Heart Association (AHA) Nutrition Committee, plasma concentrations of homocysteine between 5 and 15 $\mu\text{mol/L}$ are considered normal. Elevated homocysteine levels are referred to as hyperhomocysteinemia -- moderate, between 16 and 30 $\mu\text{mol/L}$; intermediate, between 31 and 100 $\mu\text{mol/L}$; and severe, higher than 100 $\mu\text{mol/L}$. The authors of the 1999 AHA science advisory consider a basal homocysteine level below 10 $\mu\text{mol/L}$ "a reasonable therapeutic goal for subjects at increased risk." For significant reduction of serum homocysteine levels (and, presumably, "favorable impact on CHD rates"),

Homocysteine, an intermediate in protein metabolism, is involved in conversion of the amino acid methionine to cysteine or in remethylation to form methionine (Figure 1).

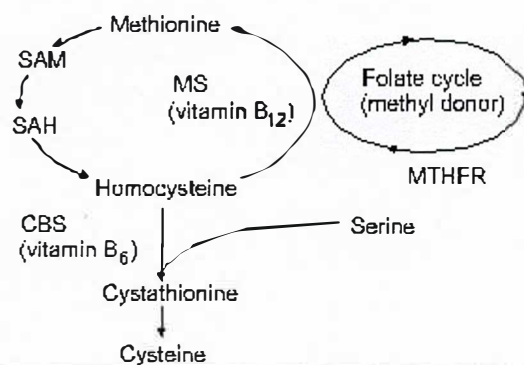


FIGURE 1. Homocysteine metabolism. An intermediate in protein metabolism, homocysteine is involved in conversion of the amino acid methionine to cysteine or in remethylation to form methionine.

(SAM=S-adenosyl methionine; SAH=S-adenosyl homocysteine; MS=methionine synthase; MTHFR=methylene tetrahydrofolate reductase; CBS=cystathionine b-synthase).

Increases in homocysteine concentrations are often the result of decreased activity of key enzymes involved in either of these metabolic pathways. The most common inherited form of hyperhomocysteinemia results from an alteration in the gene encoding the enzyme methylene tetrahydrofolate reductase. A mutation in the methylene tetrahydrofolate reductase gene leading to mild to moderate hyperhomocysteinemia has been found in 15 percent of patients with premature cerebrovascular disease.

Less often, the cause of hyperhomocysteinemia is heterozygous cystathionine b-synthase deficiency. Homocystinuria is a rare, but severe homozygous form of cystathionine b-synthase deficiency in which total homocysteine concentrations generally exceed 100 $\mu\text{mol per L}$ but can reach 500 $\mu\text{mol per L}$ if the disorder is untreated. Individuals with this inherited disorder are known to have very premature coronary artery disease.

Hyperhomocysteinemia can be acquired as the result of dietary deficiencies of folate, vitamin B12 and/or vitamin B6. These nutrients are necessary cofactors for the optimal function of methylene tetrahydrofolate reductase and cystathionine b-synthase. Deficiencies in the absorption or transport of these vitamins can also cause hyperhomocysteinemia.

Causes of Hyperhomocysteinemia

- Acquired causes
- Vitamin deficiencies
- Folic acid

- Vitamin B
- Vitamin B12
- Chronic diseases
- Chronic renal failure
- Hypothyroidism
- Psoriasis
- Malignancies (including acute lymphoblastic leukemia)
- Medications
- Anticonvulsants
- Methotrexate
- Nitrous oxide
- Inherited causes
- Cystathionine b-synthase deficiency
- Methylene tetrahydrofolate reductase deficiency or defect
- Methionine synthase defect
- Vitamin B12 transport defect
- Vitamin B12 coenzyme synthesis defect

There is adequate evidence that folic acid supplementation can decrease the elevated homocysteine levels in the blood. Folic acid is thought to protect against heart disease because it breaks down homocysteine and allows it to be cleared from the blood stream. Rimm advocates folate intake of at least 400 µg/d. To this, the science advisory authors add, patients should be encouraged to take 2 mg/d of vitamin B6 and 6 µg/d of vitamin B12 in vitamin-fortified foods and/or vitamin supplements. Older people often malabsorb food-bound B12, and thus it is important to assess for vitamin B12 deficiency in elderly patients. Treatment with folic acid can obscure unsuspected vitamin B12 deficiency and allow progression of neurologic complications.

The optimal total dose of folic acid appears to be within the range of 650 to 1,000 µg per day. In the United States, the average folic acid intake is approximately 200 µg per

day. Although many recommend getting the required folic acid from natural or even fortified foods, this has been shown to increase the folic acid intake to less than 400 μg per day. It is currently recommended that people increase their intake of folate-rich foods and that they consider adding a daily multivitamin containing 400 μg of folic acid. This recommendation is especially important for pregnant women and for persons considered to be at high risk for cardiovascular disease.

In fact, many authorities feel that high-risk patients with homocysteine levels above 12 $\mu\text{mol per L}$ should take a daily multivitamin containing 400 μg of folic acid plus an additional 800 μg of folic acid per day. The homocysteine concentration should be rechecked in eight weeks to assess the response to therapy. If the homocysteine level returns to normal, the supplemental folic acid may be discontinued. In such cases, the multivitamin should be continued, and the homocysteine concentration should be rechecked in approximately eight to 12 weeks to ensure that the level is stable. If the homocysteine level is not less than 12 $\mu\text{mol per L}$ after eight weeks of supplementation, the folic acid supplementation can be increased to 2 mg per day for an additional eight weeks, with repeat homocysteine testing performed at the end of treatment. Persistently elevated homocysteine levels warrant a careful assessment of patient compliance or testing for other possible causes of hyperhomocysteinemia.

Hyperhomocysteinemia confers an independent increased risk of atherosclerosis in end-stage renal disease and is closely linked to plasma folate and pyridoxine concentrations. A high level of total plasma homocysteine is a risk factor for atherosclerosis, which is an important cause of death in renal failure. We evaluated the role of this as a risk factor for vascular complications of end-stage renal disease. A high total plasma homocysteine concentration is an independent risk factor for atherosclerotic complications of end-stage renal disease. Such patients may benefit from higher doses of B vitamins than those currently recommended. Stress in type 2 diabetes: relation to diastolic dysfunction and hypertension.

Folic acid

Fortified foods such as breads and cereals are good dietary sources of folic acid. Good sources of folate are dark green leafy vegetables (such as asparagus and broccoli), Brewer's (nutritional) yeast, liver, orange juice, beets, dates, and avocados. Poor sources of folate are chicken, milk, most fruits and meats. Supplements generally contain folic acid but folate supplements (L-methyl folate) are now available, especially in prenatal vitamins. Green and black tea reduce the bioavailability of folic acid supplements..²⁰

Homocysteine is the product of demethylation of methionine. It is also an intermediate in the synthesis of L-cysteine from L-methionine (building blocks for proteins). When folic acid concentrations are inadequate, these reactions slow and homocysteine concentrations may accumulate in the bloodstream. Studies have linked high homocysteine levels to coronary heart disease, cerebral vascular disease (including strokes), and peripheral vascular disease.³ Studies have shown that folic acid, along with other B vitamins, lower homocysteine levels but these studies have not shown that lowering homocysteine improves disease outcome or prevents disease development.

A new study in British Medical Journal reviews the scientific evidence published in previous studies that supports the use of folic acid as a way of reducing the risk of heart disease and strokes. Lead author and heart expert David Wald and colleagues clarify existing debate over the relationship between elevated homocysteine levels, a risk factor for heart disease, and folic acid supplementation:

"Since folic acid reduces homocysteine concentrations, to an extent dependent on background folate levels, it follows that increasing folic acid consumption will reduce the risk of heart attack and stroke by an amount related to the homocysteine reduction achieved. We therefore take the view that the evidence is now sufficient to justify action on lowering homocysteine concentrations.

- Since homocysteine, one of the factors which may affect cardiovascular disease and cognitive function, increases with age, folic acid supplementation or multivitamins are recommended for the elderly.

- Increased blood homocysteine concentrations are associated with increased risk of cardiovascular disease incidence and mortality in the general population and with even greater risk in persons with diabetes mellitus. Hyperhomocysteinemia in macrovascular and microvascular diabetes complications could be of importance in their prevention through dietary (folic acid and vitamin B12 supplements) and pharmacological modifications of blood homocysteine concentrations.

Folic acid is one of the B vitamins and is also known as vitamin B9. It is found in various forms in common foods, especially green, leafy vegetables. Folic acid reduces homocysteine concentrations, to an extent dependent on background folate levels, it follows that increasing folic acid consumption will reduce the risk of heart attack and stroke by an amount related to the homocysteine reduction achieved. We therefore take the view that the evidence is now sufficient to justify action on lowering homocysteine concentrations."

Our observed inverse association between folate intake and risk of cerebral infarction is consistent with results from most previous prospective studies. Folate intake was statistically significantly inversely associated with the risk of ischemic stroke in the Health Professionals Follow-up Study and with total stroke in the First National Health and Nutrition Examination Survey Epidemiologic Follow-up Study . In addition, two prospective studies on serum or plasma folate found a statistically significant lower risk of stroke for individuals in the highest versus lowest category of blood folate . The Nurses' Health Study and the Bronx Longitudinal Aging Study (reported no association between folate intake or serum folate concentration, respectively, and risk of stroke. In the present study, the inverse association between folate intake and cerebral infarction appeared to be confined to men with no history of coronary heart disease at baseline. This may be due to the facts that men with coronary heart disease have higher serum homocysteine concentrations and that folate intake among those men may not be sufficient to protect against stroke. Prospective data on vitamin B₆ and vitamin B₁₂ in relation to risk of stroke are limited. In the Health Professionals Follow-up Study, no association was found between vitamin B₆ intake and stroke However, men in the highest

quintile of vitamin B₁₂ intake had a statistically no significant lower risk of ischemic stroke compared with men in the lowest quintile (relative risk = 0.73, 95 percent CI: 0.52, 1.03) . Serum or plasma vitamin B₁₂ concentrations were not related to risk of stroke in the Northern Sweden Health and Disease Cohort or the Bronx Longitudinal Aging Study . Hence, our null findings for vitamin B₆ and vitamin B₁₂ intakes are generally consistent with previous studies.

The absorption of vitamin B₁₂ is dependent on the intrinsic factor secreted by the healthy gastric mucosa. Intrinsic factor secretion is low from atrophic gastric mucosa resulting in negligible absorption of vitamin B₁₂ despite a normal dietary supply. Hence, a possible relation between vitamin B₁₂ intake and risk of stroke may be attenuated toward unity in subjects with atrophic gastritis. We therefore excluded from the analyses men with a low serum pepsinogen I level and thus atrophic gastritis but still found no association between vitamin B₁₂ intake and stroke risk. The lack of observed association between vitamin B₁₂ intake and the risk of stroke may not be surprising given that vitamin B₁₂ has a relatively small effect on homocysteine concentrations , and only a minor proportion of study participants had vitamin B₁₂ deficiency , being usually related to a problem of absorption (due to atrophic gastritis) rather than nutrition. We found, however, that folate intake was more strongly inversely related to cerebral infarction in men with a high vitamin B₁₂ intake. A meta-analysis of randomized trials estimated that folic acid supplementation could be expected to reduce homocysteine concentrations by 13–25 percent (depending on dose) and that vitamin B₁₂ produced 7 percent further reduction in homocysteine concentrations, whereas vitamin B₆ had no significant additional effect.

Quinlivan et al. investigated the relation between both folate and vitamin B₁₂ status and plasma homocysteine concentrations in two groups of healthy subjects pre- and postsupplementation with folic acid. They observed that, after supplementation, the usual dependency of homocysteine on folate diminished, and vitamin B₁₂ became the main determinant of homocysteine concentrations. Homocysteine is formed from methionine. Thus, increased intake of methionine could lead to an increase in blood homocysteine and consequently to an increased risk of stroke. In this study, we observed no significant

association between methionine intake and stroke. Our study has certain strengths and limitations that deserve mention. An advantage of this study is the large number of stroke cases, especially cerebral infarctions, which provided ample statistical power to detect associations. Furthermore, because no national folic acid fortification of cereal grain products has been initiated in Finland, folate fortification cannot have affected our results. The detailed data on multiple cardiovascular risk factors allowed adjustment for potential confounders, but there is still the possibility of residual or unmeasured confounding. Moreover, because of the observational design of our study, we cannot rule out the possibility that it may not be folate that is etiologically important but something else in high folate foods. Misclassification of dietary intake could have led to an underestimation of the associations of B vitamin and methionine intakes with risk of stroke in this study. Dietary intake was assessed at baseline only, which may have contributed to misclassification because of dietary changes during follow-up. Finally, our results may not be generalizable to nonsmokers, particularly as smokers have a higher risk of stroke and tend to have marginal folate status. In summary, our results in men suggest that a high dietary folate intake may reduce the risk of cerebral infarction, particularly among those with a high vitamin B₁₂ intake and among those with no history of coronary heart disease. Although these observational data do not prove a causal relation, they indicate that high consumption of folate-rich foods (e.g., whole grains, green leafy vegetables, oranges, and legumes) may play a role in the prevention of stroke.

In the late 1960s, McCully became the first to hypothesize that elevated plasma homocysteine concentrations could cause atherosclerosis. Many studies have shown that hyperhomocysteinemia may be an independent risk factor for thromboembolic disease. However, the results among studies and populations have not been consistent. Furthermore, the possible mechanisms of hyperhomocysteinemia in vascular diseases are controversial. Dietary folate, and vitamin B₆ and B₁₂ intake deficiency, may play a role in this complicated process. Homocysteine, a sulfur-containing amino acid, is an intermediate product in methionine metabolism. Most of the dietary methionine is converted to S-adenosylmethionine and then to S-adenosylhomocysteine. Hydrolysis of S-adenosylhomocysteine leads to adenosine and homocysteine. Homocysteine may then

be metabolized either by transsulfuration or transmethylation, depending on the availability of methionine (Vigneaud, Ressler & Rachele, 1950; Carson & Neill, 1962).

Plasma homocysteine levels increase with age in both genders; men have higher plasma homocysteine levels than women. However, both genetic and environmental factors play important roles in this metabolic pathway. For environmental factors, nutritional intake such as dietary deficiencies of folate, vitamin B6 and B12 are associated with raised plasma homocysteine concentrations. Riboflavin is one of the cofactors for MTHFR that may be correlated with high plasma homocysteine when it is deficient.^{3,4}

Several studies have shown that excessively high concentrations of plasma homocysteine may be associated with premature vascular diseases and thromboembolic vascular lesions. Plasma homocysteine is considered to be an independent, graded and strong risk factor for cardiovascular disease.^{5–10}

Wilcken and Wilcken¹¹ first proposed the possible association between plasma homocysteine level and vascular disease in 1976. Since then, many studies have shown that high plasma homocysteine level is an independent predictor for the subsequent development of cardiovascular disease.^{6,7} However, the mechanisms behind the influence of high plasma homocysteine on cardiovascular disease remain unclear. Plasma homocysteine levels correlate negatively with plasma folate, vitamin B6 and B12 levels that indicate inadequate dietary intake in patients with cardiovascular diseases.

SUBJECTS AND METHODS

SUBJECTS AND METHODS

Study Design

It was an observational study with a case-control design.

Study Place and Duration

It was a collaborative study conducted in the Department of Biochemistry and Molecular Biology, Rajshahi University, Biomedical Research Group (BMRG), BIRDEM. The study subjects were collected from the Out-Patient Departments of in Rajshahi Diabetic Centre after approval of institutional heads and Ethical Review Committee as appropriate. All the samples for relevant biochemical, hematological and immunological test and all types of laboratory facilities for estimation biochemical parameters using standard procedures was available in Rajshahi Diabetic Centre and Biomedical Research Group, BIRDEM, Dhaka. The study was carried out during the period of 2008 to 2010.

Study Population

Two Groups of subject were included in this study.

Group-I 108 Diabetic subject without CVDs.

Group-II 101 Diabetic subject with CVDs.

Inclusion Criteria

For Group II: Diabetic subjects, aged between 40-70 yrs, with evidence of CVDs which included:

- 1 Myocardial infarction (chest pain associated with electro cardiographic [ECG] evidence of myocardial infarction or raised cardiac enzymes or both);
- 2 Unstable angina (cardiac pain associated with dynamic ECG abnormalities);
- 3 Angiographically proven coronary artery disease (>50% stenosis in one or more major epicardial vessel in multiple projections).

For Group I: Diabetic subjects, aged between 40-70 yrs, without any other diseases and any other evidence of CVDs which included:

- 4 All the controls were free from any overt disease, i.e., had no clinical or investigative evidence of vascular, renal, hepatic or metabolic disease.

Exclusion criteria

Both for Group I and Group II:

- 1 Cardiomyopathy, pregnancy, serious organ disease (any renal, hepatic or thyroid disease), systemic illness, chronic alcohol abuse, serious psychiatric illness, anticonvulsant therapy and recent exposure to nitrous oxide (within 3 months).

Preparation of the subjects

The exact nature and purpose of the study were explained to the Diabetic CVD patient attending the Out-Patient Departments of in Rajshahi Diabetic Centre. Only those patient who gave informed written consent (Appendix-I) were included in this study. All subjects was collected by face to face interview and provided with questionnaires for detailed medical, personal, social, family history and dietary history also included by 24 hr recall and food frequency method. Blood pressure measurements were performed in all the subjects.

Anthropometric Data

Weight: Body weight was measured on a lever balance (Detecto-Medic, Detecto Scales, Inc, USA). The balance was calibrated every day before use. The body weight was measured bare footed to the nearest 0.1 kg with clothes on. The average weight (0.5 kg) of the clothes was later subtracted from the measured weight. The measurement of weight was done after the bladder has been emptied and before a meal.

Height: Heights of the subjects were measured barefooted in the standing position with a standard scale (Detecto-Medic, Detecto Scale Inc., USA). During measuring height some precautions were taken. When measuring height, the subjects stands straight with the head positioned such that the Frankfurt plane is horizontal, feet together, knees straight, and heels, buttocks and shoulder blades in contact with the vertical surface of the standiometer.

Body Mass Index (BMI): Body mass index was calculated from the body weight and height of the subjects using the following formula weight in kg divided by height in meter Square.

$$\text{BMI} = \frac{\text{Weight in kg}}{(\text{Height in meter})^2}$$

Measurement of Blood Pressure was done as per ACOG guideline (Fernando, 1993). BP was measured with the patient in lying position keeping sphygmomanometer at the level of the heart. When BP was found to be $\geq 140/90$ mm of Hg, it was confirmed on two different occasions at least 6 hours apart (point of muffling i.e. K IV). In this study hypertension was defined as BP equal to or greater than 140/90 mm of Hg.

Collection of Blood sample

Subjects were requested to fast overnight (8-12 hours) and not to take any kind of medicine on the previous day. They were then requested to attend Rajshahi Diabetic center on the next morning in empty stomach. The motivated subjects went there by their own arrangements. Each patient was explained that 10 ml blood would be necessary for the study. On obtaining their consent taking all aseptic precautions 8 ml fasting blood was collected from ante-cubical vein using disposable plastic syringe. Subjects were then given 75 g glucose orally with 250-300 ml water for OGTT (following the WHO criteria) and 2 ml blood was taken 2hr after glucose load and tested for serum glucose on the same day. The fasting blood sample was allowed to clot for 30 minutes at room temperature. Serum was separated by centrifugation at 3000 rpm for 10 minutes. Then ~400 ml of this separated serum sample was analyzed for fasting glucose, TG, total cholesterol, HDL-cholesterol and LDL-cholesterol. Rest of the serum sample (1ml) was preserved at -27°C temperature for estimation of homocysteine concentration.

General principle of collection and preservation of samples

With all aseptic precautions 8 ml of venous blood was taken from the subject after an 8-10 hr fasting sample taken. Collected blood samples were kept in capped and airtight glass test tubes. The sample containers and test tubes were deionized as follows:

- 1 The container and test tubes was washed with detergent.
- 2 Those was then rinsed with double distilled deionized water several times and then dried in an oven at 60⁰C.

After collection of samples the test tubes was sealed with parafilm. Serum was separated by centrifugation (10 minutes at a rate of 3000 rpm) immediately after the blood is taken. The separated serum was allequoted and preserved at -27⁰C until analysis. The height, weight, and blood pressure measurements were performed in all the subjects. Fasting serum sample was collected in all the subjects for analysis.

Dietary Assessment Technique:

Dietary vitamin B₆, B₁₂, Folate intake was calculated by food frequency questionnaire and 24 hour recall method.

The purpose of the diatory assessment is to identify a perso"s eating habits and to estimate the average daily nutrient intake .Though a verity of meathods, information can be obtained on the amount and type of food. eaten.

24-Hour recall : Trined interviewer asks an individual to recall all foods consumed in the 24 hours.

Foods records: Individuals are asked to record food intake over a specified period of time.

Weigher food records: The subject is instructed to weight and record all ingredients and foods consumed.

Diatory history : The dietary history method of assessment is used to evaluate usual in an individual over a long period of time.

Food frequency questionnaire : A food frequency questionnarire (FFQ) is used to determine the frequency of consumption of certain foods.

Assessment of B₆ and B₁₂ rich dietary intake:

- 1 Vitamin B₆, B₁₂, Folate rich dietary practices of the respondents were assessed through food frequency method.
- 2 There was about 100 questions in the questionnaire regarding intake of vitamin B₆, B₁₂, Folate rich vegetables, green leafy vegetables, fruits, cereals, legume and animal foods etc.
- 3 By using food frequency method, dietary history (daily/weekly/monthly/1st 6 months of pregnancy/never) and frequency of vitamin B₆, B₁₂, Folate rich foods intake among the type 2 DM with and without CVD population were assessed.
- 4 In order to estimate the amount of usual vitamin B₆, B₁₂, Folate intake during pregnancy, the fractional portion size of each food consumed per day was multiplied by its vitamin B₆, B₁₂, Folate content, obtained from the national food composition table (Gopalon, Helen Keller; Swaminathan).
- 5 The values were then summed up to obtain an estimate of an individual's total daily vitamin B₆, B₁₂, Folate intake.

Biochemical analysis

- 1 Glycemic status of the study subjects were measured by serum fasting glucose load using Glucose Oxidase method (Randox , UK)
- 2 Lipidemic status was assessed by -
 - Serum total cholesterol using enzymatic endpoint method (cholesterol Oxidase/ Peroxidase) (Randox Laboratories, UK)
 - Serum triglyceride using enzymatic-colorimetric (GPO-PAP) method (Randox laboratories, UK)
 - Serum high density lipoprotein (HDL-cholesterol) using enzymatic-colorimetric (cholesterol CHOD-PAP) method (RANDOX laboratories, UK)
 - The LDL-cholesterol level in serum was calculated using Friedewald's formula.
- Serum homocysteine concentration was measured by Fluorescence Polarization Immunoassay (FPIA) technology (Abbott Laboratories, USA).
- 1) Dietary history was measured by food frequency meethod.

Operational Definition:

DM: Diabetes mellitus (DM) is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.

CVD: The blood circulates through the body. Diabetes can change the chemical makeup of the substances found in blood, causing blood vessels to narrow or clog up totally. This is called atherosclerosis, or hardening of the arteries.

Categorization of Education level:

- 1 Illiterate: Subjects with no knowledge of signature.
- 2 Secondary: Subjects with educational level up to SSC.
- 3 Higher secondary: Subjects with educational level up to HSC.
- 4 Graduate: Subjects with educational level above HSC.

Categorization of Economic Status:

- 5 Lower class: Subjects with income <3000 Tk/ month.
- 6 Middle class: Subjects with income of 3001-5000 Tk/ month.
- 7 Upper middle class: Subjects with income of 5001-10000 Tk/ month
- 8 Upper class: Subjects with income of >10000Tk/ month.

Statistical Analysis

Data were compiled in predesigned case record forms and those were analyzed by using the Window based SPSS 11.0 Software (SPSS, Inc. Chicago. IL. USA). Pearson's correlation and multivariate regression, as appropriate, were used to analyze the association between the other variables. Significance of differences will be analyzed by Student's t- test, as appropriate. P value of <0.05 will be considered sufficient for rejecting the null hypothesis of no difference among Groups.

RESULTS

In this study a total of 209 subjects participated. Among them 108 subjects were diabetic without CVDs (Groups-I) and 101 subjects were diabetic with CVDs (Group-II).

Clinical characteristics of the study subjects (Table:1)

Table:1 shows that the two Groups were age (yrs, M±SD) - matched and 55.4% male subjects were in Group II. BMI (kg/m², M±SD); systolic blood pressure (mm of Hz) and diastolic blood pressure (mm of Hz) were significantly higher in Group II [(29±4.7); (130.5±12.9) and (88.4±8.6)] as compare to Group I [(24.1±3.8); (120.7±13) and (82.7±8)]; (p=0.013; p<0.001 and p=0.007) respectively.

Table 1 Clinical characteristics of the study subjects

Variable	Group I (n=108)	Group II (n=101)	P value
Age (yrs)	43.7±8.2	43.3±8.8	0.532
Sex			
Male	58(53.7%)	56(55.4%)	
Female	50(46.3%)	45(44.6%)	0.800
BMI (kg/m ²)	24.1±3.8	29±4.7	0.013
SBP (mm of Hz)	120.7±13	130.5±12.9	<0.001
DBP (mm of Hz)	82.7±8	88.4±8.6	0.007
Habit of exercise			
Yes	90(83.3%)	70(69.3%)	0.017
No	18(16.7%)	31(30.7%)	

Results are expressed as Mean±SD, or number (%) as appropriate, Significance level was calculated by student 't' and chi square test, n= number of subjects. BMI=Body Mass Index, SBP=Systolic Blood pressure, DBP=Diastolic Blood Pressure.

Similarly 83% subjects regularly maintained 30 minute physical exercise in Group I (p.017). 49% subjects Group II and 46% were in Group I, had a habit of regular drinking tea or coffee.

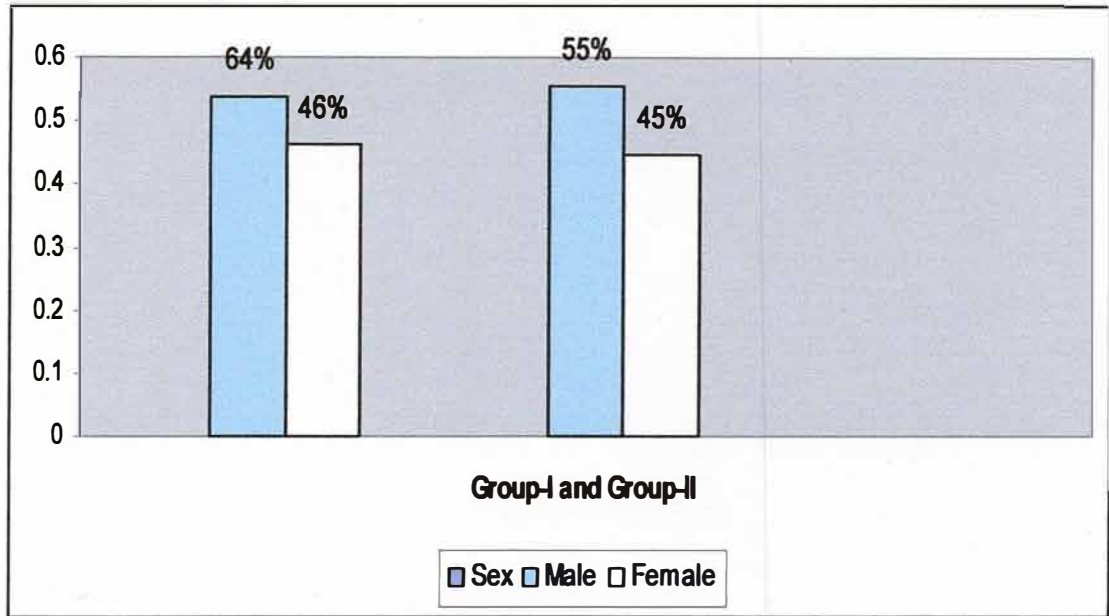


Figure 1: The proportion of male and female subjects in the different Groups.

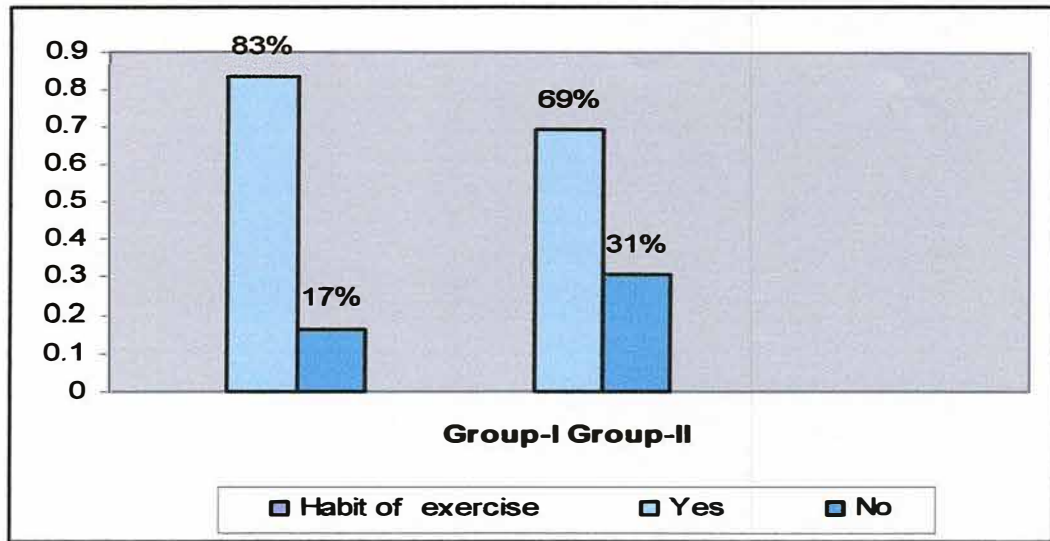


Figure 2: The proportion of physical activities according to exercise level in the different Groups.

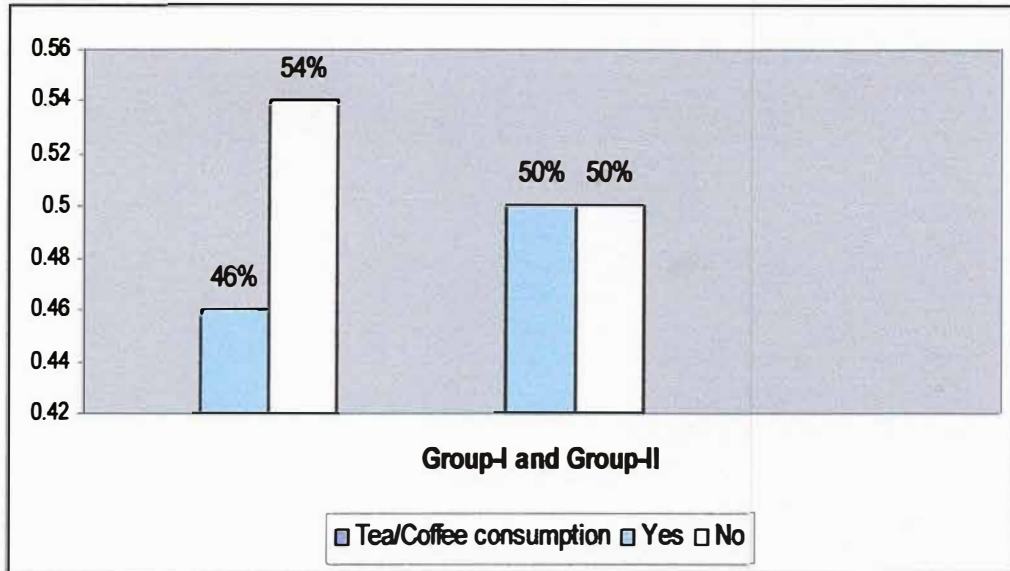


Figure 3: The proportion of habit of tea coffee consumption in the different Groups.

RESULTS

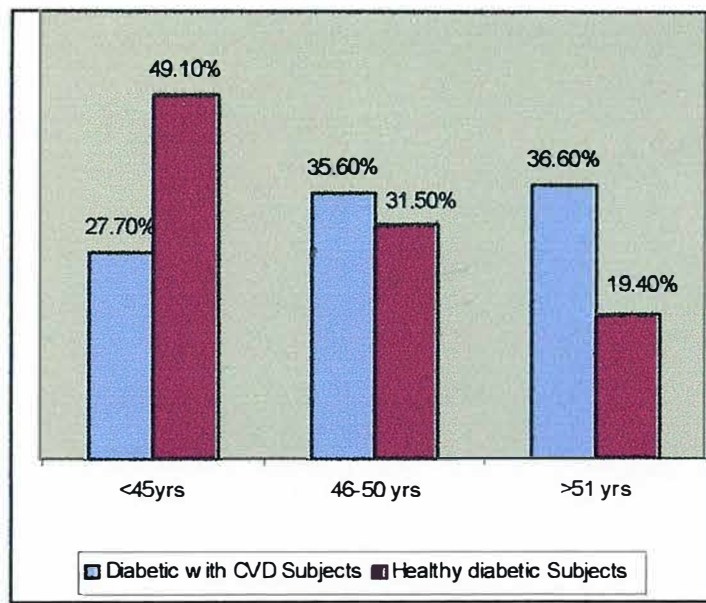


Figure 4: Age group wise the proportion of diabetic subjects with CVDs.

Biochemical characteristics of the study subjects (Table 2)

Table:2 shows that biochemical characteristics in the study subjects, Fasting serum glucose level (mmol/l, M±SD) were significantly higher in Group II compared to Group I (18.5±6.01 vs 11.2±4.2; P<0.001).

Serum glucose at 2 h after 75 g glucose administration were found to be (18.3±5.9) in Group I and (17.7±5.7) in Group II; (p=0.477). There was no significant difference in the two Groups.

Serum total cholesterol (mg/dl, M±SD) were significantly higher in Group II compared to Group I (226±47 vs 207±34.2; P<0.001).

Serum triglyceride (gm/dl, M±SD) were significantly higher in Group II compared to Group I (218±85.5 vs 172±67.3; P<0.001).

Serum HDL levels also significantly higher in Group I (47±8.1) as compare to Group II (39.8±10); (p=0.004) and

Serum homocysteine (µmol/L, M±SD) were also significantly higher then compare to Group I (7.6±1.5) and Group II (14.5±5.8); (p<0.001).

Table 2: Biochemical characteristics of the study subjects

Variable	Group I (n=108)	Group II (n=101)	P value
FBG mmol/l	11.2±4.2	18.5±6.01	<0.001
ABG mmol/l	18.3±5.9	17.7±5.7	0.477
Total cholesterol (mg/l)	207±34.2	226±47	0.001
Triglyceride (mg/l)	172±67.3	218±85.5	<0.001
LDL-cholesterol	139±39.5	141.4±43.9	0.689
HDL-cholesterol (mg/l)	47±8.1	39.8±10	0.004
Total Homocysteine (µmol/L)	7.6±1.5	14.5±5.8	<0.001

Results are expressed as Mean±SD, n= number of subjects. FBG=Fasting Blood Glucose, ABG=After blood Glucose, LDL=Low density lipoprotein, HDL=High density lipo protein.

The proportion of higher Homocysteinemic subjects (Table: 3)

This table shows that 37.6% subjects were in Group II their homocysteine level was below cut off value (<12 $\mu\text{mol/L}$) and 62.4% subjects were above cut off value of homocysteine level.

Table 3: The proportion of higher Homocysteinemic subjects

Cut off value of Homocysteine	Group I	Group II	P value
<12 $\mu\text{mol/L}$	108 (100%)	38 (37.6%)	0.001
>12 $\mu\text{mol/L}$	0 (0%)	63 (62.4%)	

Results are expressed as n(%), n= number of subjects. The distribution was calculated by cross tab analysis.

The dietary vitamin (B₁₂, B₆ and Folate) levels of the study subjects (Table:4)

The daily mean intake of dietary folate (mg/day, M±SD) in the study subjects as follows: as Group I (7.7±1.8) and Group II (1.8±0.93) respectively (p<0.001). Daily vitamin B₆ as follows: as Group I (1.6±0.38) and Group II (1.0±0.61) respectively (p<0.001). And daily vitamin B₁₂ as follows: as Group I (277±74.1) and Group II (119.1±29.8) respectively (p<0.001). There was significant difference in dietary intake folate, vitamins B₆ and B₁₂ in between the two Groups

Table 4: Dietary vitamin (B₁₂, B₆ and Folate) levels of the study subjects

Variable	Group I (n=108)	Group II (n=101)	P value
Dietary Folate (mg/day)	7.7±1.8	1.8±0.93	<0.001
Dietary Vitamin B ₆ (mg/day)	1.6±0.38	1.0±0.61	<0.001
Dietary Vitamin B ₁₂ (mg/day)	277±74.1	119.1±29.8	<0.001

Results are expressed as Mean±SD, n= number of subjects.

The correlation of Homocysteine with other variables of the study subjects (Table:5)

Table 5 demonstrates the data on the coefficient correlation of homocysteine with other variables. From the data, a highly positive correlation of homocysteine was found with BMI, duration of DM, habit of exercise fasting blood glucose level and serum triglyceride ($r=0.468, p=0.050$; $r=0.392, p<0.001$; $r=0.181, p=0.009$; $r=0.332, p<0.001$ and $r=0.146; p=0.035$) and negative correlation was found between dietary folate, vitamin B6 and B12 ($r=-0.563, p<0.001$; $r=-0.379, p<0.001$ and $r=-0.484$) respectively ($p<0.001$) among the Group II.

Other variables such as age, family history of CVD, tea coffee consumption and total cholesterol did not have any significant positive or negative correlation with homocysteine level.

Table 5: Correlation of Homocysteine with other variables of the study subjects

Variable	Group I (n=108)		Group II (n=101)	
	r	p	r	p
Age (yrs)	-0.146	0.133	0.002	0.212
BMI (kg/m ²)	-0.059	0.544	0.468	0.050
Duration of DM	0.178	0.065	0.392	<0.001
Family history of CVD	-0.074	0.444	0.080	0.250
Habit of Exercise	0.092	0.346	0.181	0.009
Tea/Coffee consumption	0.091	0.347	0.128	0.065
FBG (mmol/l)	0.016	0.870	0.332	<0.001
Total cholesterol (mg/l)	-0.218	0.023	0.060	0.389
Triglyceride (mg/l)	0.051	0.598	0.146	0.035
Dietary Folate (mg/day)	-0.147	0.129	-0.563	<0.001
Dietary Vitamin B ₆ (mg/day)	0.030	0.757	-0.379	<0.001
Dietary Vitamin B ₁₂ (mg/day)	-0.043	0.660	-0.484	<0.001

Pearson's parametric correlation coefficient's test was done as a test of significance. DM=Diabetic mellitus

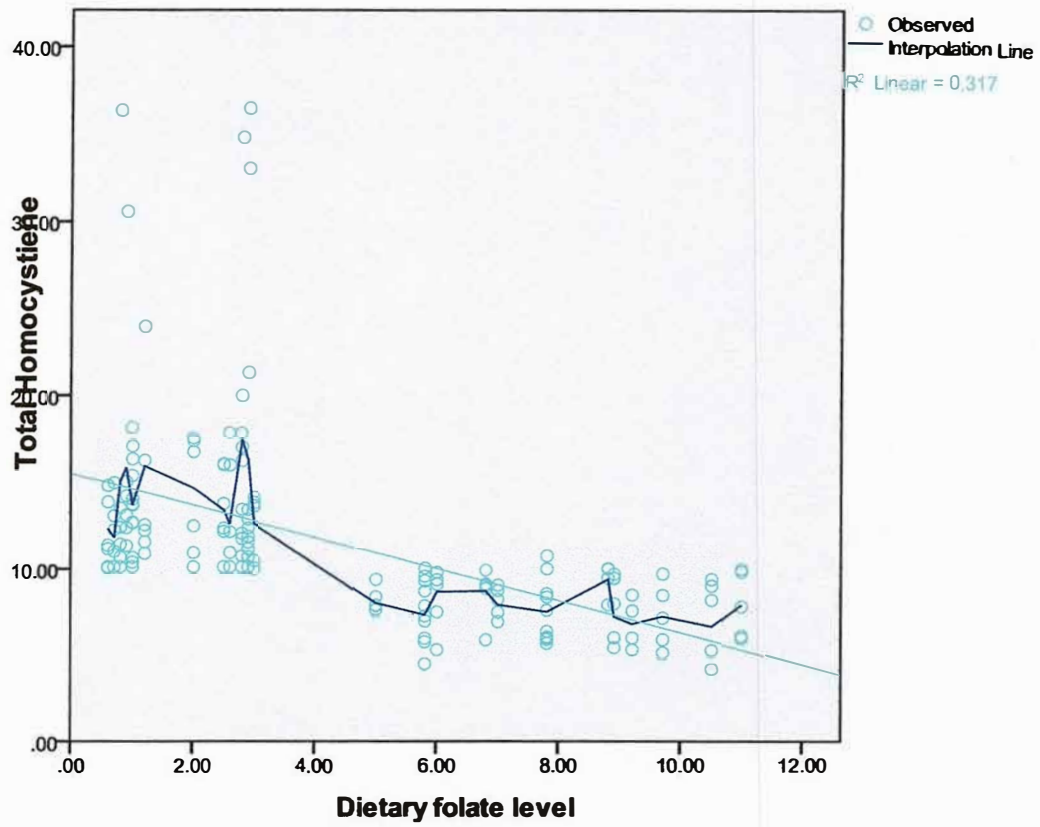


Figure 5: Correlation of total homocysteine level with dietary folate level

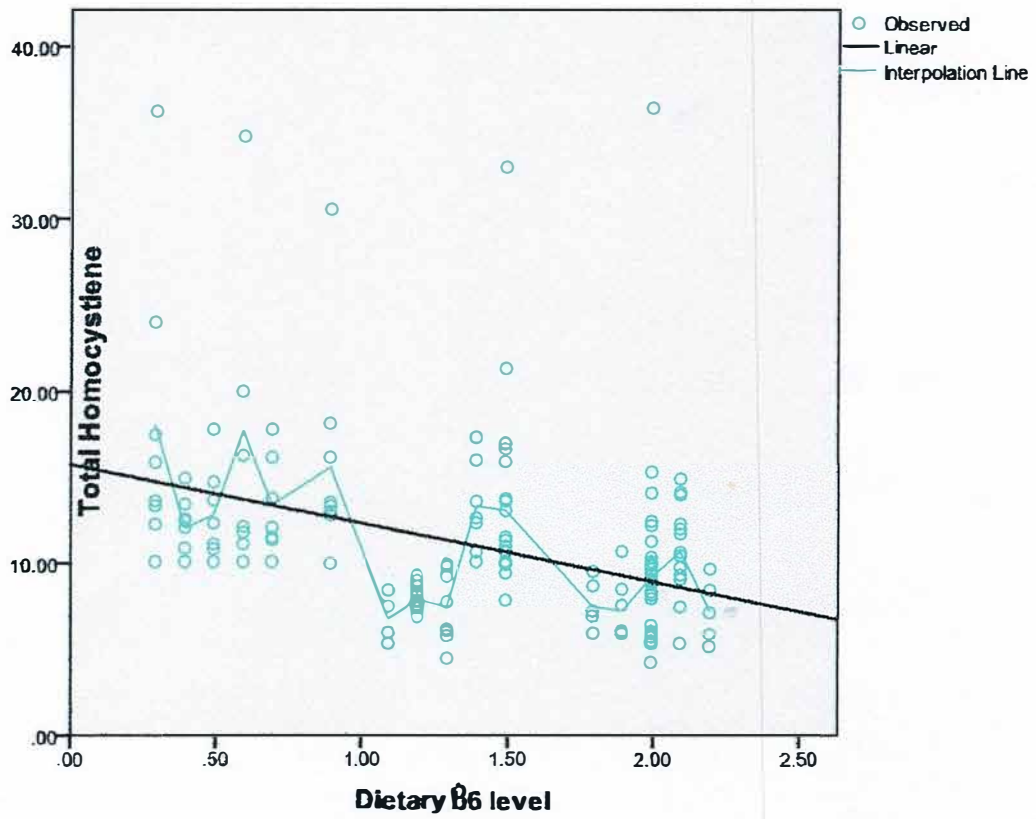


Figure 6: Correlation of total homocysteine level with dietary Vitamin B₆ level

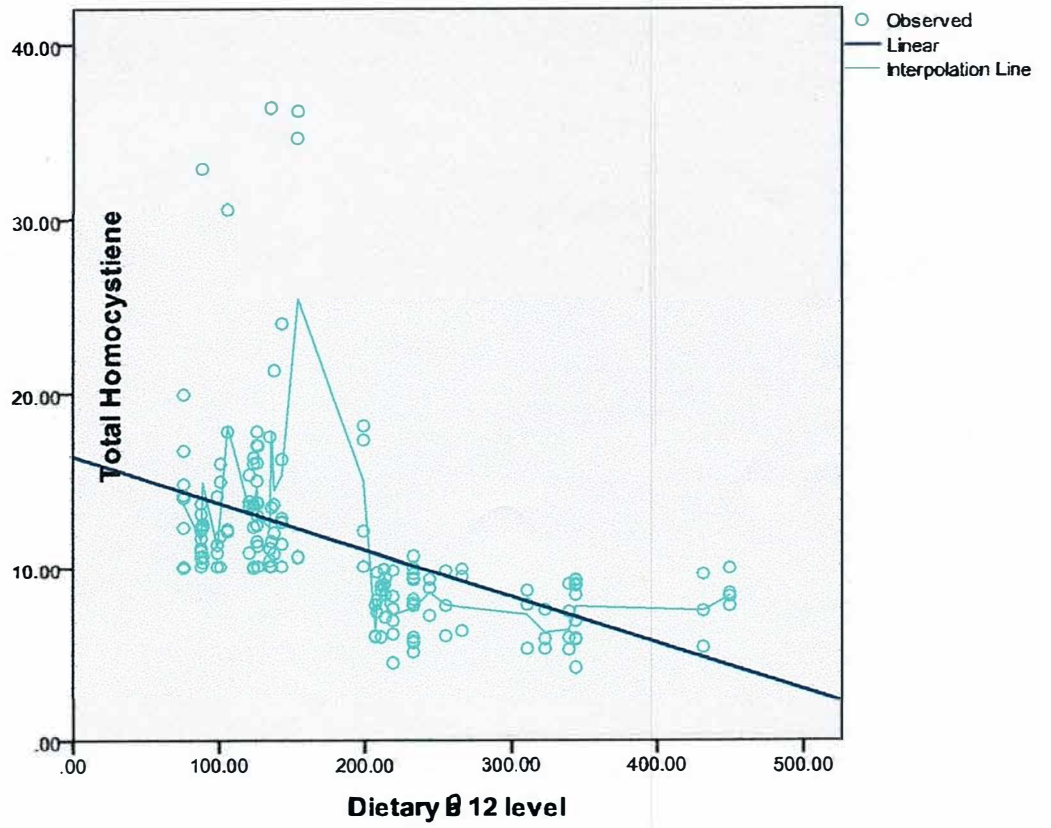


Figure 7: Correlation of total Homocysteine level with dietary Vitamin B₁₂ level

The association of homocysteine with other factors Ωφas explored by multiple linear regression analysis (Table:6)

Multiple regression analysis was carried out in Group II subjects with homocysteine level as the dependent variable (DV) and other parameters (sex, exercise, tea coffee consumption and betel nut chewing) as independent variable. No significant association with variable was observed.

Table 6: Association of homocysteine with other factors as explored by multiple linear regression analysis

Variables	SE	β value	P value
Sex	0.591	0.038	0.487
Tea coffee consumption	0.642	0.076	0.161
Betel nut	0.590	-0.026	0.635
Exercise	0.709	0.077	0.166

β for standardized regression coefficient. Homocystine was taken as dependent variable whereas other variables were taken as independent variable; n = number of subjects

adjust

The association of homocysteine with other factors as explored by multiple linear regression analysis (Table:7)

Multiple linear regression analysis was also performed taking all the Group I and Group II subjects (Table 7) with homocysteine level as the dependent variable (DV) and age, BMI, Group and daily dietary intake of folate and vitamins as independent variables. A significantly positive association with Groups ($\beta=0.605$; $p<0.001$) has found and significant negative association was found with dietary folate and vitamin B₆ ($\beta=-0.408$; $p<0.001$ and $\beta=-0.128$; $p=0.053$).

Table 7: Association of Homocysteine with other factor as explored by multiple linear regression analysis

Variables	SE	β value	P value
Age	0.037	0.042	0.482
BMI	0.072	0.035	0.546
Group	0.616	0.605	<0.001
Dietary Folate	0.154	-0.408	<0.001
Dietary Vitamin B ₆	0.597	-0.128	0.053
Dietary Vitamin B ₁₂	0.005	-0.106	0.241

β for standardized regression coefficient. Homocysteine was taken as dependent variable whereas other variables were taken as independent variable; n = number of subjects

DISCUSSION

Atherosclerosis is too complex to be explained by any one mechanism or cause. To date, many factors have been identified which have been shown to be a risk factor for the development of the CVD. At the same time, it is now being increasingly appreciated that the traditional risk factors for cardiovascular disease (CVD) may account for only one half to two thirds of the actual risk.

Smoking, obesity, hypercholesterolemia, family history, physical inactivity, diabetes mellitus, hypertension, and other co morbidities for CVD. It becomes important to try and identify other risk factors, especially those that can be easily modified or corrected. Some of the factors whose role is being seriously investigated include estrogen deficiency, lipoprotein (a), plasma fibrinogen, plasminogen-activator inhibitor type 1, endogenous tissue plasminogen activator (tPA), C-reactive protein, and homocysteine.

In the late 1960s, McCully became the first to hypothesize that elevated plasma homocysteine concentrations could cause atherosclerosis. Many studies have shown that Hyperhomocysteinemia may be an independent risk factor for thromboembolic disease. However, the results among studies and populations have not been consistent. Furthermore, the possible mechanisms of hyperhomocysteinemia in vascular diseases are controversial. Dietary folate, and vitamin B₆ and B₁₂ intake deficiency, may play a role in this complicated process. Homocysteine, a sulfur-containing amino acid, is an intermediate product in methionine metabolism. Most of the dietary methionine is converted to S-adenosylmethionine and then to S-adenosylhomocysteine. Hydrolysis of S-adenosylhomocysteine leads to adenosine and homocysteine. Homocysteine may then be metabolized either by transsulfuration or transmethylation, depending on the availability of methionine (Vigneaud, Ressler & Rachele, 1950; Carson & Neill, 1962).

Plasma homocysteine levels increase with age in both genders; men have higher plasma homocysteine levels than women. However, both genetic and environmental factors play important roles in this metabolic pathway. For environmental factors, nutritional intake such as dietary deficiencies of folate, vitamin B₆ and B₁₂ are associated with raised plasma homocysteine concentrations. Riboflavin is one of the cofactors for MTHFR that

may be correlated with high plasma homocysteine when it is deficient (Jacques et al, 1996; Guttormsen et al, 1996).

Several studies have shown that excessively high concentrations of plasma homocysteine may be associated with premature vascular diseases and thromboembolic vascular lesions. Plasma homocysteine is considered to be an independent, graded and strong risk factor for cardiovascular diseases (Clarke et al, 1991; Stampfer et al, 1992; Perry et al, 1995; Mayer EM, Jacobsen DW, Robinson, 1996; Malinow, 1996; Selhub et al, 1995).

Wilcken and Wilcken¹¹ first proposed the possible association between plasma homocysteine level and vascular disease in 1976. Since then, many studies have shown that high plasma homocysteine level is an independent predictor for the subsequent development of cardiovascular disease (Stampfer et al, 1992; Perry et al, 1995). However, the mechanisms behind the influence of high plasma homocysteine on cardiovascular disease remain unclear. Plasma homocysteine levels correlate negatively with plasma folate, vitamin B₆ and B₁₂ levels that indicate inadequate dietary intake in patients with cardiovascular diseases.

However, the genes involved in the metabolism of these nutrients (particularly the MTHFR gene) may have substantial ethnic variation and thus, the gene-nutrient interaction underlying hyperhomocysteinemia needs to be studied in individual population groups.

In the present diabetic population of northern Bangladesh, with no evidence of CVD, the control Group range of plasma Hcy was found to be 5.6 to 9.4 $\mu\text{mol/L}$ which shows that 62.4% subjects have tHcy values above the cut-off levels. The data is compatible with the previous reports where a group association of CVD with hyperhomocysteinemia has been demonstrated.

The major objective of the present study was to explore the determinants of hyperhomocysteinemia in our population. Analysis of group difference shows that BMI, blood pressure, hyperglycemia and dyslipidemia are significantly higher in Group II compared to Group I and those are potential determinants of hyperhomocysteinemia in this population. In addition it seems that lifestyle (habit of exercise) and dietary factors (folate, vit B₆ and B₁₂ intake) are important determinants of the disorder. The association

of these risk factors with hyperhomocysteinemia is further evident on correlation analysis where BMI, duration of DM, habit of exercise, FBG and TG show a significant positive correlation and dietary folate, Vit B₆ and vit B₁₂ show a negative correlation with plasma homocysteine.

On further analysis by multiple linear regressions tHcy was found to have significant negative association only with dietary folate and vitamin B₆ when the effects of age, BMI and groups were adjusted. No significant association with sex, tea/coffee consumption, betelnut or habit of exercise was found.

The association of hyperhomocysteinemia with low intake of dietary folate and Vit B₆ has been postulated in a prospective intervention study. The present study reinforces the concept that attention towards adequate intake of folate and vit B₆ may be a major step to prevent this important risk factor for CVD.

CONCLUSIONS

From the present data it may be concluded that:

- a. Hyperhomocysteinemia is an important risk factor of cardiovascular disease in Bangladeshi diabetic population;
- b. Dietary deficiency of folate and Vit B₆ are the major determinants of hyperhomocysteinemia in type 2 diabetic subjects.

RECOMMENDATIONS

- Plasma level of homocysteine should be routinely checked during clinical evaluation of a patient;
- Adequate dietary folate and vit B₆ should be ensured (by nutrition or supplementation) to prevent hyperhomocysteinemia.
- Large scale studies (including those involving genetics) should be conducted in this area.

SUMMARY AND CONCLUSIONS

7 SUMMARY AND CONCLUSION

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease that is independent of major risk factors such as diabetes, hypertension, hypercholesterolemia, and smoking. Although the mechanisms by which homocysteine promotes atherothrombosis are unknown, the epidemiological evidence for the association of hyperhomocysteinemia with atherothrombotic disease is strong (Ueland, Refsum, Brattstrom, 1992; Boushey et al, 1995; Welch, Loscalzo, 1998). Recent retrospective and prospective studies, it is now widely accepted that increased total plasma homocysteine is a risk factor for cardiovascular disease. Impaired enzyme function as a result of genetic mutation or deficiency of the essential B vitamins folic acid B₁₂ and B₆ can lead to hyperhomocysteinemia. Several evidence suggests that it may play a role in atherothrombotic disease. Gradually increased homocysteine causes dysfunction of vascular endothelium.

It was an observational study with a case-control design. Two Groups of subject were included in this study. 108 subjects were diabetic without CVDs in Group I and 101 subjects were selected diabetic with CVDs in group II. A total of 209 subjects included in this study and its association with clinical, socio-economical and biochemical risk factors. Purposively and their economical, and clinical characteristics were noted in a pre-designed case record form. Nutritional intake was assessed by food frequency questionnaire (24 hr dietary recall) method. Each subject went through OGTT following appropriate preparation and DM was diagnosed as per WHO Study Group Criteria. Blood glucose was measured by glucose oxidase method (Randox, UK) using a semi auto analyzer (300 MICROLAB). Lipids were measured by enzymatic methods using the same analyzer in the lab of Rajshahi Diabetic center. Plasma homocysteine concentration was measured by Fluorescence Polarization Immunoassay (FPIA) technology (Abbott Laboratories, USA). Significance of differences will be analyzed by Student's t- test, as appropriate. Pearson's correlation and multivariate regression, as appropriate, were used to analyze the association between the other variables. Two Groups were age (yrs, M±SD) - matched and 55.4% male subjects were in Group II. BMI (kg/m², M±SD); systolic blood pressure (mm of Hg) and diastolic blood pressure (mm

of Hz) were significantly higher in Group II [(29±4.7); (130.5±12.9) and (88.4±8.6)] as compare to Group I [(24.1±3.8); (120.7±13) and (82.7±8)]; (p=0.013; p<0.001 and p=0.007) respectively.

Fasting serum glucose level (mmol/l, M±SD) were significantly higher in Group II compared to Group I (18.5±6.01 vs 11.2±4.2; P<0.001). Serum glucose at 2 h after 75 g glucose administration were found to be (18.3±5.9) in Group I and (17.7±5.7) in Group II; (p=0.477). There was no significant difference in the two Groups. Serum total cholesterol (mg/dl, M±SD) were significantly higher in Group II compared to Group I (226±47 vs 207±34.2; P<0.001). Serum triglyceride (gm/dl, M±SD) were significantly higher in Group II compared to Group I (218±85.5 vs 172±67.3; P<0.001). Serum HDL levels also significantly higher in Group I (47±8.1) as compare to Group II (39.8±10); (p=0.004).

Serum homocysteine (µmol/L,, M±SD) were also significantly higher then compare to Group I (7.6±1.5) and Group II (14.5±5.8); (p<0.001). 37.6% subjects were in Group II their homocysteine level was below cut off value (<12 µmol/L) and 62.4% subjects were above cut off value of homocysteine level.

The daily mean intake of dietary folate (mg/day, M±SD) in the study subjects as follows: as Group I (7.7±1.8) and Group II (1.8±0.93) respectively (p<0.001). Daily vitamin B₆ as follows: as Group I (1.6±0.38) and Group II (1.0±0.61) respectively (p<0.001). And daily vitamin B₁₂ as follows: as Group I (277±74.1) and Group II (119.1±29.8) respectively (p<0.001). There was significant difference in dietary intake folate, vitamins B₆ and B₁₂ in between the two Groups.

On the coefficient correlation of homocysteine with other variables. From the data, a highly positive correlation of homocysteine was found with BMI, duration of DM, habit of exercise fasting blood glucose level and serum triglyceride (r=0.468, p= 0.050; r=0.392, p<0.001; r=0.181,p=0.009; r=0.332, p<0.001 and r=0.146; p=0.035) and negative correlation was

found between dietary folate, vitamin B6 and B12 ($r=-0.563, p<0.001; r=-0.379, p<0.001$ and $r=-0.484$) respectively ($p<0.001$) among the Group II.

On further analysis by multiple linear regressions Hyperhomocystenemia was found to have significant negative association only with dietary folate and vitamin B6 ($\beta=-0.408; p<0.001$ and $\beta=-0.128; p=0.053$) when the effects of Age, BMI and groups were adjusted.

From the above discussion it may be concluded that, Hyperhomocysteneimia is a important risk factor of cardiovascular disease in Bangladeshi diabetic population. Dietary deficiency of folate and vit B₆ are the major determinants of hyperhomocystienemia in type 2 diabetic subjects.

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APPENDICES

APPENDIX-I

Consent form

I have been informed by **kuhilika Akter** that a research work is going to be done in Biochemistry and Molecular Biology Department of Rajshahi University on Type-2 diabetic with CVD patient. She has explained me about different aspects of this research work. I understand that this research work will bring benefit to the diabetic patients. I have also understood clearly the nature and duration of this work. I have been informed that my personal identity and other sensitive social information will be kept undisclosed. I have been told that I have to give sample of blood 5 ml after a fasting period of 12 hours and I shall to give another sample of 2 ml of blood after 75 gm glucose drink. Time for this interview will be about 45 minutes. I also understand that I will be able to ask any question about different procedures of this research work and I shall be able to ask any question in future if necessary. I also understand that I can withdraw my participation in this research work at any time without any explanation or without any reason. Considering the above points I am giving my consent in health and willingness to participate into this research work.

Signature of the participant

Signature of the witness

Address:

Address:

APPENDIX-II

প্রশ্নপত্র

ডায়াবেটিস এ আক্রান্ত অধিক হোমোসিস্টিনেমিয়া'র নির্ধারক সম্পর্কিত গবেষণা

আই ডি নং :

সাক্ষাতের তারিখ :

পর্ব - ক : সমাজ ও জনসংখ্যা বিষয়ক তথ্যাবলী

১. উত্তরদাতার নাম :

২. বর্তমান ঠিকানা :

১ = শহর

২ = গ্রাম

৩ = উপশহর

৩. বয়স (> ২০ বছর) :

৪. লিঙ্গ :

১ = পুরুষ

২ = মহিলা

৫. পেশা :

১ = বেকার

২ = চাকুরী

৩ = ব্যবসা

৪ = শ্রমিক

৫ = গৃহিনী

৬ = অন্যান্য (নির্দিষ্ট করে বলুন)

৬. শিক্ষা :

১ = নিরক্ষর

২ = প্রাথমিক

৩ = মাধ্যমিক,

৪ = উচ্চ মাধ্যমিক

৫ = স্নাতক

৬ = স্নাতকোত্তর

৭. ধর্ম :

১ = মুসলিম

২ = হিন্দু

৩ = খ্রীষ্টান

৪ = বৌদ্ধ

৮. বৈবাহিক অবস্থা :

১ = বিবাহিত

২ = অবিবাহিত

৩ = বিধবা

৪ = পৃথক থাকা/তালাক

৯. পরিবারের সদস্য সংখ্যা :

১০. পরিবারের মাসিক আয় (পরিবারের সকল সদস্যের সম্মিলিত আয়) :

পর্ব - খ : দৈহিক পরিমাপন বিষয়ক তথ্যাবলী

ওজন (কেজি) :

উচ্চতা (সে.মি):

BMI :

Waist/Hip :

পর্ব - গ : স্বাস্থ্য বিষয়ক তথ্যাবলী

১১. কতদিন ধরে ডায়াবেটিসে ভুগছেন ?

১ = < ৫ বছর ২ = ৫-১০ বছর ৩ = > ১০ বছর

১২. বর্তমানে আপনার রক্তের শর্করার মাত্রা কত?

১৩. আপনার পরিবারে অন্যকারো এই রোগ আছে?

১.বাবা ২.মা ৩.ভাই ৪. বোন

১৪. ডাক্তার কর্তৃক অনুমোদিত কোন ঔষধ সেবন করছেন?

১৫. আপনার কি উচ্চ রক্তচাপ আছে?

১ = হ্যাঁ ০ = না

১৬. কতদিন ধরে উচ্চ রক্তচাপে ভুগছেন?

১ = < ৫ বছর ২ = ৫-১০ বছর ৩ = > ১০ বছর

১৭. বর্তমানে আপনার রক্তচাপ মাত্রা কত?

১৮. আপনার পরিবারে অন্যকারো এই রোগ আছে?

১.বাবা ২.মা ৩.ভাই ৪. বোন

১৯. ডাক্তার কর্তৃক অনুমোদিত কোন ঔষধ সেবন করছেন?

২০. আপনার কি হৃদরোগ আছে?

১ = হ্যাঁ ০ = না

২১. নিবলিখিত লক্ষণগুলো অনুভব করেন?

১. নাড়ীস্পন্দন

২. বুকের ব্যাথা

৩. বুকে চাপ

৪. মাথা ব্যাথা

৫. মাথা ঘোরানো

৬. চোখের সমস্যা

৭. অস্থিরতা অনুভব

৮. ঘেমে যাওয়া

৯. বমি বমি ভাব

২২. হৃদরোগের সাথে সম্পর্কিত নিবলিখিত জটিলতা আছে?

ক. কার্ডিয়াক এরিথমিয়া খ. হৃদকার্য বন্ধ হয়ে যাওয়া গ. হৃদযন্ত্রে কোন বাধা আছে
ঘ. স্ট্রোক

২৩. অন্যান্য জটিলতা আছে?

ক. ক্যানসার খ. স্নায়ু রোগ গ. কিডনী রোগ ঘ. চোখের রোগ ঙ. ফুট আলসার

২৪. আপনার কি প্রসাবে প্রোটিন যায় ?

১ = হ্যাঁ ০ = না

২৫. ধূমপান করেন কি ?

১ = হ্যাঁ ০ = না

২৬. যদি হ্যাঁ হয় তাহলে দিনে কয়টা

২৭. চা ও কফি পান করেন কি?

১ = হ্যাঁ ০ = না

২৮. যদি হ্যাঁ হয় তাহলে দিনে কয়বার?

২৯. আপনি কি পান সুপারী খান?

১ = হ্যাঁ ০ = না

৩০. যদি হ্যাঁ হয় তাহলে দিনে কয়টা?

৩১. আপনি কি মদ্যপান করেন?

১ = হ্যাঁ ০ = না

৩২. যদি হ্যাঁ হয় তাহলে সেটা কি

১. মাঝে মাঝে ২. প্রতিদিন

৩৩. নিয়মিত ব্যায়াম করেন কি ?

১ = হ্যাঁ ০ = না

৩৪. সম্প্রতি আপনার কোনো অসুপ্রচার হয়েছে?

১ = হ্যাঁ ০ = না

৩৫. যদি হ্যাঁ হয় তাহলে সেটা কতদিন পূর্বে?

পর্ব - ঘ : খাদ্যাভাস বিষয়ক তথ্যাবলী

৩৬. সাধারণত কোন ধরনের খাদ্যে অভ্যস্ত :

১ = বেশী সবজি এবং কম শর্করা যুক্ত খাদ্য

২ = বেশী চর্বি যুক্ত খাদ্য এবং অন্যান্য শর্করা খাদ্য

৩ = পরিমিত খাদ্য

৪ = অপরিমিত খাদ্য

৩৭. সম্প্রতি আপনি কোনো ঔষধ খেয়েছেন/খাচ্ছেন?

১ = হ্যাঁ ০ = না

৩৮. যদি হ্যাঁ হয় তাহলে (ভিটামিন/উচ্চ রক্তচাপ/হৃদরোগ/ডায়াবেটিস):

ঔষধের নাম	সেবন মাত্রা	

আইডি:

রোগীর নাম:-----

- বর্তমান ঠিকানা: ফোন:
- স্থায়ী ঠিকানা: ফোন:

শারীরিক পরিশ্রম

২৪ ঘন্টায় শারীরিক পরিশ্রম

কাজ	সময় (মিনিট)	সময় (ঘন্টায়)	কাজের ধরণ	ক্যালরী / মিনিট
প্রধান কাজ - অফিসিয়াল ব্যবসা শিক্ষকতা অন্যান্য				
ঘর পরিষ্কার করা				
ঘর মোছা				
বাসন ধোয়া				
রান্না করা				
কাপড় ধোয়া				
পড়া লেখা				
ওযু করা				
নামাজ পড়া				
কোরআন তেলোয়াত				
মসলা বাটা				
বাজার করা				
ইত্ত্রি করা				
টিভি দেখা				
দৌড়ান				
ব্যায়াম				
খেলা				
গোসল করা				
কম্পিউটারে কাজ				
আড্ডা দেয়া				

বিশ্রাম - অলস ভাবে বসে থাকা অলস ভাবে শোয়া				
ঘুমানো				
সেলাই করা				
ডসড়ি ভাঙ্গা				
খাওয়া				
অন্যান্য				

Interviewer signature

আই.ডি নং :

রোগীর নাম :

খাদ্য গ্রহন তালিকা :

খাবারের নাম	রান্নার প্রক্রিয়া	বক্তিত অংশের পরিমাণ	গ্রাম/দিন	বার/দিন	বার/সপ্তাহ	বার/মাস	কখনো না বা >১/মাস
Cereals							
ভাত							
রুটি							
চিড়া							
ত্রুড়ি							
পরোটা							
লুচি							
Legumes							
বুটের ডাল							
বুট ভাজা							
মসুর ডাল							
মুগ ডাল							
মটর ডাল							
অন্যান্য							
Milk/ MilkProd							
গরুর দুধ							
ননীবিহীন গরুর দুধ							
পাউডার দুধ							
মাখন							
ঈনির							
দই							
দুধের সর							
বাদাম							
অন্যান্য							
Meat/ Meat Prod							
গরুর মাংস							
খাসীর মাংস							
মুরগীর মাংস							
গরু/খাসীর কনিজা							
Eggs/Egg							

Products								
হাঁসের ডিম								
মুরগীর ডিম								
Fish								
ইলিশ								
পাঙ্গাস								
রুই								
কাতল								
চিতল								
কৈ								
টেংরা								
চিংড়ী								
মাগুর								
ডশং								
অন্যান্য								
Vegetables								
পুইশাক								
নানশাক								
নাউশাক								
কচুশাক								
পালংশাক								
কলমিশাক								
পাটশাক								
কুমড়াশাক								
আলু								
মিষ্টি আলু								
বেগুন								
এলা								
মিষ্টিকুমড়া								
পটল								
গীম								
গাজর								
বাধাকপি								
শালগম								
টমেটো								
মটরভুটি								
ড়িচিন্সা								

বরবটি							
ফুলকপি							
কাঁচা পেঁপে							
ওলকপি							
কচুর মুখি							
করলা							
কাকরোল							
অন্যান্য							
Fruits							
নারকেল							
আম							
কাঁঠাল							
কলা							
পাকা পেঁপে							
পেয়ারা							
অন্যান্য							
Oils/Fats							
গয়াবিন							
সানফ্লাওয়ার							
গরিষা							
ঘি							
ডালডা							
পামওয়েল							
মার্জারিন							
রসুনের তেল							
Sweets							
ডমষ্টি							
পায়েস							
পুডিং							
সুজি							
সেমাই							
অন্যান্য							

Interviewer signature

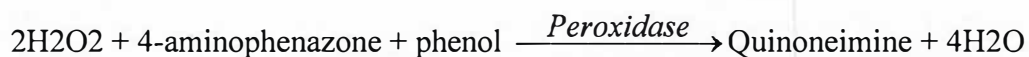
APPENDIX-III

Estimation of serum blood glucose

Serum glucose was estimated by enzymatic colorimetric (GOD-PAP) method

Principle (Barham, Trinder, 1972)

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red violet quinoneimine dye as indicator.



Reagents

Contents	Initial concentration of solution
Buffer	
Phosphate Buffer	0.1 mol/l, pH 7.0
Phenol	11 mol/l
GOD-PAP Reagent	
4-aminophenazone	0.77 mmol/l
Glucose oxidase	≥1.5 kU/l
Peroxidase	≥1.5 kU/l
Standard	
Glucose	5.55 mmol/l (100 mg/dl)

Additional Reagent

Uranyl Acetate 0.16% cat NO DP 647 2x 500 ml

Materials required

Microcentrifuge tube

Micropipettes and pipettes with disposable tips

Microlab- 300 (Semiautoanalyzer)

Procedure

Procedure for glucose GOD-PAP assay without deproteinization. The instrument was calibrated before estimation.

Serum and reagent were taken in specific cup. They were arranged serially into the Microlab, 300 (Semi autoanalyzer). The Auto lab was programmed for the estimation of glucose and allowed to run with following procedure:

5 μ l sample and 500 μ l reagent were mixed and incubated at 370 C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

$$\text{Glucose concentration (mmol/l)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times 5.55$$

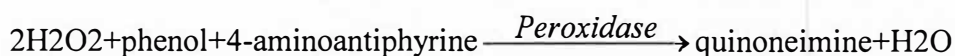
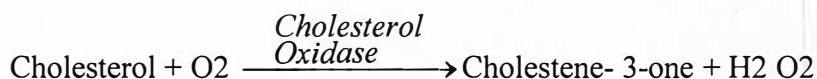
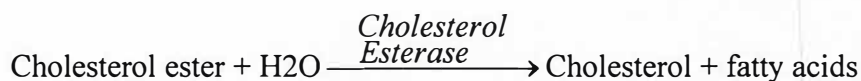
APPENDIX- IV

Estimation of serum Total Cholesterol (Bio Systems S.A. Barcelona Spain)

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/Peroxidase) method in Microlab, 300 (Semiautoanalyzer) using reagent of (BioSystems S.A. Barcelona Spain)

Principle

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.



Reagent composition

Contents	Initial Concentration of Solution
Reagent	
4-Aminoantipyrine	0.30 mmol/l
Phenol	6 mmol/l
Peroxidase	≥ 0.5 U/ml
Cholesterol esterase	≥ 0.15 U/ml
Cholesterol oxides	≥ 0.1 U/ml
Pipes Buffer	80 mmol/l; pH 6.8
Standard	5.17 mmol/l (200 mg/dl)

Materials

Microcentrifuge tube

Micropipettes and pipettes

Disposable tips

Micro lab, 300 (Semiautoanalyzer)

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the MICROLAB. 5 μ l sample and 500 μ l reagent were mixed and incubated at 37°C for 5 minutes within the Microlab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration of cholesterol in sample was calculated by using software program with the following formula.

$$\text{Cholesterol concentration (mg/dl)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times \text{concentration of standard.}$$

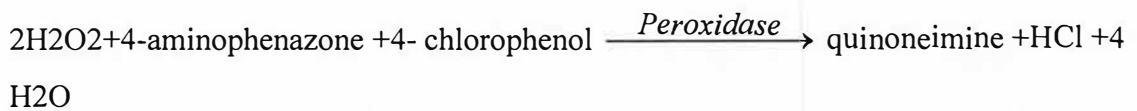
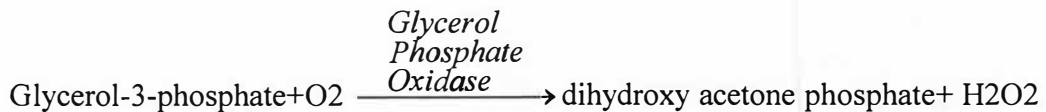
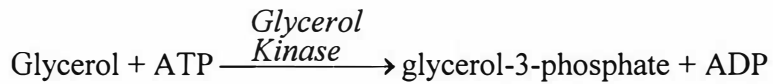
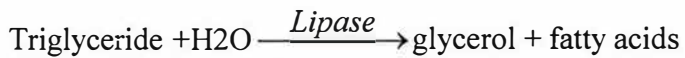
APPENDIX- V

Estimation of serum Triglycerides

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method Micro lab, 300 (Semi autoanalyzer) (BioSystems SA Barcelona Spain).

Principle

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen- peroxide, 4- aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.



Reagents

Contents	Concentrations in the Test
Buffer	
Pipes Buffer	40 mmol/l, pH 7.6
4-choloro-phenol	5.5 mmol/l
Magnesium-ions	17.5 mmol/l
2. Enzyme Reagent	
4-aminophenazone	mmol/l
ATP	1.0 mmol/l
Lipases	>150 U/ml
Glycerol-3-phosphate oxidase	1.5 U/ml
Peroxidase	0.5 U/ml
3. Standard	2.29 mmol/l (200 mg/dl)

Materials

Micropipettes and pipettes

Disposable tips

Micro lab, 300 (Semiautoanalyzer)

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the MICROLAB. 5 μ l sample and 500 μ l reagent were mixed and incubated at 37°C for 5 minutes within the MICROLAB.

The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Triglyceride concentration was calculated by using software program in MICROLAB with the following formula.

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times \text{Concentration of the standard.}$$

APPENDIX- VI

Estimation of Serum High Density Lipoprotein (HDL)

Serum High density lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method in Micro lab, 300 (Semiautoanalyzer) Barcelona (Spain).

Principle

HDL (High Density Lipoproteins) are separated from chylomicrons, VLDL (very low density lipoproteins) and LDL (Low density lipoproteins) by the addition of a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum or serum. After centrifugation, the cholesterol contents of HDL fraction, which remains in the supernatant, are determined by the enzymatic colorimetric method using CHOD- PAP.

Reagent composition

Contents

Buffer

Enzymes

Standard 50 mg/dl (1.29 mmol/l)

Materials

Microcentrifuge tube, Micropipettes and pipettes

Disposable tips

Micro lab, 300 (Semiautoanalyzer).

Procedure

Samples (200 μ l) and precipitating reagents (500 μ l) were taken in a microcentrifuge tube. Then it was mixed and allowed to sit for 10 minutes at room temperature. Then it was centrifuged at 4000 rpm for 10 minutes.

The supernatant was used as sample for determination of cholesterol content by the CHOD-PAP method. The sample and reagents were taken in specific cup or cell. They were arranged serially then ID number for test was entered in the MICROLAB. Then 5

μl sample and 500 μl reagent were mixed and incubated at 37°C for 5 minutes within the MICROLAB. The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration was calculated by using software program.

APPENDIX- VII

Estimation of LDL-Cholesterol

The LDL-Cholesterol level in serum was calculated by using by Friedewald formula (Friedewald WT. 1972)

Formula

$$\text{LDL cholesterol} = \text{Total cholesterol} - \left(\text{HDL Cholesterol} + \frac{1}{5} \times \text{Triglyceride} \right)$$

APPENDIX- VIII

Estimation of fasting serum Homocysteine

The AxSYM Homocysteine assay is a Fluorescence Polarization Immunoassay (FPIA) for the quantitative measurement of total L-homocysteine in human serum or plasma on the AxSYM system.

Principle:

Bound homocysteine (oxidized form) is reduced to free homocysteine that is enzymatically converted to S-adenosyl-L-homocysteine (SAH) as follows:

Reduction: Homocystine, mixed disulfide and protein-bound forms of Homocysteine in the sample are reduced to form free Homocysteine by the use of dithiotreitol (DTT).

Homocysteine-SS-Homocysteine

R1-SS-Homocysteine (R1=thiol residue) $\xrightarrow{\text{DTT}}$ Homocysteine Protein-SS-Hcy

Enzymatic conversion: Total free Homocysteine is converted to SAH by the use of SAH Hydrolase and excess adenosine.

Homocysteine + Adenosine $\xrightarrow{\text{SAH Hydrolase}}$ SAH

Under physiological conditions, SAH hydrolase converts SAH to homocysteine. Excess adenosine in the pretreatment solution drives the conversion of Homocysteine to SAH by the recombinant SAH hydrolase.

Reagents:

- S-adenosyl-L-cysteine Fluorescein Tracer in phosphate buffer with protein (bovine) stabilizer.

- S-adenosyl-L-homocysteine Hydrolase (recombinant) in phosphate buffer with protein (recombinant) stabilizer.
- Anti-S-adenosyl-homocysteine (mouse monoclonal) in phosphate buffer with protein (porcine) stabilizer.
- Pretreatment solution containing dithiothreitol (DTT) and adenosine in citric acid.

Calibrators and Controls:

Solution 4 (line diluent)

Procedure: 1(one) ml of venous blood was collected in a heparin or EDTA containing tube. Serum or plasma specimens may be used with the AxSYM Homocysteine assay. The samples were centrifuged at 1000 x g for 10 minutes. 50 µl of sample is the minimum volume required to perform the assay. Samples have been shown to be stable at -20°C for 8 months if measurement not done immediately. The refrigerated samples were mixed thoroughly after thawing to ensure consistency of the results.

The AxSYM Homocysteine reagents and samples were pipetted in the following sequence:

Sampling Center

- Sample and all AxSYM Homocysteine Reagents required for one test were pipetted by the sampling probe into various wells of a Reaction Vessel (RV).
- Sample, Pretreatment Solution, Solution 4 (Line diluent) and SAH Hydrolase Enzyme were pipetted into one well of the RV to make up the predilution mixture.

The RV was immediately transferred into the Processing center. Further pipetting was done in the processing center by the Processing probe.

Processing Center

- An aliquot of the predilution mixture, Antibody, and Solution 4 (Line diluent) were delivered to the cuvette of the RV.
- Tracer, Solution 4, and a second aliquot of the predilution mixture were transferred to the cuvette.

- SAH and labeled Fluorescein Tracer compete for the sites on the monoclonal antibody molecule.
- The intensity of polarized fluorescent light was measured by the FPIA optical assembly.

Expected Values: A majority of scientific literature agrees upon a range of normal values (adult male and female) between 5 and 15 $\mu\text{mol/L}$.

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