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Original Article

Association of Hyperhomocysteinemia with Early Onset Atherosclerotic Peripheral Arterial Occlusive Diseases

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Key Words : *Homocysteine, Atherosclerosis, Peripheral arterial occlusive diseases.*

Abstract:

Background: *Homocysteine is increasingly recognized as an independent risk factor for vascular disease specially peripheral arterial occlusive diseases. In the present study, an attempt was made to find out the association between the raised fasting plasma total homocysteine level and early onset atherosclerotic peripheral arterial occlusive diseases (PAOD).*

Methods: *In this prospective observational study a total of 50 study subjects were included. All patients were clinically and angiographically documented for atherosclerotic PAOD. All patients underwent surgical intervention as well as estimation of serum total homocysteine level. Arterial segment was sent for histopathological examination to find out whether atherosclerosis was present or not. Patients were divided into two groups- Group-1 included patients of 20-40 years of age and group-II included those of 41-60 years of age. The groups were compared to see association between elevated level of plasma homocysteine and atherosclerotic peripheral arterial occlusive diseases in elderly as well as in early age.*

Results: *41 (82%) patients were male and 9 (18%) patients were female. Serum homocysteine level was higher in group I than group II (71.4% vs. 40.9%). Besides, the level of mean serum homocysteine level was significantly (p=0.02) higher in group I than group II (21.18±9.53 vs. 17.24±8.92 µmol/L).*

Conclusion: *In conclusion, this study suggests that serum homocysteine has an association with early onset atherosclerotic PAOD. Therefore, a raised serum homocysteine level can be used as an independent biochemical predictor of early onset atherosclerotic PAOD.*

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Introduction:

Homocysteine is a sulfur-containing essential amino acid which is an intermediary product of methionine. B vitamins have a primary role as co-factors and substrates in homocysteine metabolism such that there is an inverse relationship between plasma homocysteine concentration and of folic acid, vitamin B6, and vitamin B12.[1]

Homocysteine is present in plasma in four forms: about 1% circulates as free thiol; 70-80% is disulphide –bound to plasma protein, chiefly albumin; and the remaining 20-30 % combines with itself to form the dimer homocysteine or with other thiols, including cysteine. The term 'total plasma homocysteine' refers to the combinded pool of all four forms of homocysteine.[2] Homocysteine and its derivatives cause endothelial dysfunction, intimal-medial thickening, oxidation of Low Density Lipoprotein (LDL), and a pro-coagulant state and eventually lead to premature atherosclerosis.[3] The normal fasting levels of homocysteine are between 5 and 15 µmol/L. Hyperhocysteinemia may be classified as mild to moderate (16 to 30 µmol/L), intermediate (31 to 100 µmol/l) and severe (more than 100 µmol/L).[3]

Hyperhomocysteinemia is now recognized as an independent risk factor of atherosclerosis. Homocysteine is an unstable amino acid, which

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undergoes autodigestion to produce free oxygen radicals. Hyperhomocystinemia thus causes increased production of free oxygen radicals and an oxidative stress.[4] A series of cross-sectional and case control studies and a meta-analysis of 27 observational studies strongly support that the increased level of plasma homocysteine is an independent risk factor for Coronary Artery Disease (CAD), cerebrovascular disease (CVD), and peripheral vascular disease.[5] Homocysteine induced vascular pathologies include fragmentation of the intimal elastic lamina, disruption of elastic fibers, smooth muscle hyperplasia, and arterial and venous thrombosis.[2]

Homocysteinemia is the result of deficiency in vitamin B6, B12 or folate, or a combination of them. Because these vitamins are essential cofactors of key enzymes related to metabolism of homocyteine, deficiency of these vitamins would impair the activity of these enzymes and lead to accumulation of homocysteine.[6] Atherosclerosis is an inflammatory process that is initiated as a response to injury and there is growing evidence of its relationship to inflammatory parameters. The association of inflammation with vascular disease is clearly established in non-diabetic individual.[7]

At the National Institute of Cardiovascular Diseases and Hospital (NICVD), there are a lot of patients suffering from Peripheral Arterial Occlusive Diseases (PAOD). While a significant number of these patients are actually suffering from Thrombo-Angitis Obliterans (TAO) or Buerger's disease, there are others in whom the angiographic and histomorphologic picture of Buerger's are not found. Rather, typical atheromatous tissue is found to be the cause of PAOD in these patients which has been confirmed macroscopically and histopathologically. These young patients of atherosclerotic PAOD pose considerable etiopathological dilemma. Considering the known association of homocysteine with PAOD, it may be speculated that raised homocysteine may have an important causative association with atherosclerotic PAOD in these patients.

Homocysteine has been a known risk factor of atherosclerosis. Raised serum level of homocysteine thus predisposes to atherosclerotic CAD and PAOD. Although the process of atherosclerosis begins early in life, its clinical manifestations usually occur in the elderly population. Thus, like CAD, atherosclerotic PAOD is basically a disease of the elderly.[8]

No previous study investigating the particular role of raised homocyteine in young patients with atherosclerotic PAOD in Bangladeshi population could be found. The present study may provide important insight in to the etiopathology of atherosclerosis in this patient population. Therefore, the study was undertaken to evaluate the possible association between higher serum level of homocystine and early onset atherosclerotic PAOD.

Study Methods

This is a Prospective observational study. Patients admitted in vascular surgery department of National Institute of Cardiovascular Diseases, Dhaka, Bangladesh during the period of January 2014 to December 2014. Patients with PAOD who were aged between 20 and 60 years and fulfilled the inclusion & exclusion criteria were considered for this study and a total 60 patients were enrolled. The inclusion criteria were patient's Age between 20 and 60 years and Patients with clinically and angiographically documented PAOD. The exclusion criteria were patient's Age <20 and >60 years; patients with Buerger's pattern angiographic picture, redo vascular surgery, emergency vascular surgery and PAOD patients presenting with extensive tissue loss/gangrene. It was a Purposive sampling technique. Therefore 50 patients were included in this study and the total sample size was 50. Ethical clearance for the study was taken from the Ethical Committee, NICVD.

Preoperative Variables were patient's Age (in years) and Sex (Demographic variables) others were Pulse, Blood pressure, Intermittent claudication, Rest pain, Ulcer and Smoking along with Fasting plasma total homocysteine level (tHcy), Blood sugar level, fasting serum lipid profile and Serum creatinine Postoperative variables: Histopathology of the occluded arterial segments.

Each of the patients was assessed by a complete medical history, thorough clinical examination and necessary preoperative investigations. Fasting total plasma homocysteine level was measured in

each patient. Other related investigations such as fasting blood sugar, serum creatinine, fasting serum lipid profile level of all patients were measured and recorded. Peripheral angiography was done to detect the site, extent, severity and pattern of stenosis or occlusion of affected arteries.

Demographic data such as age, sex, occupation was noted. Following risk factors were looked for: Smoking, hypertension, diabetes, dyslipidemia, family history of PAOD. Pulse, BP and other vital parameters were recorded.

A complete drug history was obtained. All prescribed medications were continued except aspirin & clopidogrel which were stopped 5 days preoperatively. An informed consent was taken from all patients for operation. Biopsy of the affected vessels harvested intraoperatively was sent for histopathological diagnosis.

Arterial segments containing atherosclerotic plaque were prepared and stained with Hematoxylin and Eosin stain to see the cellularity and nature of the plaque, Silver stain to see the elastic fibers of the arterial wall and Masson's trichrome stain to see the collagen content.

Data was collected purposively. The numerical data obtained from the study was analyzed and significance of difference was estimated by using statistical method. Qualitative data was expressed in frequency with corresponding percentage and quantitative data was expressed in mean and standard deviation. Comparison between groups was made by Chi square test and student's t test. All data from the study were analyzed by SPSS® statistical soft ware for windows. Multivariate regression analysis was done to establish dependent variable.

Patients were divided into two groups; Group A included patients aged between 20-40 years Group B included those aged between 41-60 years.

Results:

A total of 50 patients were included who were divided into two groups; Group-I (20-40 years of age) consisted of 28 patients and Group-II (41-60 years of age) was 22 patients. All the variables like baseline characteristics and outcome variables were compared between these two groups. Almost equal distribution of patients in group I and group II; 56% and 44% respectively. The study demonestrated that 41 (82%) patients were male and 9 (18%) patients were female. The mean age was 39.4±9.1 years. Analysis revealed statistically insignificant mean age difference (p=0.39) between male and female (39.8±9.2 vs 37±8.5) patients in unpaired t-test. 92.9% and 81.8% patients in the group I and group II presented with rest pain respectively. Ulcer was present in 10.7% and 9.1% patients in group I and group II respectively. Systolic blood pressure were (115.93±10.83 vs. 114.57±13.89 mmHg) in group I and group II respectively. Diastolic blood pressure were (76.67±8.98 vs. 73.91±10.76 mmHg) in group I and group II respectively. The above all parameters were observed in both study groups with insignificant association (p>0.05). Besides, pulse were (78.0±8.62 vs. 73.22±4.64 bpm) in group II and group I respectively. Considering the risk factor smoking was found 13 (46.1%) patients in group I and 11 (50.8%) patients in group II. Difference between the two groups was statistically insignificant (p=0.81). Analysis revealed that vasoactive drug was significantly (p=0.01) used in group I than group II (92.9% vs. 59.1%). The use

Table-I

Comparison of age and sex between study & control groups (N=50).

Age in years	Male (n=41)		Female (n= 9)		Total (n= 50)		p value
	Number	%	Number	%	Number	%	
20 – 40	23	56.1	5	55.6	28	56.0	
41 – 60	18	43.9	4	44.4	22	44.0	
Mean ± SD	39.8±9.2		37±8.5		39.4±9.1		0.39^{ns}

ns = Not significant (p>0.05)

p value reached from unpaired t-test

of antiplatelet was more in group I than group II (89.3% vs. 68.2%) but the difference between two groups was not statistically significant (p=0.06). biochemical investigations like fasting blood sugar, total cholesterol, triglyceride, LDL cholesterol and creatinine level are higher in group II patients than group I patients with statistically insignificant difference (p>0.05). HDL cholesterol level was found to be higher in group I than group II patients with statistically insignificant difference (p=0.80).

Table I shows an almost equal distribution of patients in group 1 and group II; 56% and 44% respectively. The table also demonstrates that 41 (82%) patients were male and 9 (18%) patients were female. The mean age was 39.4±9.1 years. Analysis revealed statistically insignificant mean age difference (p=0.39) between male and female (39.8±9.2 vs 37±8.5) patients in unpaired t-test.

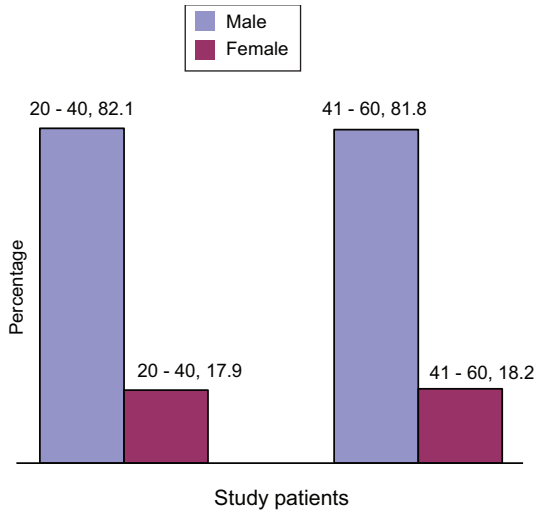

Fig.-1: *Sex distribution among the study patients by bar diagram (N=50).*

Group-I (20-40 years of age) and Group-II (41-60 years of age)

Table-II

Comparison of risk factor distribution between two groups (N=50).

Risk Factors	Group I (n = 28)		Group II (n = 22)		p value
	Number	%	Number	%	
Smoking					
Smoker	13	46.1	11	50.0	0.81^{ns}
Not smoker	15	53.6	11	50.2	

Group-I (20-40 years of age) and Group-II (41-60 years of age)
ns = Not significant (p>0.05)
Data were analyzed using Pearson Chi-Square ($\div^2$) test.

Table III

Comparison of medications between two groups (N = 50).

Drugs	Group I (n = 28)		Group II (n = 22)		Total (n= 50)		p value
	Number	%	Number	%	Number	%	
Antiplatelet	25	89.3	15	68.2	40	80.0	0.06^{ns}
Anticoagulant	17	60.7	14	63.6	31	62.0	0.83^{ns}
Vasoactive	26	92.9	13	59.1	39	78.0	0.01^{s}
Antispasmodic agents/ smooth muscle relaxants	15	53.6	16	72.7	31	62.0	0.16^{ns}

Group-I (20-40 years of age) and Group-II (41-60 years of age)
ns = Not significant (p>0.05), s= Significant (p<0.05)
P value reached from Chi-Square ($\div^2$) test and Fisher's Exact test.

Table IV

Comparison of biochemical parameters between two groups (N = 50).

Biochemical parameters	Group I (n = 28) Mean ± SD	Group II (n = 22) Mean ± SD	p value
FBS (mmol/L) 5.18±1.60	6.42±1.21	0.98^{ns}	
Total Cholesterol mg/dl	191.96±47.50	199.87±49.44	0.56^{ns}
Triglyceride mg/dl	188.70±69.66	208.57±93.93	0.39^{ns}
LDL cholesterol mg/dl	111.44±36.77	113.65±52.38	0.86^{ns}
HDL cholesterol mg/dl	39.4±5.8	37.8±5.5	0.80^{ns}
Creatinine mg/dl	1.02±0.23	1.16±0.29	0.24^{ns}

Group-I (20-40 years of age) and Group-II (41-60 years of age)
ns = Not significant (p>0.05), s= Significant (p<0.05)
P value reached from unpaired t test.

Table-V

Comparison of homocysteine level between two groups (N = 50).

Serum homocystine µmol/L	Group I (n = 28)		Group II (n = 22)		Total (n= 50)		p value
	Number	%	Number	%	Number	%	
Elevated (>15)	20	71.4	9	40.9	29	58.0	
Normal (5-15)	8	28.6	13	59.1	21	42.0	
Mean ± SD	21.18±9.53		17.24±8.92		21.48±9.54		0.02^{s}

Group-I (20-40 years of age) and Group-II (41-60 years of age)
ns = Not significant (p<0.05), s= Significant (p<0.05)
P value reached from unpaired t test.

Table-VI

Factors related to early on-set atherosclerotic PAOD.

Variables of interest	Univariate analysis			Multivariate analysis		
	OR	95% CI of OR	p value	OR	95% CI of OR	p value
Smoking	1.89	0.797-3.538	0.17^{ns}	1.59	0.699-3.443	0.16^{ns}
Dyslipidaemia	1.02	0.989-1.431	0.36^{ns}	1.00	0.987-1.132	0.42^{ns}
Creatinine (mg/dl)	1.07	0.989-1.931	0.36^{ns}	1.00	0.987-1.032	0.42^{ns}
Hyperhomocysteinemia > 15 µmol/L	2.01	1.142-3.144	0.02^{s}	1.77	1.26-3.261	0.03^{s}

Dependent variable: Early on-set atherosclerotic peripheral arterial occlusive disease (PAOD) and smoking, dyslipidaemia, creatinine and hyper Hcy>15 µmol/L were taken as independent variables.
s= Significant (p<0.05), ns= Not significant (p>0.05)

Table II compares the risk factor between the study groups. Considering the risk factor smoking was found 13 (46.1%) patients in group I and 11 (50.8%) patients in group II. Difference between the two groups was statistically insignificant (p=0.81).

Table III showed medications of the study patients. Analysis revealed that vasoactive drug was significantly (p=0.01) used in group I than group II (92.9% vs. 59.1%). The table also projects that the use of antiplatelet was more in group I than group II (89.3% vs. 68.2%) but the difference between two groups was not statistically significant (p=0.06). Rest of the drug on the above table was used in both group of patients.

The above table reveals that raised serum homocystine level was higher in group I than group

II (71.4% vs. 40.9%). Besides, the level of mean serum homocystine level was significantly (p=0.02) higher in group I than group II (21.18±9.53 vs. 17.24±8.92 μmol/L). So it can be concluded that elevated plasma homocystine is responsible development of early onset atherosclerotic peripheral arterial occlusive disease (PAOD). Therefore, the aim of the study which was to evaluate the association between hyper-homocysteinemia and early onset atherosclerotic PAOD was statistically validated.

Univariate and Multivariate analysis: Table VI projects the Odds Ratio of binary logistic regression analysis for characteristics of the subjects likely to develop early on-set atherosclerotic PAOD. The variables revealed to be significantly associated with early on-set atherosclerotic PAOD by univariate analysis were all entered into the model directly. In univariate analysis, hyper-homocystinemia was observed as significant predictors for developing early on-set atherosclerotic PAOD with OR being 2.01. It was also observed in multivariate analysis, hyperhomocystinemia was found to be the independent predictors for developing early on-set atherosclerotic PAOD with OR being 1.77. Smoking, dyslipidaemia and serum creatinine were not observed as independent predictors for developing early on-set atherosclerotic PAOD (p>0.05).

Discussion:

No previous study could be found looking into the association between hyperhomocysteinemia and early onset atherosclerotic PAOD in Bangladesh. Reports on this subject are also few in published literatures worldwide. A few studies were found in India on hyperhomocysteinemia and peripheral vascular diseases. In these studies, age specification was limited and no study was found dealing specifically with early age. To the best of our knowledge, the present study is the first to evaluate the association between hyperhomocysteinemia and early onset atherosclerotic PAOD particularly in Bangladeshi population.

In this study mean age of male patients in both groups were 39.8 ± 9.2 and of female patient were 37 ± 8; in total it was 39.4±9.1 years. Analysis revealed statistically insignificant mean age difference between male and female patients.Overall, male- female ratio was 4.5 : 1. In a similar study conducted on atherosclerotic coronary artery diseases by Rahman MA, et al., the majority of patients were male and male-female ratio was 3.6 : 1, which was consistent with this study.[17,19]

The comparison of clinical parameters between the two groups showed 92.9% and 81.8% patients in group I and group II had rest pain and 10.7% and 9.1% patients had ulcer. Differences between the two groups were not statistically significant. Comparison of risk factor between the two groups revealed that smoking and dyslipidaemia were equally prevalent. A study conducted by Hackman et al. , showed negative association of smoking and serum lipid levels, which was consistent with this study. Analysis of medications revealed that the use of vasoactive drugs were significantly higher in group I than in group II. The study also found that the use of antiplatelet was more in group I than in group II but the difference was not statistically significant. The rest of the drugs were used more or less similarly in both groups. Analyses of biochemical parameters demonstrate that fasting blood sugar, total cholesterol, triglyceride, LDL cholesterol and creatinine level were higher in group II than in group I with statistically insignificant difference. [6,14,16]

Atherothrombotic vascular events were linked to elevated tHcy in patients with homocystinuria in 1969, but it was not until 1976 that a controlled study showed a clear association between moderately raised tHcy and atherosclerotic disease . Since then, a possible association between tHcy and atherothrombotic vascular disease has been examined in more than 12000 patients in more than 100 cross-sectional, case-control, and prospective cohort studies. A systematic review of data from these observational studies is complicated by variations in study design, type, and number of patients and controls; in the measurement of tHcy, in definitions of hyperhomocysteinaemia and definitions and measurement of other vascular risk factors and possible confounding factors, in methods of follow-up, types and definitions of vascular outcome events (and surrogate outcome measures); and in statistical analyses. Despite these reservations, a positive association was found between raised

plasma homocysteine and atherothrombotic vascular disease.[7]

The strongest epidemiological evidence for an association between hyperhomocysteinaemia and vascular risk comes from large prospective observational cohort studies.[7] The unequivocally positive studies-with relative risks (RR) whose lower 95% confidence interval is above 1-are the Physicians' Health Study, the British United Provident Association study (BUPA), the Tromso study, the British Regional Heart Study, and the study of Nygard et al. 1997. A strong graded relation has been reported between increasing tHcy and overall mortality in individuals with angiographically demonstrated coronary heart disease.[3] However, other prospective studies, including further reports from the Physicians' Health Study, have not found a significant association between hyperhomocysteinaemia and myocardial infarction or stroke.Published cross-sectional and case-control studies demonstrate a clear association between tHcy and the anatomical extent of carotid, coronary, aortic, and peripheral vascular disease.[7]

TT Genotype in the MTHFR gene is a strong predictor of raised tHcy in the general population but is not associated with an increased risk of vascular disease. [10,17]

Most retrospective case-control and cross-sectional studies have demonstrated a positive association between raised tHcy and an increased risk of initial and recurrent venous thrombosis, and this has been confirmed by a meta-analysis (RR=2·95 [2·08–4·17]) in patients with fasting hyperhomocysteinaemia.[7,17]

There is a large body of epidemiological evidence that links increasing tHcy with an increasing risk of atherothrombotic vascular disease. There is no complete consistency usually because of differences in study design, statistical power, and chance. A systematic review of studies with the same types of patients and controls and the same methods and outcome events could provide a more accurate estimate of the true association between tHcy and vascular risk.[11-13]

Assuming that a strong, dose-dependent, and positive association does exist, there is reasonable evidence, from regression analyses, that the association is independent of other factors known to be associated with raised tHcy (eg, age, sex, smoking, low physical activity, blood pressure, and cholesterol).[3]

Furthermore, homozygotes for the three distinct autosomal-recessive inborn errors of homocysteine metabolism (CâS deficiency, MTHFR deficiency, and the cobalamin metabolic defects that impair methionine synthase activity have tHcy concentrations that are 10–50 times higher than those in the general population and a very high risk of premature atherothrombotic vascular disease.[7]

The number of patients with elevated serum homocysteine level was more in group I than in group II (71.4% vs. 40.9%). Likewise, the mean level of serum homocystine level was also significantly (p=0.02) higher in group I than group II (21.18±9.53 vs. 17.24 ± 8.92 μmol/L). This data suggests a positive association between raised plasma homocysteine and early onset atherosclerotic peripheral arterial occlusive diseases (PAOD). Therefore, the aim of the study which was to evaluate the association between hyperhomocysteinemia and early onset atherosclerotic PAOD was statistically validated. A study conducted by Ian M Graham et al., showed an elevated plasma tHcy level is now established as a strong and indipendent factor associated with all categories of atherosclerotic disease in both men and women , which is consistent with this study. This study ascertained smoking, dyslipidemia and serum creatinine as dependent variables of early onset atherosclerotic peripheral arterial occlusive disease (PAOD) were while hyperhomocysteinemia (tHcy>15 μmol/L) was taken as independent variable.The binary logistic regression analysis of Odds Ratio for characteristics of the subjects likely to develop early onset atherosclerotic PAOD. The variables revealed to be significantly associated with early onset atherosclerotic PAOD by univariate analysis were all entered into the model directly. In univariate analysis, hyperhomocystinemia was observed as significant predictor for developing early onset atherosclerotic PAOD with OR being 1.78. It was also observed in multivariate analysis, hyperhomocystinemia was found to be the independent predictor for developing early on-set atherosclerotic PAOD with

OR being 1.22. Smoking, dyslipidaemia and serum creatinine were not observed as independent predictors for developing early on-set atherosclerotic PAOD (p>0.05). Univariate regression analysis of Das et al. revealed that an odd ratio of 1.4 for hypercholesterolemia, 0.6 for hyper triglyceridemia , 0.7 for low HDL cholesterol, 1.5 for high LDL cholesterol, 2.6 for smoking and 2.4 for diabetes. None of these risk factors had significant association with PAOD which is consistent with this study.[15,18]

Conclusion:

In conclusion, this study suggests that serum homocysteine has an association with early onset atherosclerotic PAOD. Therefore, a raised serum homocysteine level can be used as an independent biochemical predictor of early onset atherosclerotic PAOD. This study provided evidence that elevated level of fasting plasma total homocysteine level is not only signiûcantly associated with early onset PAOD but also directly with the severity of PAOD.

Conflict of Interest - None.

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