

## RESEARCH ARTICLE

## Lack of association of methylenetetrahydrofolate reductase 677C>T mutation with coronary artery disease in a Pakistani population

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### ABSTRACT

Pakistanis belong to the South Asian population which has the highest known rate of coronary artery disease. Folic acid deficiency also appears to be highly prevalent in this population. Methylenetetrahydrofolate reductase (*MTHFR*) 677C>T polymorphism decreases the activity of this enzyme and can be associated with mild to moderate hyperhomocysteinemia in homozygotes, particularly when there is folic acid deficiency, as well as with coronary artery disease. To assess the value of genotyping the *MTHFR* 677C>T dimorphism, we carried out a case-control study of dimorphism 677C>T for putative association with myocardial infarction (MI) among Pakistani nationals. We investigated a sample population of 622 Pakistanis consisting of 225 controls and 397 patients with clinical diagnosis of acute MI (AMI). *MTHFR* C677T alleles were determined by assays based on polymerase chain reaction and restriction endonuclease analysis. Frequencies of C alleles were 0.87 among controls and 0.86 among AMI patients. The *MTHFR* 677C>T dimorphism showed no association with MI ( $\chi^2 = 0.25$ , 1df,  $P=0.62$ ), serum levels of folate and vitamin B12 and plasma level of vitamin B6. A significant association, however, was found between homozygous 677T genotype and plasma levels of homocysteine. Multivariate analysis of the data showed that in case of log homocysteine, age and *MTHFR* genotypes were significantly different ( $P<0.001$ ). In case of B12, smoking and age were found to be statistically significant ( $P<0.001$ ), while in case of serum folate only smoking was found to be significant ( $P<0.001$ ). The results indicate that *MTHFR* 677C>T polymorphism, though associated with homocysteine levels, confers no significant risk of coronary artery disease in the Pakistani population investigated here. We suggest that the higher incidence of AMI in South Asia occurs through mechanisms other than the *MTHFR* related pathways.

**KEYWORDS:** *Coronary artery disease, folic acid, homocysteine, methylenetetrahydrofolate reductase, mutation, myocardial infarction, Pakistani population*

### INTRODUCTION

Pakistani people belong to a population which has the highest rate of coronary artery disease (CAD; Nishtar, 2002). Moreover, the relative risk of CAD in South Asian men is highest at early ages (Balarajan, 1991; McKeigue, 1992) suggesting that both intrinsic and extrinsic factors contribute to the development of CAD in this population.

Methylenetetrahydrofolate reductase (*MTHFR*) catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-

methyltetrahydrofolate, a carbon donor in the methylation of homocysteine to methionine. A C677T mutation in the *MTHFR* gene (*MTHFR*) converts an alanine residue to a valine residue and functionally impairs the enzyme (Frosst et al, 1995). The aberration leads to hyperhomocysteinemia, an independent risk factor for cardiovascular disease (Aguilar et al, 2004).

Although numerous reports have brought somewhat conflicting views about the involvement of *MTHFR* in CAD, it is now commonly accepted that the *MTHFR* gene and its

molecular variant 677C>T represent one of the best examples to date of genetic influences that constitute inherited predispositions to CAD and allied clinical manifestations in humans (Kluijtmans, 1996). Association (case-control) studies are increasingly preferred as methods for understanding genetic influences of molecular variants (Lander, 1996; Risch and Merikangas, 1996). Data from genetically isolated populations are important in the resolution of problems related to the concept of linkage disequilibrium (Schork, 1997).

Chambers et al (2000) pointed out that Indian Asians residing in the United Kingdom have twice the rate of coronary heart disease deaths compared to European Caucasians, and hyperhomocysteinemia in this population could be a contributing factor. However, *MTHFR* 677T mutation was not found to be associated with hyperhomocysteinemia or increased CAD risk in Indian Asians compared to Europeans (Chambers *et al.*, 2000). In a similar case-control study on Indians by Mukharjee et al (2002) a low prevalence of *MTHFR* 677T mutation was observed in both normal healthy subjects and CAD patients, however, an association of the mutant allele with CAD was found in women.

While mild hyperhomocysteinemia has been reported to be quite common among South Asians including the Pakistanis (Sastri et al, 2001; Aamir et al, 2004; Iqbal et al, 2005), the role of *MTHFR* mutation in causing this hyperhomocysteinemia is still unclear.

Since folate, vitamin B6 and vitamin B12 deficiencies are very common in Pakistani patients with acute myocardial infarction (AMI; Iqbal et al, 2005), the role of *MTHFR* mutations could be of more significance in causing hyperhomocysteinemia, and thereby, increasing the risk for the development of CAD.

The present study was undertaken to assess the usefulness of *MTHFR* marker 677C>T in the Pakistani population as an independent risk factor for CAD in Pakistanis. A case-control study of dimorphism 677C>T was carried out for a putative association with AMI (a presentation of CAD) and with plasma or serum levels of homocysteine, folate, vitamin B12 and vitamin B6 (pyridoxal phosphate, PLP) amongst Pakistani nationals.

## MATERIALS AND METHODS

### Patients and methods

Three hundred and ninety seven consecutive Pakistani patients with AMI (age range: 30-74 years) admitted to the National Institute of Cardiovascular Diseases (NICVD), Karachi from January 2001 to June 2001, and the Armed Forces Institute of Cardiology, Rawalpindi from August 2003 to September 2003, were included in this study. They were enrolled within the index admission after confirmation of AMI diagnosis based on WHO criteria of clinical history suggestive of myocardial ischaemia, ECG indications of myocardial damage, and elevation of biochemical markers (creatinine kinase and creatine kinase-MB). All patients were assessed as having risk factors for CAD, such as diabetes mellitus, hypertension, obesity,

hypercholesterolemia and smoking. Criteria for diabetes were set as an abnormal fasting blood glucose level > 125 mg/dl at admission, or having taken hypoglycemic agents. All those with systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure of 90 mmHg or those on regular antihypertensive medications were classified as hypertensive. Those with serum cholesterol levels greater than 200 mg/dl were considered to have hypercholesterolemia. A body mass index (BMI) of greater than 30 was classified as obese. Subjects were considered smokers if they had been smoking cigarettes regularly (one or more per day). Individuals who were pregnant, using antiepileptics, taking oral contraceptives, having malabsorption syndrome, suffering from tuberculosis, liver disease, uremia, or cancer or using vitamin B-complex supplements during the previous 6 months were excluded from the study.

Similarly, 225 normal healthy subjects, who had been matched for sex, BMI, and for age within 5 years and belonging to the same socio-economic class, were selected as controls during the same period from the personnel of the Aga Khan University and Civil Hospital, Karachi, and Armed Forces Institute of Cardiology and Military Hospital, Rawalpindi. They were also assessed for the above mentioned risk factors. However, more stringent criteria were used for the selection of normal healthy control subjects. In addition to being matched for BMI, sex, age and socioeconomic background, they had no evidence of CAD and hypertension on the basis of their clinical history and physical examination. Informed consent was obtained from the participants and the study was approved by the Ethical Review Committee of the Aga Khan University.

### DNA analysis

DNA was extracted from 5 ml blood samples according to published methods (Sambrook et al, 1989). C677T of the *MTHFR* gene was amplified by polymerase chain reaction (PCR) according to protocol conditions and primer sequences published previously (Frosst et al, 1995), except that PCR volumes were scaled down to 10 µl. A Perkin-Elmer Gene Amp PCR system 9700 was utilized (Wellesley, MA, USA). C677T PCR products were digested with *Hinf*I (New England Biolabs, Beverly, MA) at 37°C for 3 hr, size-separated by gel electrophoresis using 2% (w/v) agarose at 150 volts for 1 hr, and visualized under UV light after staining with ethidium bromide. 677C alleles were identified as 198 base pairs (bp) fragments and 677T alleles as 175 and 23 bp fragments (Frosst et al, 1995).

### Estimation of vitamin B12, folate, B6 and homocysteine in serum/plasma

Fasting venous blood was obtained from cases as well as controls. Serum samples were analyzed for folate and vitamin B12 using radioassays (Chanarin, 1989; Quadros, 2000). Plasma samples from both cases and controls were screened for pyridoxal phosphate (PLP; coenzymic form of vitamin B6) and homocysteine. PLP in plasma was determined using a method described previously (Iqbal, et al, 2003). Determination of plasma homocysteine was carried out using a kit based on fluorescence polarization immunoassay (Abbott Laboratories, Ltd., Pakistan).

### Data analysis

Statistical analyses were done with the help of an SPSS® (Statistical Package for Social Sciences) software version 13.0 for Windows® Apache Software Foundation, USA). Distribution differences of *MTHFR* genotypes in AMI patients as compared to controls, as well as Hardy-Weinberg proportions of allele distribution, were assessed using chi-square test. Homocysteine values were converted to logs, as the data were not normally distributed. Means were compared using one way analysis of variance (ANOVA). Significant differences were identified using a Post-Hoc test (Dunnett T3) for *MTHFR* genotypes, when homogeneity of variance was found to be significant.

Multivariate analysis was performed using values for log homocysteine, vitamin B12, vitamin B6 and folate as outcome variables, whereas cases and controls, *MTHFR* genotypes, age and smoking status were independent variables. A p-value less than 0.05 was considered significant.

### RESULTS

Table 1 shows the demographic and clinical characteristics of patients and normal healthy control subjects. Patient and control groups had female to male ratios of about 1:3. The mean age was slightly higher for patients. Mean BMI values for the two groups were similar.

Table 2 shows the distributions of 677C and 677T alleles, as well as those of genotypes of *MTHFR* 677C>T dimorphism in AMI patients and controls. Frequencies of the 677T alleles were 0.13 in controls and 0.14 in cases.

*MTHFR* genotypes were found to occur in Hardy-Weinberg proportions in the normal as well as the patient groups ( $P=0.53$  and  $P=0.45$  with 2 df, respectively).

We also explored association of *MTHFR* genotypes with several phenotypic variables, including plasma homocysteine, serum folate, serum B12 and plasma PLP (Table 3). While studying the association between *MTHFR* genotypes and homocysteine, log transformation of homocysteine revealed that homocysteine value in the TT group was significantly different from that of both CC ( $P<0.03$ ) and CT groups ( $P<0.004$ ) for both patients and controls. However, between cases and controls, differences were not found to be statistically significant. In multivariate analysis, log homocysteine, B12 and folate were found to be significant. With regard to log homocysteine, age and *MTHFR* genotypes were found to be significantly different ( $P<0.001$ ). With B12, smoking and age were found to be statistically significant ( $P<0.001$ ), whereas for serum folate, only smoking was found to be significant ( $P<0.001$ ).

### DISCUSSION

Normal healthy subjects included in this study constituted a “comparison” group, rather than a true control group. Since they had no evidence of CAD on the basis of their clinical history and were matched for sex, age and BMI, they represented a valid comparison group. However, it is likely that some of them will develop CAD in future, and some might even have heart disease at a subclinical, i.e. undetectable level.

**Table 1.** Demographic and clinical characteristics of Pakistani subjects

	Controls (n=225)		AMI Patients (n=397)	
	Mean $\pm$ SD (Range)	Frequency (%)	Mean $\pm$ SD (Range)	Frequency (%)
<b>Age</b> (Years)	49 $\pm$ 8.8 (30-75)		53.5 $\pm$ 10.1 (30-74)	
<b>Gender</b> (Male) (Female)		173 (76.9) 52 (23.1)		309 (77.8) 88 (22.2)
<b>Body mass index*</b> (kg/m <sup>2</sup> )	23.86 $\pm$ 5.3 (13.48-45.28)		25.1 $\pm$ 3.9 (10.77-43.75)	
>30 (obese)		19 (8.4)		43 (10.8)
<b>Smoking status*</b> (smokers) (non-smokers)		38 (17.2) 183 (82.8)		109 (28.6) 272 (71.4)
<b>Hypertension*</b> (Yes) (No)		0 225 (100)		175 (44.9) 215 (55.1)
<b>Diabetes*</b> (Yes) (No) Glucose (mg/dl)		6 (2.6) 219 (97.4)		122 (31.4) 267 (68.6)
	96 $\pm$ 27 (51-230)		137 $\pm$ 76 (51-578)	
<b>Hypercholesterolemia*</b> (Yes) Total-cholesterol (mg/dl)		13 (5.8)		70 (17.6)
	166 $\pm$ 35 (61-230)		179 $\pm$ 49 (68-457)	

\*As defined in the *Patients and Methods* section.

**Table 2.** Distribution of *MTHFR* 677C>T genotypes and allele frequencies (Means± standard errors) in normal healthy subjects and AMI patients

Genotype*	Normal healthy subjects (n=225)	AMI patients (n=397)	Odds Ratio (OR) (95% C.I.)
	n(%)	n(%)	
CC	161(71.6)	279(70.2)	0.7 (0.2-1.8)
CT	57(25.3)	110(27.7)	0.6 (0.2-1.7)
TT	7(3.1)	8(2.0)	1
p(C allele)**	0.87 ± 0.02	0.86 ± 0.02	
q(T allele)**	0.13 ± 0.02	0.14 ± 0.02	

\* $\chi^2=1.029$ ; 2 df;  $P=0.598$ \*\* $\chi^2=0.25$ ; 1 df;  $P=0.62$ **Table 3.** *MTHFR* genotypes, age and concentrations of plasma/serum homocysteine, folate, vitamin B12 and PLP in normal healthy subjects (controls) and AMI patients

Subjects	MTHFR Genotype		
	CC	CT	TT
<b>Controls</b>			
Age (years)	49.7 ± 8.7	48 ± 8.7	40.9 ± 8.0
Fasting homocysteine (µmol/l) [Log transformed]	2.79 ± 0.33	2.86 ± 0.36	3.21 ± 0.65
Serum folate (ng/ml)	4.62 ± 2.93	5.01 ± 2.71	2.89 ± 4.1
Serum B12 (pg/ml)	547 ± 314	517 ± 260	384 ± 177
Plasma PLP (nmol/l)	21.3 ± 15.7	22.1 ± 12.2	34 ± 30.6
<b>AMI Patients</b>			
Age (years)	54 ± 10.2	52.7 ± 9.7	47.4 ± 10.6
Fasting homocysteine (µmol/l) [Log transformed]	2.83 ± 0.38	2.83 ± 0.44	3.56 ± 0.42
Serum folate (ng/ml)	3.9 ± 3.1	3.8 ± 2.7	2.3 ± 2.7
Serum B12 (pg/ml)	250 ± 171	276 ± 199	226 ± 121
Plasma PLP (nmol/l)	22.3 ± 24.8	22.3 ± 19.9	16 ± 11.8

Pakistan is facing a huge challenge in combating CAD (Ahmad, 2002; Bhopal, 2002). According to the most careful estimates, nearly 100,000 individuals in a total population of about 150 million suffered AMI in Pakistan in the calendar year 2002 (Samad, 2003). This rate is alarmingly high. High prevalence of folate, B12 and B6 deficiencies along with mild hyperhomocysteinemia could be contributing to this high CAD rate (Iqbal et al, 2005). These observations pointed towards the possible role of *MTHFR* gene in the development of CAD in this population.

Several studies have suggested that *MTHFR* 677C>T mutation confers increased risk for CAD (Kluijtmans et al,

1997). At the same time, quite a few reports show no association between this mutation and the risk of CAD, especially among Asians (Chambers et al, 2000, Wang et al, 2001, Kim et al, 2001, Mukherjee et al, 2002). In the present study, we report that the allele frequencies of the *MTHFR* 677C>T polymorphism in the Pakistani normal subjects are 0.87 ± 0.02 and 0.13 ± 0.02 for the 677C and 677T alleles, respectively, while in AMI patients, these are 0.86 ± 0.02 and 0.14 ± 0.02, respectively. This low prevalence of mutant allele (T allele) among cases and controls in our study compares well with “T” allelic frequencies of 0.18 and 0.19 in normal healthy subjects and CAD patients, respectively, in a population of Asian Indians (Mukherjee et al, 2002). Similarly, the overall homozygous

MTHFR TT variant frequency of 0.024 in this study compares well with an observed frequency of 0.02 among South Asians living in England (Cappuccio et al, 2002). Analysis of our data revealed that C677T genotype distributions were in Hardy-Weinberg proportions, both in the control group and AMI patient group.

Our data did not show any significant association between 677C>T polymorphism and AMI (a presentation of CAD) in the Pakistani population sampled. Since the overall effect of *MTHFR* mutation 677C>T on CAD has been postulated to occur through hyperhomocysteinemia, we searched for any association between plasma levels of homocysteine and *MTHFR* 677C>T polymorphism. Many reports based on data from various populations show association of homozygous *MTHFR* 677T genotypes with raised plasma homocysteine concentrations (Frosst et al, 1995; Ma et al, 1996; Kluijtmans et al, 1996; Kluijtmans et al, 1997; Verhoef et al, 1997; Brattstrom et al, 1998; Nair et al, 2002; Yilmaz et al, 2005), especially in the presence of low folate (Jasques et al, 1996; Ma et al, 1996; Christensen et al, 1997; Schwatz et al, 1997; Gemmati et al, 1999). Our results are quite consistent with these observations; in both AMI patients and controls (with serum folate levels quite low compared to most Western populations), homozygous *MTHFR* 677T genotype was found to be associated with elevated concentrations of plasma homocysteine ( $P < 0.001$ ).

Since total serum levels of homocysteine have been shown to depend on both genetic and environmental factors, the role of folate status cannot be overlooked. Although differences in serum folate in the present study are not statistically significant between genotypes, homozygous mutant individuals (TT genotype) do have lower serum folate in both controls and AMI patients (Table 3). It is possible that the mutation itself may affect serum folate levels because 5-methyltetrahydrofolate is the primary circulating form of folate and in severe *MTHFR* deficiency, the proportion of intracellular folate (5-methyltetrahydrofolate) has been shown to be reduced (Rosenblatt, 1995).

Matsushita et al (1997) reported that frequencies of homozygous *MTHFR* genotypes 677T decrease with age in the normal population, and that younger people (< 55 years) have higher frequency of homozygous *MTHFR* mutation than older ones (> 55 years). Chamber et al (2000) also reported a higher frequency of homozygosity for *MTHFR* 677T in cases with onset of coronary heart disease before the age of 50 years. In the present study, a similar nonsignificant ( $P=0.25$ ) trend was observed as homozygous *MTHFR* mutation (TT genotype) occurred in 1.5% of subjects over 50 compared to 3.5% in those below the age of 50. Further studies involving a larger sample size would be required to ascertain whether younger subjects have significantly high prevalence of TT genotype as it might account for the high risk of CAD in our younger population (Virk et al, 1995).

Frequencies of *MTHFR* 677T homozygotes in both cases and controls in our population were 2% and 3.1%,

respectively (Table 2). These values are quite comparable with those reported by Chambers et al (2000) who have found these frequencies to be 1% (in coronary heart disease patients) and 3% (in normal controls) among Indian Asians residing in the United Kingdom. Mukherjee et al. (2002) found these values to be 2% in normal control subjects, and zero in CAD patients among Asian Indians. In contrast, the frequencies of homozygous mutant gene in controls and cases in three US populations were in the range 10% - 13.1% (Ma et al, 1996; Schwartz et al, 1997; Anderson et al, 1997), 10.5% - 10.6% in Western Australians (Van Bockxmeer et al, 1997), 10% - 14% in Canadians (Christensen et al, 1997), 9% - 11% in Poles (Goracy et al, 1999), 12.3% - 12.8% in Chinese (Zheng et al, 2000), 5.8% and 12.1% in Slovaks (Raslova et al, 2001) and 10% - 14% in European Caucasians (Chambers et al, 2000), respectively.

While homozygosity for *MTHFR* 677T is associated with hyperhomocysteinemia in the Pakistani population, it appears not to be an important determinant of increased CAD mortality in this population. Mild hyperhomocysteinemia, perhaps through an interplay with other cardiovascular risk factors, such as smoking, dyslipidemia (especially low HDL cholesterol), nutritional deficiencies (folate, B12 and selenium), and raised fasting glucose could be enhancing the risk of CAD in Pakistan.

## CONCLUSIONS

- *MTHFR* mutation (677 C>T) is relatively rare among Pakistanis and the *MTHFR* TT genotype does not appear to be a risk factor for AMI in the population investigated.
- We hypothesize that the higher AMI incidence in South Asia (especially in Pakistan) occurs through mechanisms other than the *MTHFR* related pathways.
- Bearing in mind the complex nature of genetic susceptibility for chronic degenerative diseases, further studies need to be conducted in individual ethnic groups (involving younger subjects) taking into account their nutritional status in terms of vitamins B6, B12 and folic acid to verify the disease relevance of this polymorphism.

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## STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

## LIST OF ABBREVIATIONS

MI: Myocardial infarction  
 AMI: Acute myocardial infarction  
 CAD: Coronary artery disease  
 BMI: Body mass index

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