Original article

RELATIONSHIP BETWEEN METHYLENE TETRA HYDRO FOLATE REDUCTASE (MTHFR) GENE POLYMORPHISM AND HYPERHOMOCYSTEINEMIA IN STROKE

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ABSTRACT

Cardiovascular disease is a major cause of mortality in Indonesia. Hyperhomocysteinemia (hyper-hcy) is an independent cardiovascular risk factor, which may be due to methylene tetrahydrofolate reductase *(*MTHFR) deficiency, frequently linked to MTHFR gene mutation. This case-control study examined the relationship between homocysteine (hcy), folate, and vitamin B12 plasma concentrations with C677T mutation of MTHFR gene among 20 haemorrhagic and non-haemorrhagic stroke patients aged 18-55 years, in Sanglah Hospital, Denpasar. 10 age-matched controls were selected via random sampling of 1 of 4 neighbours; all subjects were Balinese. Hyper-hcy (X^2: 5.4; PR: 1.8; 95% CI: 1.0-2.7; p=0.03), hypertension (X^2: 13.12; PR 2.66; 95%CI 1.41 to 5.02; p=0.00) were associated with increased risk of stroke. There were no significant correlation between plasma hcy levels and plasma folate and vitamin B_{12} levels as co-factors of hcy metabolism. Low plasma vitamin B_{12}, smoking, alcohol drinking, and hypertension tend to be determinant factors of hyper-hcy. This study found no mutation on 677 from C to T (C677T), however there were substitution in nucleotides among stroke and controls, with or without producing chances of amino acids, including: 1) G659A substitution that caused changing in amino acid from glutamine to glycine found in 1 stroke patients with hyper-hcy; 2) A660G substitution that cause changing in amino acid from glutamine to glycine found among all control subjects and among 3 stroke patients, one of whom had hyper-hcy; and 3) A661G substitution that cause changing in amino acid from lysine to glutamine found in one stroke patients with normo-hcy. Some variations were also found in nucleotide 659 and 660, however, did not produce changing in amino acid. Whether this substitution is a kind of polymorphism that specific to Balinese ethnicity needs a further study to answer.

Keywords: methylene tetra hydro folate reductase (MTHFR), gene polymorphism and hyperhomocysteinemia, stroke

BACKGROUND

Cardiovascular disease is a major cause of mortality in Indonesia. Household survey in Indonesia (SKRT, 1995) reported that stroke and cardiovascular disease poses the top cause of death. Epidemiologic data have shown that so called conventional cardiovascular risk factors namely

dyslipidemias, hypertension and smoking were found in only around 50% of patients with atherosclerotic coronary disease.[1,2]

Recently, however, there has been increasing number of younger patients with cardiovascular disease who relate to genetic background. Some epidemiologic studies showed that increased plasma homocysteine levels (hyperhomocysteinemia) was considered an independent cardiovascular risk factor. Clarke et al. (1991) reported that hyperhomocysteinemia (hyper-hcy) is not dependent to other major cardiovascular risk factors such as dyslipidemia, hypertension and obesity. Homocysteine (hcy) is sulphur containing amino acid compound which is produced through demethylation of metheonine. Its accumulation in human body may produce an accumulation of adenosyl homocysteine (AdoHcy), a toxic substance and involve in the pathogenesis atherosclerosis.[1-3] Homocysteine is very reactive and toxic to vascular endothelium, activate autooxidation of LDL-cholesterol and produces vascular thrombosis. Some reports showed that hyper-hcy was found in 1-2% of general population, however, it was found in 15-35 % in patients with cardiovascular disease.[3-5]

Hyper-hcy may be due to methylene tetrahydrofolate reductase *(*MTHFR) deficiency and frequently linked to mutation of MTHFR gene.[1,2] This enzyme catalyzes reduction of 5,10,methyl tetrahydrofolate to 5,methyl tetrahydrofolate. This 5,methyl tetrahydrofolate is a methyl donor in the remethylation of homocysteine to metheonine. Most of genetic studies have related patients with hyper-hcy with a mutation in nucleotide 677 from C to T mutation that causes change of amino acid coding from alanine to valine.[6-8]

Until recently, there is no study reported the prevalence of MTHFR gene mutation and its relationship with hyper-hcy among stroke patients in Indonesia. This study is aiming to determine the relationship between C677T mutation of MTHFR gene and stroke. This study is also aiming at determining the relationship between homocysteine (hcy) and stroke, relationship between plasma hcy lavel and co-factor namely folate and vitamin B12 plasma concentrations. This study may describe the role of MTHFR in hyper-hcy, and the role of hyper-hcy in the pathogenesis of atherosclerotic cerebral disease.

PATIENTS AND METHODS

A case-control study was performed among stroke patients in Department of Neurology ward at Sanglah Hospital Denpasar. Stroke patients both haemorrhagic and nono-haemorrhagic diagnosed based on clinical and CT-scan findings, aged 18-55 years were selected as cases. Controls with the same range of age were selected from population where cases originated, four of their neighbours, were listed, one of whom were selected via random sampling. Those who had relative relationship were excluded as controls. Controls were un-matched selected from cases. During the first phase of the study 20 stroke patients as cases and 10 subjects as controls were recruited.

Ten ml blood samples of both cases and controls were taken for fasting blood sugar, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride, blood urea nitrogen and creatinine total plasma homocysteine. Blood samples were also taken for *polymerase chain reaction* (PCR) examination. All subjects underwent an interview

using a special questionair to explore demographic data, history of smoking, family history of stroke. Blood sampling and interview were performed by a group of field workers taken from local faculty of medicine students, who underwent special training to follow the study procedures. All study procedures were guided by a predetermined study protocol and supervised by research team.

Waist to hip ratio was measured using waist circumference at the level of umbilicus and the greatest circumference of the hip. Resting blood pressure was taken at sitting position after 5 minutes rest. Blood samples for MTHFR gene examination were sent to Biomolecular Lab Faculty of Medicine Udayana University Denpasar Bali. Gene amplification of MTHFR was done using PCR technique using HintfI as restriction enzyme to cut PCR product 198 bp on the position 677 to show substitution from C (cytosine) to T (thymin). In order to determine base sequence coding amino acids a sequencing technique was carried out in Lab Eykman Jakarta. Plasma hcy was taken after overnight fast as total concentrations using *Fluoroscence Polarization Immuno Assay* (FPIA).

Hyperhomocysteinemia is defined if total plasma fasting hcy levels 12 µmol/L or more. Hypertension is defined if systolic blood pressure 140 mm Hg or more and/or diastolic blood pressure 90 mm Hg or more. Gene mutation of MTHFR was focused on base substitution on 677 positions.

Data was presented as mean and standard deviation and percetage using descriptive statistics. Prevalence of hyper-hcy and hypertension among cases and controls was analysed using 2X2 table and prevalence ratio as the strength of relationship. Independent student's t test was used to determine difference of continuous variables between cases and controls. Significant level was set at p-value less than 5%.

RESULTS

Twenty cases and 10 controls, all subjects were Balinese, included in this study. Table 1 showed clinical characteristics with regard to traditional cardiovascular risk factors. It was shown that some factors including gender, age, blood pressure, fasting blood sugar, total-cholesterol and LDL-cholesterol were clinically significant different.

Table 1. Clinical characteristic among cases and controls

Characteristics	Cases N = 20	Controls N = 10	Mean difference
Age (years)	44.9 (6.4)	48.1 (6.5)	3.2
Gender (F/M)	8/2	17/3	
Smoking	5/10	6/20	
Coffee drinking	6/10	8/20	
BMI (kg/m2)	24.3 (1.5)	22.2 (1.90	2.1
Systolic BP (mmHg)	110 (83)	144 (22)	34
Diastolic BP (mmHg)	73 (8)	87 (12)	14
MAP (mmHg)	85 (5)	106 (85)	21
Fasting BS (mg/dl)	85 (9)	102 (36)	17
Waist to hip ratio	0.94 (0.07)	0.95 (0.05)	0.01
Lipid profile			
Total-cholesterol (mg/dl)	165 (14)	181 (34)	15
Triglyceride (mg/dl)	109 (18)	99 (36)	-10
HDL-cholesterol (mg/dl)	54 (7)	49 (12)	-5
LDL-cholesterol (mg/dl)	88 (18)	112 (33)	23

This study showed that a significantly lower plasma vitamin B12 levels in strokr patients than controls was found. Plasma hcy tend to be higher in stroke patients than controls, see table 2.

Table 2. Plasma folate, vitamin B12 and homocysteine levels in stroke and controls

Vitamins	Stroke N=20	Controls N=10	P-value	MD*	95%CI
Folate (ng/ml)	10.45 (4.62)	10.42 (1.39	0.98	0,03	-2.28 to 2.34
B12 (pg/ml)	532 (233)	779 (165)	0.003	-274	-400 to. -95
Hcy (µg/ml)	10.3 (3.0)	9.2 (1.2)	0.27	1.1	-0.48 to 2.73

*) mean difference

This study showed that in addition to hyper-hcy (X^2: 5.4; PR: 1.8; CI95%: 1.0 to 2.7; p=0.03), hypertension (X^2: 13.12; PR 2.66; 95%CI 1.41 to 5.02; p=0.00) was associated with increased risk of stroke, see table 3 and table 4.

Table 3. Relationship between hyperhomocysteinemia and stroke

		Groups		Total
		stroke	controls	
Hyper-Hcy	yes	8	0	8
	no	12	10	22
Total		20	10	30

X^2: 5.4 PR: 1.8; CI95%: 1.0 to 2.7, p=0.03

Table 4 Relationship between hypertension and stroke

		groups		Total
		stroke	controls	
Hypertension	Yes	14	0	14
	No	6	10	16
Total		20	10	30

X^2: 13.12; RP 2.66; 95%CI 1.41 s.d. 5.02, p=0,00

There were no significant correlation between plasma hcy levels and plasma folate (r=0.17, p=0.36) and vitamin B12 levels (r=0,05 ,p=0,78) as co-factors of hcy metabolism in stroke and control subjects, see scattered diagram in figure 1 and 2.

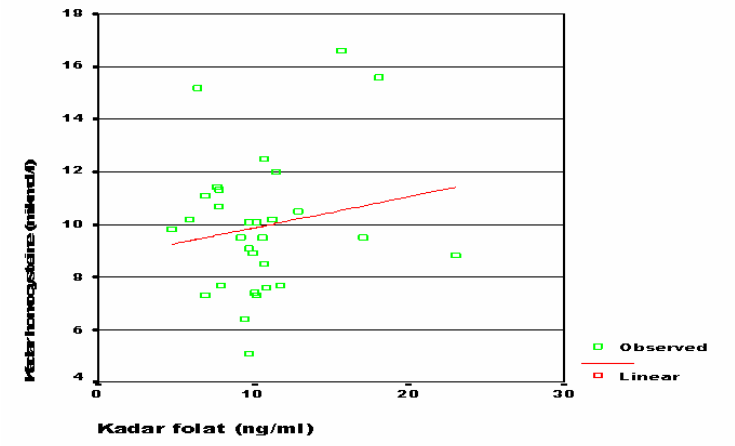

Figure 1. Relationship between plasma Hcy and plasma folate in stroke and controls subjects (r=0.17, p=0.36)

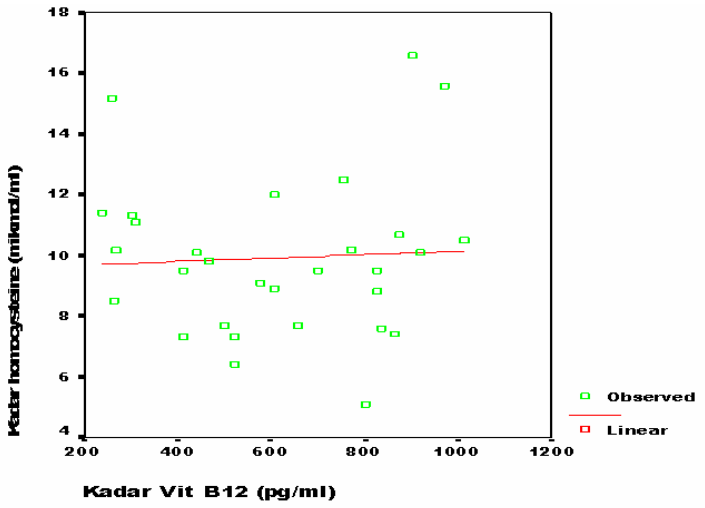

Figure 2. Relationship between plasma vitamin B12 and plasma Hcy ind both stroke and control subjects (r=0.05; p=0.78)

In order to know whether the amplification works, electrophoresis using agarose gel 2% and its results was visualized using UV-transiluminator and the picture was taken using Polaroid DS-40 camera. The results of DNA amplification both in stroke and control subjects can be seen in figure 3 and 4.

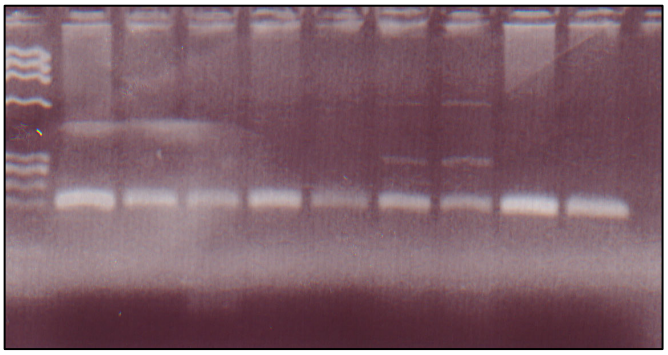

Figure 3. Electrophoresis of total DNA amplification using agarosa 2% for 30 minutes.

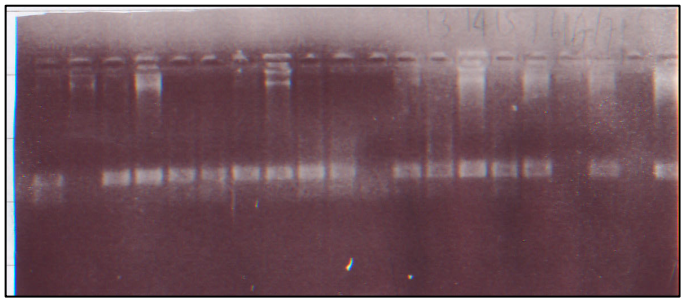

Figure 4. Electrophoresis of total DNA amplification in 15 samples

Sequencing for MTHFR gene mutation was done in 10 stroke patients and 3 controls. .This study found no mutation on 677 from C to T (C677T), however there were substitution in nucleotides among stroke and controls, with or without producing chances of amino acids. Results of sequencing can be seen in figures 5 trough 8.

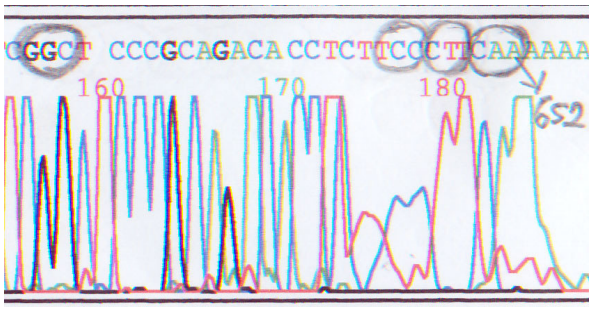

Figure 5. Sequencing of MTHFR gene among controls

Figure 5 showed substitution of the nucleotide in the position 660 (G660A), yielding changing of amino acid from glutamine (GAG) to glisine (GGA) and A659G, however no changing of coding amino acid. These substitution occured in all controls. These substitutions were referred to the gene bank.

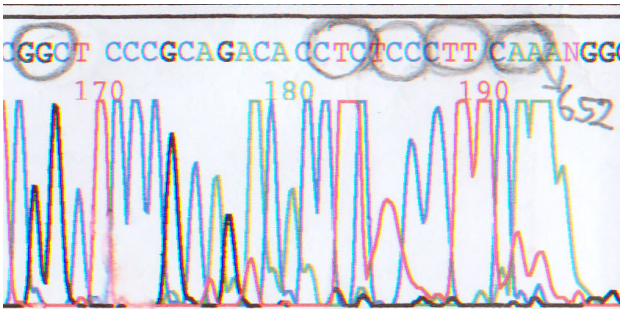

Figure 6. Sequencing of MTHFR gene (variation 1)

Figure 6 showed substitution of the nucleotide in the position 661 from A to G (G660A), yielding changing of amino acid from lysine (AAG) to glutamine (GAG). This substitution occured in one stroke patients with hyper-hcy.

Other variation of A659G and G660A were found, however, no changing of coded amino acids was produced.

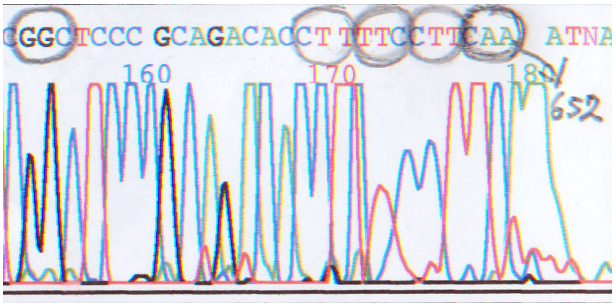

Figure 7. Sequencing of MTHFR gene (variation 2)

Figure 7 showed a substitution of nucleotide 660 from G to A (G660A), howeve no changing of coded amino acid was produced (still glutamine). This variation was found in 5 stroke patients, one of whom had hyper-hcy.

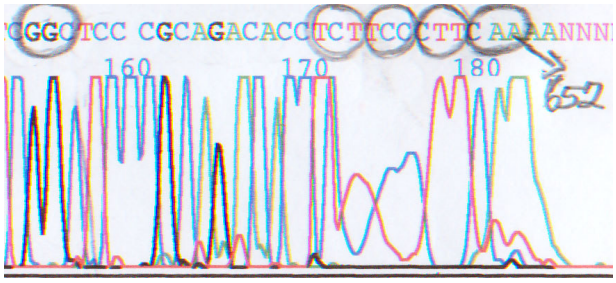

Figure 8. Sequencing of MTHFR gene (variation 3)

Figure 8 showed 2 substitutions in the nucleotide 659 from A to G (A659G), howeve no changing of coded amino acid was produced (still glutamine), since glutamine was coded by GAG dan juga GAA. A second substitution was found in nucleotide 660 from G to A (G660A), with a change of coded amino acid from glutamine (GAGto glisine (GGA).These substitutions occurred in 4 stroke patients, 2 of whom had hyper-hcy. Detail of distribution of MTHFR gene mutation was decribed in table 5 through 7

Table 5. Distribution of MTHFR gene mutation among controls

No of samples	N	MTHFR gene			
		G659A	G660A	A661G	C677T
1, 3, 10	3	+-*)	++**)	--***)	--

*) substitution of bases in the nucleotide on a given position, however, no changing of coded amino acid was produced, **) substitution of bases in the nucleotide on a given position and a changing of coded amino acid was produced, and ***) no substitution of bases in the nucleotide on a given position and no changing coded amino acid was produced.

Table 6. Distribution of MTHFR gene mutation among stroke patients with normo-hcy

No of simples	N	MTHFR gene			
		G659A	G660A	A661G	C677T
1 and 19	2	+-*)	++**)	--***)	--
3 and 20	2	--	+-	--	--
10	1	+-	+-	++	--
11 and 14	2	+-	++	--	--

*) substitution of bases in the nucleotide on a given position, however, no changing of coded amino acid was produced, **) substitution of bases in the nucleotide on a given position and a changing of

coded amino acid was produced, and ***) no substitution of bases in the nucleotide on a given position and no changing coded amino acid was produced.

Table 7. Distribution of MTHFR gene mutation among stroke patients with Hyper-hcy

No. of samples	N	MTHFR gene			
		G659A	G660A	A661G	C677T
5	1	+- *)	++ **)	-- ***)	--
9	1	--	+-	--	--
16	1	++	--	--	--

*) substitution of bases in the nucleotide on a given position, however, no changing of coded amino acid was produced, **) substitution of bases in the nucleotide on a given position and a changing of coded amino acid was produced, and ***) no substitution of bases in the nucleotide on a given position and no changing coded amino acid was produced.

DISCUSSION

This study shows that hyper-hcy increases risk of stroke. In addition to hyper-hcy, hypertension also increases risk of stroke. Clarke et al. (1991) reported that hyper-hcy is independent to other major vascular risk factor, and some authors called hyper-hcy as non-traditional risk factor. Homocysteine (Hcy) is a sulphur containing amino acid compound which is produced through demethylation of metheonine. Its accumulation in human body may produce an acumuulation of adenosyl homocysteine (AdoHcy), a toxic substance and involve in the pathogenesis atherosclerosis.[1-3] Hcy is an amino acid which is very reactive and toxic to vascular endothelium, activate autooxidation of LDL-cholesterol and produces vascular thrombosis. Recent studies reported that hyper-hcy causes early vascular disease.[5] Vitamin B12 and folate are co-factors needed in the remethylation of homocysteine. Disturbance in this remethylation cycle will increase plasma homocysteine level at fasting condition.[9,10]

In this study there were some variation of bases substitution on nucleotide of MTHFR gene with/or without changes of coded amino acid both in stroke patients and controls. These substitutions may not be considered as mutation of the gene since it reffered to the exixting gene bank. In addition, lack of samples and inconsistency between pattern of substitution and hyper-hcy, making it is difficult to conclude whether these substitution are really mutation or only a kind of ethinic polymorphism specific to Balinese. This study involves all Balinese subjects. A meta-analysis 72 individual study aiming to determine the presence of MTHFR gene mutation and its relationship with cardiovascular disease was done by Wald et al., 2002. This study reported that in stroke patients (7 studies) there was increased incidence of hyper-hcy in TT homozygous than wild type CC mutation (OR 1.65) and heterozygous CT than wild wild type CC mutation (OR1.15). Among 8 studies, in patients with stroke it was found that every 5 µmol/l increase of homocysteine levels 42% risk of stroke after adjustment of age, gender, smoking, blood pressure and serum cholesterol.[11,12]

Our study shows that there is no correlation between plasma homocysteine levels and co-factors (plasma vitamin B_{12} and folate). In stroke patients lower plasma vitamin B_{12} than control is found.

Low vitamin B_{12} among strok may to some extend explain the role of low vitamin B_{12} in the pathogenesis of hyper-hcy in our setting.

We conclude that in adition to hyper-hcy hypertension was associated with increase risk of stroke. There were substitution in nucleotides among stroke and controls, with or without producing chances of amino acids. Whether this substitution is a kind of polymorphism that specific to Balinese ethnicity needs a further study to answer.

REFERENCES

1. Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homosisteine as a risk factor for cardiovascular disease: probable benefits of increasing folic acid intake. JAMA 1995;274:1049-57.

2. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homosisteine as a risk factor for cardiovascular disease: probable benefits of increasing folic acid intake. JAMA 1995;274:1049-57.

3. Clarke R, Dally, Robinson K, naughten E, Cahalane S, Fowier B, Graham. Hyperhomosisteinemia : An independent risk faktor for vascular disease. N Eng J Med 1991;324:1149-55.

4. Duell PB, Malinow MR. Homosisteine: an important risk faktor for atherosclerosis vascular disease. Curr Opin Lipidol 1997;8:28-34.

5. McKully KS. Vascular pathology of hyperhomocysteinemia: implication for the pathogenesis of atherosclerosis. Am J Pathol 1959;56:111-28.

6. Franco RF, Araujo AG, Guerreiro JF, Elion J, Zago MA. Analysis of the 677 C → T mutation of the methylenetetrahydrofolate reductase gene in different ethnic group. Thromb Haemost 1998;79:119-21.

7. Frost P, Blom HJ, Milos R, Goyette P , Sheppard CAA, Matthews RG, BoersGJH, den Heijer M, Kluijtmans LAJ, van der Heuvel LP, Rozen R. A candicate genetic risk factor for vascular disease: a common mutation in Methyletetrahydrofolate reductase. Nat Genet 1995;10:111-3.

8. Kluijtmans LAJ, Van den Heuvel LPWJ, Boers GHJ, Froost P, Stevens EMB, Van Oost BA, Den Heijer M, Trijbels FJM, Rozen R and Blom HJ. Molecular genetic Analysis in mild hyperhomosisteinemia: a common Mutation in Methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 1996;58:35-41.

9. Jacobsen, DW. Homosisteine and vitamins in cardiovascular disease. Clin Chem 1998;44:1833-43.

10. Kuller LH, Evans RW. Homosisteine, vitamins and cardiovascular disease. Circulation 1998;98:196-9.

11. Markus HS, Ali N, Swaminathan R, Sankaralingam A, Molloy J, Powell J. A Common polymorphism in the methylene tetra hydrofolate reductase gene, homocysteine and ischemic cerebrosvascular disease, stroke. Sep 1998;28(9):1739-43.

12. Wald DS, Law M, Morris JK. Homosisteine and cardiovascular disease: evidence on causalty from meta-analysis BMJ 2002; 325: 1-7.