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Relationship between total plasma homocysteine, polymorphisms of homocysteine metabolism related enzymes, risk factors and coronary artery disease in the Australian hospital-based population

Xing L. Wang [a,*], Natalia Duarte [a], Hua Cai [a], Tetsuo Adachi [b], Ah Siew Sim [a], Greg Cranney [a], David E.L. Wilcken [a]

[a] *Department of Cardiovascular Medicine, University of New South Wales, Prince Henry/Prince of Wales Hospitals, Sydney, Australia*
[b] *Laboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu, Japan*

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Abstract

Modest elevations of circulating homocysteine are common in patients with vascular disease. We explored interrelations between total plasma homocysteine levels and mutations in genes for three key enzymes in methionine-homocysteine metabolism. Methyltetrahydrofolate reductase (MTHFR) 677C→T, cystathionine beta synthase (CBS) 68-bp insertion at exon 8, and methionine synthase (MS) 2756A→G were typed in 685 Australian caucasian patients aged ⩽65 years with and without angiographically documented coronary artery disease (CAD). We also assessed associations between homocysteine levels and extracellular superoxide dismutase (EC-SOD) and other CAD risk factors. There were significant correlations between plasma total homocysteine, and EC-SOD ($r=0.170$, $p=0.001$ for men; $r=0.241$, $p=0.003$ for women) and LDL ($r=0.153$, $p=0.001$ for men; $r=0.132$, $p=0.081$ for women). Levels were also significantly higher among patients with unstable angina (15.30 ± 0.44 μmol/l for men, 14.44 ± 0.74 μmol/l for women) than those without angina (13.98 ± 0.38 μmol/l for men, 13.41 ± 0.98 μmol/l for women) or with stable angina (14.00 ± 0.37 μmol/l for men, 12.88 ± 0.71 μmol/l for women). There were no significant associations between the levels and the presence or severity of CAD. The mutant MTHFR homozygotes tended to have higher levels and those with the MS and CBS mutations tended to have lower levels. We conclude that there is a significant correlation between plasma homocysteine levels and EC-SOD suggesting that elevated homocysteine may exert oxidative stress and that levels are associated with unstable angina, but not the occurrence or extent of coronary stenosis. The contributions to total plasma homocysteine levels of the common mutations of genes coding for the enzymes controlling homocysteine metabolism are modest.

Keywords: Methionine synthase; Total plasma homocysteine; Methyltetrahydrofolate transferase; Cystathionine beta synthase; Coronary artery disease; Extracellular superoxide dismutase

1. Introduction

Modest elevation of circulating homocysteine, commonly referred to as hyperhomocysteinaemia, is generally considered to be a risk factor for coronary, cerebral, and peripheral vascular disease [1–7] and for venous thrombosis [8]. Levels are more markedly increased in chronic renal failure [9–12] and in these patients there is also greatly increased cardiovascular risk [11,12]. Whilst the mechanisms mediating vascular changes are still unclear, there is evidence that elevated homocysteine levels may cause endothelial injury, promote thrombosis and increase smooth muscle cell proliferation and oxidative stress [13–17].

Homocysteine is formed during the metabolism of the essential sulphur-containing amino acid methionine [18]. Most exogenous methionine ($\sim 90\%$) is taken up by cells to be incorporated into protein, whilst the remaining 10% is metabolised to homocysteine via *S*-adenosylmethionine (AdoMet) and *S*-adenosylhomo-

* Corresponding author. Present address: Cardiovascular Genetics Laboratory, Ground Floor, Edmund Blacket Building, Prince of Wales Hospital, Randwick, NSW 2031, Australia. Tel.: +61-2-93824835; fax: +61-2-93824826.

E-mail address: x.l.wang@unsw.edu.au (X.L. Wang)

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cysteine [19]. The homocysteine formed is either converted into cysteine via the transsulphuration pathway mediated by cystathionine beta synthase (CBS), the rate limiting enzyme in the pathway, or it is re-methylated back to methionine by a folate- and vitamin B12-dependent enzyme, methionine synthase (MS). CBS is located on chromosome 21 [20] and MS on chromosome 1 [21]. MS is one of the only two B12-dependent mammalian enzymes, and it catalyses the re-methylation of homocysteine to methionine by concurrent demethylation of 5-methyltetrahydrofolate to tetrahydrofolate [20,22]. The production of 5-methyltetrahydrofolate is mediated by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), the gene for which is located on chromosome 1 [23].

Defective activity caused by mutations in these enzymes results in rare inborn errors of metabolism associated with homocystinuria in which levels of plasma homocysteine are greatly increased [18,24–27]. In these inborn errors the phenotypic features include precocious vascular disease [18,22]. However studies of common DNA variants at these loci have been inconsistent in relation to their contribution to mild homocysteinaemia. A common 677C→T substitution producing an alanine to a valine residue mutation at the MTHFR gene results in thermolabile MTHFR which has reduced enzymatic activity and an increased folate requirement. There are reports that the mutation is associated with mild homocysteinaemia in a healthy young population, whilst others report variable levels among older patients with CAD [13,28–30]. These findings are consistent with the notion that the association between the thermolabile MTHFR and mild homocysteinaemia is dependent on plasma folate levels and that hyperhomocysteinaemia only occurs with folate levels below the population median [31]. Furthermore, most population studies have failed to show that the MTHFR mutation occurs more frequently in patients with vascular disease, than in those without [32–35], findings confirmed now in a meta analysis of about 6,000 vascular patients and controls genotyped for the mutation [31].

Most major mutations at the CBS gene identified so far are too rare for effective population studies with sufficient power [18,26,27] to determine whether or not the alleles are more frequent in the general vascular disease population. However, there is a 68-bp insertion at exon 8 reported to be relatively common and the insertion polymorphism always occurs together with the 899C→T mutation [36]. Several mutations have been identified at the MS gene which lead to deficient MS enzyme activity and markedly elevated homocysteine levels (cobalamin G disease) [37,38]. There is also a 2756A→G which is relatively common although it is not known whether or not it results in altered enzyme activity.

To assess the contribution of these DNA variants at the MTHFR, MS and CBS loci to circulating total homocysteine levels and their interrelations with the occurrence and severity of significant CAD, we measured total homocysteine levels in those CAD patients for whom we also had genotype information at the MTHFR, CBS and MS loci. We also evaluated interactions between homocysteine levels and other conventional risk factors and explored the possibility that modest homocysteine elevation may be associated with demonstrable oxidative stress. We assessed this by measuring EC-SOD levels in the patients and determining whether or not they were related to circulating homocysteine in the same blood samples.

2. Materials and methods

2.1. The patient population

We studied 685 caucasian patients aged 65 years or less, both men (505) and women (180), consecutively referred to the Eastern Heart Clinic at Prince Henry Hospital for coronary angiography. A written consent was obtained from every patient after a full explanation of the study, which was approved by the Ethics Committee of the University of New South Wales.

A 4 ml venous blood was drawn into an EDTA sample tube before the angiogram after at least a 6 h fast. The blood sample was centrifuged within 2 h and plasma and cellular components stored separately at −70°C in aliquots until analysis.

2.2. Measurement of lipid variables

The hospital's clinical chemistry department measured lipoprotein and apolipoprotein analysis total cholesterol (TC), HDL-cholesterol and triglyceride levels using standard enzymatic methods. The LDL-cholesterol levels were calculated using the Friedewald formula. Levels of apo AI, apo B and Lp (a) were measured in our laboratory using in-house ELISA methodology as described previously [39].

2.3. Measurement of plasma total homocysteine and EC-SOD levels

Total plasma homocysteine levels were measured using an IMx automated fluorescence-based enzyme immunoassay. In this method, mixed disulphide and protein bound molecular forms of homocysteine in the blood sample are reduced to free homocysteine by use of dithiothreitol. As a result, all molecular forms are converted into one form, total homocysteine. The homocysteine in the test sample is converted to *S*-adenosyl-L-homocysteine using SAH hydrolase and excess

adenosine. After addition of an anti-SAH antibody and a fluoresceinated tracer (*S*-adenosyl-cysteine) the amount of homocysteine is determined by fluorescence polarisation immunoassay (FPIA). This method correlates highly with HPLC measurement ($r = 0.991$). This close correlation between HPLC and IMx methods is also confirmed in our laboratory ($r = 0.993$). The range of measurements is 0.5–50 μmol/l with a sensitivity of < 0.5 μmol/l. Within and between assay CVs were 1.9 and 4.1%, respectively, in our laboratory when assessed by two control samples in every run (20 samples per run). Cross-reactivity with glutathione, L-cysteine, adenosine, L-cystathionine were below 0.1%, and with *S*-adenosyl-L-methionine it was 12.9%. Plasma levels of EC-SOD were measured by ELISA as previously described [40].

2.4. Detection of mutations at MTHFR, CBS and MS loci

DNA was extracted from the frozen cellular blood component by a salting-out method. The extracted DNA was stored at 4°C until analysis. The DNA samples were subjected to amplification by the polymerase chain reaction (PCR) and followed by the relevant restriction enzyme digestions. The methods used to determine the MTHFR 677C→T mutation, the methionine synthase 2756A→G mutation, and the 68-bp insertion at exon 8 of CBS gene were described previously [33,36,37].

2.5. Biometric measurements

The patient's height and weight were routinely measured before the coronary procedures by either a registered nurse or an attending doctor who also interviewed the patients to record the medical history. The waist and hip circumferences were measured specifically for the project and waist/hip ratio was calculated. The body mass index (BMI) was obtained from the ratio of weight (kg) to height squared (m).

2.6. Documentation of CAD and other medical conditions

The severity of coronary artery disease was determined from the number of significantly stenosed major coronary arteries. Each angiogram was classified as revealing either normal coronary arteries or having no coronary lesion with more than 50% luminal stenosis or as having one, two, or three major epicardial coronary arteries with more than 50% luminal obstructions. We obtained each patient's medical history using a questionnaire with standardised choices of answers to be ticked during the interview as described previously [39].

2.7. Statistical analysis

One-way ANOVA was employed to assess the associations between the genotype distributions, presence and severity of CAD and quantitative variables. We used a general factorial design of ANOVA to assess the independent contribution of each variable to total plasma homocysteine levels. We coded those with normal coronary arteries and those without significantly diseased vessels (< 50% luminal obstruction) as 0, and those with 1 or more significantly diseased vessels (50% luminal obstruction) 1 as an ordinal dichotomous variable. In an expanded model to include the number of significantly diseased vessels, we assigned 0, 1, 2 and 3 as the ordinal variable for, respectively, the absence of significant disease and significant disease of increasing severity. We used logistic regression analysis to evaluate associations between CAD as the response variable and homocysteine levels and other risk factors as predictors. A forward-stepwise model was used to explore significant predictors and their interactive terms. Two-tailed *p* values are reported.

3. Results

3.1. Correlations between plasma total homocysteine levels and other quantitative variables

Male patients tended to have higher homocysteine levels (mean ± SEM: 14.73 ± 0.27 μmol/l, $n = 505$) than female patients (13.77 ± 0.46 μmol/l, $n = 180$, $p = 0.159$). The difference became statistically significant when other variables were controlled for ($p = 0.013$). Using 15 μmol/l as a cut-off point to classify mild hyperhomocysteinaemia [31], 33.9% of the patients (36.0% in men and 27.9% in women) had hyperhomocysteinaemia. To assess the association between plasma homocysteine and EC-SOD levels, patients with the Arg213→Gly mutation at EC-SOD gene were excluded from analysis since the physiological significance of this small proportion of patients (3.2%) is unknown [41]. This group of patients always has EC-SOD levels above 400 ng/ml. Only patients with common EC-SOD phenotypes (EC-SOD levels < 400 ng/ml) were included in the statistical analysis. The mean ± SEM plasma EC-SOD levels were 86.6 ± 5.1 ng/ml for men and 113.6 ± 13.2 ng/ml for women [41]. There was a significant correlation between total homocysteine and EC-SOD levels in both men ($r = 0.170$, $p = 0.001$) and women ($r = 0.241$, $p = 0.003$). This correlation was more significant for the population as a whole ($r = 0.168$, $p = 0.0001$). There were also significant correlations between levels of homocysteine and levels of total cholesterol ($r = 0.091$, $p = 0.018$) and LDL cholesterol ($r = 0.147$, $p = 0.0001$). However, when the analysis was

Table 1
Mean ± SEM (μmol/l) total homocysteine levels (*N*) in man and women smokers[a]

	Smoking status			*P*
	Non-smoker	Current smoker	Ex-smoker	
Male	15.62 ± 0.56 (122)	14.30 ± 0.56 (119)	14.52 ± 0.38 (264)	0.181
Female	13.26 ± 0.65 (88)	15.29 ± 1.05 (34)	13.44 ± 0.83 (55)	0.248

[a] *p* values were obtained by one-way ANOVA.

confined to men and women separately, these latter associations were significant in men ($r = 0.098$, $p = 0.030$ for total cholesterol and $r = 0.153$, $p = 0.001$ for LDL) but not in the smaller number of women ($r = 0.087$, $p = 0.244$ for total cholesterol and $r = 0.132$, $p = 0.081$ for LDL cholesterol).

There was a positive correlation between total plasma homocysteine and waist/hip ratio of marginal significance ($r = 0.076$, $p = 0.052$) but homocysteine levels were not correlated with age, BMI or other lipoprotein and apolipoprotein levels assessed in this patient population. There was no consistent association between cigarette smoking status and homocysteine levels (Table 1). The total life-time smoking dose was also not correlated with homocysteine levels ($r = 0.023$, $p = 0.554$). The association between total homocysteine and LDL and EC-SOD remained independent and statistically significant ($p = 0.008$ and $p = 0.006$, respectively) after controlling for other variables which included gender, genotypes of MTHFR, CBS and MS genes and CAD. Furthermore, in the multivariate regression model, waist/hip ratio became significantly and positively correlated with total plasma homocysteine levels ($p = 0.031$).

3.2. Total homocysteine levels and mutations

As shown in Tables 2–4, none of the measured genotypes of the three key enzymes for homocysteine metabolism contributed significantly to plasma homocysteine levels. However there were trends in that those with the thermolabile MTHFR mutation had higher total homocysteine levels (Table 2). The G allele of the MS gene tended to have lower homocysteine levels in males but not in females (Table 3). And homocysteine levels in male patients with the 68-bp insertion at exon 8 of the CBS gene also tended to have lower homocysteine levels than those without the insertion. These quantitative differences were also seen when we used the 15 μmol/l cut-off level for elevated homocysteine. Among TT homozygotes of the MTHFR gene 39% had mild hyperhomocysteinaemia compared to 35% for rest of the patients. Among mutant GG homozygotes for the MS gene 22.2% had hyperhomocysteinaemia compared to 36% of the wild-type AA homozygotes. And none of the CBS I/I patients had plasma homocysteine above 15 μmol/l whereas 34.8% of those with D/D and 28.8% of those with I/D had levels above the cut-off. However, the differences in total homocysteine levels among those with different genotypes remained non-significant after controlling for other variables including lipid profiles and EC-SOD levels.

Table 2
Mean ± SEM (μmol/l) total homocysteine levels (*N*) in man and women with MTHFR genotypes[a]

	MTHFR Genotypes			*P*
	TT	TC	CC	
Male	15.37 ± 0.88 (44)	14.72 ± 0.43 (184)	14.68 ± 0.44 (169)	0.774
Female	15.07 ± 1.60 (15)	13.28 ± 0.72 (74)	14.15 ± 0.76 (66)	0.506

[a] *p* values were obtained by one-way ANOVA.

Table 3
Mean ± SEM (μmol/l) total homocysteine levels (*N*) in man and women with methionine synthase genotypes[a]

	Methionine Synthase			*P*
	GG	GA	AA	
Male	12.31 ± 1.45 (18)	14.33 ± 0.48 (166)	15.13 ± 0.35 (301)	0.100
Female	14.89 ± 1.90 (10)	12.36 ± 0.82 (53)	14.22 ± 0.57 (109)	0.147

[a] *p* values were obtained by one-way ANOVA.

3.3. Total homocysteine levels and occurrence and severity of CAD

Patients with triple vessel disease tended to have higher homocysteine levels than those with no diseased vessels, but none of the differences were statistically

Table 4
Mean ± SEM (μmol/l) total homocysteine levels (*N*) in man and women cystathinine β-synthase genotypes[a]

	Cystathinine β-synthase genotypes			*P*
	D/D	I/D	I/I	
Male	14.79 ± 0.34 (302)	13.53 ± 0.85 (48)	10.47 ± 4.16 (2)	0.240
Female	13.81 ± 0.56 (112)	14.78 ± 1.79 (11)	12.82 ± 5.95 (2)	0.863

[a] *p* values were obtained by one-way ANOVA.

Table 5
Mean ± SEM (μmol/l) total homocysteine levels (*N*) in man and women with different number of significantly diseased vessels[a]

Number of significantly diseased vessels	Males	Females
0	14.80 ± 0.60 (107)	13.09 ± 0.66 (91)
1	14.66 ± 0.52 (142)	14.96 ± 1.02 (38)
2	14.42 ± 0.57 (117)	13.86 ± 1.16 (29)
3	15.02 ± 0.52 (139)	14.42 ± 1.34 (22)
p	0.445	0.889

[a] *p* values were obtained by one-way ANOVA.

significant for men or for women (Table 5). There were minimal differences between patients with different numbers of significantly diseased vessels. This was also true when patients were grouped into those without CAD or without significant CAD (< 50% luminal stenosis) and those with significant CAD (Table 6). The frequencies of patients with mild hyperhomocysteinaemia also did not differ among patients with different numbers of diseased vessels as assessed by a chi-square comparison.

3.4. Total homocysteine levels and other medical conditions

Levels of total homocysteine were higher in male and female patients with a history of unstable angina, than in those with no angina ($p = 0.028$, Table 7), but the differences were small. Of the patients with unstable angina 39.5% had plasma homocysteine levels above the 15 μmol/l cut-off level compared to only 31.9% of the patients without angina ($\chi 2 = 5.931$, $df = 2$, $p = 0.052$). As shown in Table 6, total homocysteine levels tended to be higher in patients with a positive family history of CAD ($p = 0.014$). However there were no clear trends or significant differences in homocysteine levels between patients with or without a past history of myocardial infarction, of diabetes or of hypertension requiring treatment.

Table 7
Mean ± SEM (μmol/l) total homocysteine levels (*N*) in man and women with Angina pectoris[a]

	Angina			*P*
	No	Stable	Unstable	
Male	13.98 ± 0.38 (185)	14.00 ± 0.37 (245)	15.30 ± 0.44 (250)	0.028
Female	13.41 ± 0.98 (39)	12.88 ± 0.71 (57)	14.44 ± 0.74 (81)	0.323

[a] *p* values were obtained by one-way ANOVA.

4. Discussion

An important positive finding in the present study is the first identification of a clear cut association between levels of total plasma homocysteine and EC-SOD. While more studies are needed to explore the mechanisms involved, it is possible that even a mildly elevated homocysteine is a source of oxidative stress and stimulates expression of EC-SOD. EC-SOD is the major enzyme opposing the oxidative effects of superoxide [42,43] and excessive superoxide production is a likely contributor to homocysteine induced atherogenesis. Our study of the same patient population demonstrated reduced EC-SOD levels in patients with myocardial infarction. The EC-SOD levels were also lower among

Table 6
Mean ± SEM (μmol/l) total homocysteine levels (*N*) in man and women with presence or absence of medical conditions[a]

	Sex	Presence or absence of following medical conditions		*P*
		Yes	No	
Family history of CAD	Male	15.22 ± 0.37 (315)	13.91 ± 0.38 (191)	0.014
	Female	13.69 ± 0.55 (125)	13.95 ± 0.85 (56)	0.801
Myocardial infarction	Male	14.52 ± 0.42 (207)	14.82 ± 0.35 (308)	0.580
	Female	13.97 ± 0.83 (56)	13.64 ± 0.53 (134)	0.736
Diabetes	Male	14.07 ± 0.29 (57)	14.80 ± 0.29 (444)	0.644
	Female	14.52 ± 1.29 (23)	13.59 ± 0.50 (154)	0.781
Hypertension	Male	14.65 ± 0.43 (206)	14.76 ± 0.36 (294)	0.903
	Female	14.17 ± 0.64 (93)	13.20 ± 0.67 (84)	0.565
Significant CAD	Male	14.72 ± 0.31 (398)	14.80 ± 0.60 (107)	0.900
	Female	14.47 ± 0.66 (89)	13.09 ± 0.65 (91)	0.140
Presence of CAD	Male	14.68 ± 0.30 (435)	15.06 ± 0.74 (70)	0.630
	Female	14.48 ± 0.60 (106)	12.83 ± 0.73 (73)	0.082

[a] *p* values were obtained by one-way ANOVA.

smokers and male patients [41]. An increased homocysteine by enhancing oxidative stress could stimulate more EC-SOD production. Furthermore, plasma EC-SOD is derived from vascular wall, with which it is in equilibrium [44,45]. It is also possible that elevated circulating homocysteine may decrease the affinity of EC-SOD to vascular wall extracellular proteoglycan resulting in more EC-SOD being released into the circulation.

While the present study defines a trend of higher total plasma homocysteine levels in patients with significant coronary stenosis, particularly in women (Tables 5 and 6) none of the differences achieved statistical significance. However, we did find significantly higher total homocysteine levels in patients with unstable angina (Table 7) and in those with a positive family history of CAD (Table 6). These findings suggest that any contribution of mild hyperhomocysteinaemia to CAD is at most modest [31,46,47]. While the mechanisms of hyperhomocysteinaemia related atherogenesis remain speculative, our findings of higher levels in unstable angina patients are consistent with the hypothesis that more markedly elevated homocysteine may promote progression of atherogenesis by increased thrombosis. The hyperhomocysteinaemia could also act indirectly through other risk factors, e.g. oxidative stress, or be merely consequence of other CAD risk factors in CAD patients as reviewed by Brattstrom and colleagues [31].

One of the major aims of the present study was to assess the genotype contributions to circulating homocysteine levels in this patient population. Although total homocysteine levels tended to be higher in patients with the thermolabile MTHFR genotype (TT) as expected, the difference was small and did not achieve statistical significance (Table 2). The lower levels of homocysteine in patients homozygous for the rare MS allele (GG) were also not statistically significant (Table 3). The same was true for patients with the 68bp insertion of the CBS gene (Table 4). The findings remained the same after controlling for other variables including gender, disease status, cigarette smoking and lipid profiles.

It is established that the 677C→T mutation at MTHFR results in thermolability of the enzyme with a reduced activity in the absence of an above average folate intake [23,25,28]. Thus the association between the thermolabile MTHFR variant and elevated homocysteine levels is a function of folate status. A positive association is only present if plasma folate levels are below average Western population levels [31]. Although we did not measure folate levels in the patients of our study they do constitute a well nourished group likely to have had an adequate folate intake. This would be consistent with our results revealing only a trend for an increase in homocysteine associated with the mutation. With regard to the 919D→G mutation it is not known whether it results in changes in MS enzymatic activity or levels [37]. However our population study shows that the mutation is not associated with either CAD [48] or total plasma homocysteine levels. We also found that the 68-bp insertion at exon 8 of CBS gene made no significant contribution to either total homocysteine levels or CAD risk. These findings indicate that genetically determined factors, e.g. enzyme activities, and dietary determined factors, e.g. availability of B12 and folate, are all contributing significantly to homocysteine levels. Therefore a single defect may not be sufficient to result in a significant change in the homocysteine level, particularly if it is at a single point of the metabolic cycle subject to the influences of other enzymes and cofactors.

In conclusion, our study shows that patients with elevated total plasma homocysteine levels are more likely to experience unstable angina but finds only weak associations between mildly elevated homocysteine levels and the occurrence and extent of coronary stenosis. It also shows that the contributions of the mutations we assessed of MTHFR, MS and CBS genes to plasma homocysteine levels are minimal in our CAD patients. There was however a significant correlation between total homocysteine levels and plasma EC-SOD suggesting that the vascular effects of homocysteine could be caused by excessive oxidative stress. Our findings are consistent with the hypothesis that mildly elevated homocysteine may make only a modest contribution an increased risk of atherogenesis. The finding of no association between the homocysteine and the extent of coronary stenosis but of a significant association with increased risk of unstable angina could support a role for mildly elevated homocysteine in promoting thrombosis.

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