

Homocysteine is a risk factor for cerebral small vessel disease, acting via endothelial dysfunction

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Summary

Cerebral small vessel disease (SVD) causes focal lacunar infarction and more diffuse ischaemia, referred to as leukoaraiosis. Endothelial dysfunction has been proposed as a causal mechanism in the disease. Homocysteine is toxic to endothelium. We determined whether elevated homocysteine levels and the methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism are risk factors for SVD as a whole, and for two different SVD subtypes: isolated lacunar infarction and ischaemic leukoaraiosis. We also determined whether any association was mediated by endothelial dysfunction, as assessed by circulating endothelial markers. One hundred and seventy-two Caucasian patients with SVD and 172 community controls of similar age and sex were studied. Serum homocysteine measurement and MTHFR genotyping was performed. Levels of intercellular adhesion molecule 1 (ICAM1) and thrombomodulin were measured in a subgroup. Mean homocysteine levels were higher in SVD than

controls [14.55 $\mu\text{mol/l}$ [95% confidence interval (CI) 13.78–15.35] versus 12.01 $\mu\text{mol/l}$ (95% CI 11.42–12.64), $P < 0.0005$]. Homocysteine was a stronger risk factor in those with ischaemic leukoaraiosis [12.92 (95% CI 4.40–37.98), $P < 0.0005$] per μmol increase in log homocysteine concentration ($P < 0.0005$) in comparison with isolated lacunar infarction [4.22 (95% CI 1.29–13.73), $P = 0.02$] after controlling for both conventional risk factors and age. The MTHFR 677T allele was a risk factor only in the ischaemic leukoaraiosis group [odds ratio (OR) 2.02 (95% CI 1.31–3.1), $P = 0.001$]. Inclusion of the endothelial markers ICAM1 and thrombomodulin in a logistic regression model resulted in the association between homocysteine and SVD no longer being significant. In conclusion, hyperhomocysteinaemia is an independent risk factor for SVD, particularly ischaemic leukoaraiosis, and this effect may be mediated via endothelial dysfunction. Homocysteine-lowering therapy may be particularly effective in this subgroup.

Keywords: cerebral small vessel disease; homocysteine; MTHFR; endothelial dysfunction; cerebrovascular disease

Abbreviations: CI = confidence interval; ICAM1 = intercellular adhesion molecule 1; MTHFR = methylene tetrahydrofolate reductase; OR = odds ratio; SVD = small vessel disease; TM = thrombomodulin

Introduction

Lacunar infarction, resulting from disease of the cerebral small vessels, accounts for a quarter of ischaemic strokes. Infarcts can be isolated or accompanied by diffuse changes, referred to as leukoaraiosis, in the periventricular white matter seen on CT or MRI. Both lacunar infarction and leukoaraiosis are thought to be caused by cerebral small vessel disease (SVD). At autopsy, thickening and hyaline deposition of the small perforating end-arterioles supplying the white matter can be seen, and in some cases of larger

symptomatic lacunar infarction localized microatheroma was found at the origin of the deep perforating arterioles (Fisher, 1968, 1979). The neuropathological appearance corresponding to leukoaraiosis is neuronal loss, ischaemic demyelination and gliosis (Pantoni and Garcia, 1997). Both clinical and pathological studies support the hypothesis that lacunar infarction and ischaemic leukoaraiosis represent different forms of small vessel disease. It is hypothesized that if ischaemia is acute this causes small focal regions of

parenchymal damage in perforating arteriole territories (lacunar infarction), while if it is more chronic it results in diffuse ischaemic injury (leukoaraiosis). Leukoaraiosis itself is a radiological definition and can also be caused by non-ischaemic pathologies, although ischaemia is believed to account for the vast majority of cases. The term 'ischaemic leukoaraiosis', defined as radiological leukoaraiosis with a clinical lacunar syndrome, has been introduced to identify a group of patients in whom leukoaraiosis is likely to have an ischaemic basis (Jones *et al.*, 1999).

The pathogenesis of cerebral SVD is not fully understood. Hypertension is the major risk factor but fails to account for much of the risk (Boiten and Lodder, 1995). It has been proposed that newer risk factors, including genetic predisposition, are important (Carmelli *et al.*, 1998). Several lines of evidence have suggested that chronic endothelial dysfunction plays a pivotal role. This may be responsible for breakdown of the blood-brain barrier (Tomimoto *et al.*, 1996; Lin *et al.*, 2000) and impaired cerebral autoregulation (Bakker *et al.*, 1999; Terborg *et al.*, 2000), which have been observed in SVD. Endothelial dysfunction can be assessed *in vivo* by measuring soluble plasma markers (Cines *et al.*, 1998). These are released into the circulation in response to endothelial activation by a number of different proatherogenic stimuli. Surface expression of intercellular adhesion molecule 1 (ICAM1) is a precondition for the adhesion and transendothelial migration of lymphocytes (Bevilacqua *et al.*, 1989) and blood levels reflect an endothelial inflammatory response. Thrombomodulin (TM) is normally expressed on the endothelial cell surface, where, with thrombin, it regulates the activity of protein C. Increased plasma levels are thought to reflect endothelial damage (Ishii *et al.*, 1991). Levels of both ICAM1 and TM are elevated in patients with SVD (Kario *et al.*, 1996; Fassbender *et al.*, 1999a; Hassan *et al.*, 2003). Homocysteine could also be an important risk factor for SVD. This hypothesis is biologically plausible because homocysteine has been shown to have important effects on endothelial function *in vitro* (Wall *et al.*, 1980; Stamler *et al.*, 1993) and *in vivo* (Tawakol *et al.*, 1997; Woo *et al.*, 1997). Moderate hyperhomocysteinaemia can be caused by genetic or environmental factors or a combination of both. The most frequent genetic defect involves the enzyme methylene tetrahydrofolate reductase (MTHFR). A common polymorphism (C677T) is associated with hyperhomocysteinaemia, the highest levels of homocysteine being found in those with the TT genotype (Frosst *et al.*, 1995).

Whilst many studies have described the relationship between homocysteine and stroke in general (Hankey and Eikelboom, 2001), and intra- or extracranial large vessel disease (Yoo *et al.*, 1998; McQuillan *et al.*, 1999; Spence *et al.*, 1999), there have been fewer studies which have specifically examined the role of homocysteine in SVD or its different subtypes. Some studies have suggested that homocysteine is a more potent risk factor for SVD in comparison with other stroke subtypes (Evers *et al.*, 1997; Fassbender *et al.*, 1999b), whilst others have not been able to confirm this

(Lindgren *et al.*, 1995; Eikelboom *et al.*, 2000). Potential explanations for these disparate findings include small sample sizes and differences in study design, particularly the selection of controls. Heterogeneity within SVD could also be an issue, particularly if homocysteine was more closely related to the presence of diffuse leukoaraiosis or focal lacunar infarction. Therefore in this study we determined whether homocysteine (and the MTHFR C677T polymorphism) is a risk factor for SVD as a whole, or for different SVD subtypes. We also examined if the effects of homocysteine were mediated by endothelial dysfunction, determined using the circulating markers of endothelial function ICAM1 and TM.

Methods

Study population

One hundred and seventy-two consecutive Caucasian patients with cerebral SVD attending outpatient stroke clinics were enrolled. Cerebral SVD was defined as a presentation with a clinical lacunar syndrome (Bamford *et al.*, 1987) with a compatible lesion on neuroimaging. In the cerebral SVD group many patients (particularly those with leukoaraiosis) had other features associated with SVD, including cognitive impairment (8%), gait disorder excluding hemiplegia (16%), and a past or current history of mood disturbance (22%). All patients had standard stroke investigation, including brain imaging, and carotid artery imaging with duplex or magnetic resonance angiography. Exclusion criteria included subcortical infarction ≥ 1.5 cm diameter, cortical infarction of any size, a potential source of cardiac source of embolism (Adams *et al.*, 1993), and large vessel cerebrovascular disease, defined as carotid or vertebral artery stenosis $>50\%$. One hundred and seventy-two Caucasian community controls without a history or signs of cerebrovascular disease were also recruited by sampling of family doctor lists from the same geographical regions as the patients. Sampling was stratified to provide a distribution of age and sex similar to that in the patient group. Brain imaging was not performed in controls.

All patients and controls were reviewed by one physician and underwent a standardized clinical assessment. Hypertension was defined as a systolic blood pressure >160 mm Hg or diastolic pressure >95 mm Hg (World Health Organization-International Society of Hypertension, 1993), or current treatment with antihypertensive drugs. A positive smoking history was recorded for those who had smoked at any time in their lives. Diabetes mellitus was defined as a previous diagnosis of insulin or non-insulin-dependent diabetes. The study protocol was approved by local research ethics committees and informed consent was obtained from all participants.

Blood sampling and laboratory methods

In all patients, non-fasting blood was taken at least 2 months after the last clinical ischaemic event because both depression of homocysteine levels (Stein and McBride, 1998) and transient elevation of some endothelial markers have been reported in the first few weeks after acute stroke (Fassbender *et al.*, 1995; Lindsberg *et al.*, 1996). Twenty millilitres of blood was obtained and divided into polypropylene tubes for serum and siliconized glass tubes containing 0.105 M sodium citrate for plasma collection. Specimens were

Table 1 Clinical characteristics of cases and controls

	Controls (n = 172)	Cerebral SVD (n = 172)	P
Mean age (years)	66.3 (10.2)	67.1 (10.3)	0.43
Male sex	100 (58.1)	102 (59.3)	0.83
Hypertension	72 (41.8)	130 (75.6)	<0.0005
Ever smoked	105 (61.5)	127 (73.8)	0.01
Diabetes mellitus	4 (2.3)	13 (7.6)	0.03
Myocardial infarction	9 (5.2)	5 (2.9)	0.28
Cholesterol (mmol/l)	5.6 (1.0)	5.6 (1.1)	0.91
Creatinine (μmol/l)	92.1 (31.9)	86.7 (22.4)	0.07

centrifuged at 3000 g for 10 min and the isolated serum and plasma was stored at -80°C . Total serum homocysteine concentration was measured using reversed-phase high-performance liquid chromatography with fluorescent detection after manual preparation of derivatives. The intra-assay (inter-assay) coefficients of variation were 3% (5%). DNA was extracted from leucocytes and MTHFR genotyping was performed as described previously (Frosst *et al.*, 1995). Circulating markers of endothelial function were measured using enzyme-linked immunosorbent assay-based commercially available kits in the first consecutive 110 cases and 50 controls. The methods and results of these assays have been reported in detail in a related study concerning the role of endothelial dysfunction in cerebral SVD (Hassan *et al.*, 2003).

Assessment of scans

MRI scans were performed in 142 patients (82.5%). In all of the cases with MRI, axial T2-weighted images were evaluated blind to clinical and laboratory data by a single observer using a semiquantitative rating scale. The Fazekas scale was used to score leukoaraiosis as this scale been shown to reflect pathological severity of cerebral SVD in a post-mortem validation study (Fazekas *et al.*, 1993). An additional category was included to allow differentiation of more severe cases of leukoaraiosis. Leukoaraiosis was rated as: 1 = absent or mild (equivalent to Fazekas periventricular score ≤ 2); 2 = moderate (Fazekas scale 3); 3 = severe (more than half of the hemispheric white matter involved). In addition we scored the number of lacunar infarcts: 1 = ≤ 2 lesions; 2 = 3–5 lesions; 3 = > 5 lesions. Separate scores were generated for small (≤ 5 mm maximal diameter) and large (6–14 mm maximal diameter) focal lesions.

Subtyping of SVD

To explore possible pathogenic differences between patients with isolated lacunar infarcts and those with ischaemic leukoaraiosis, cerebral SVD patients with MRI were subtyped according to their scan appearances. Isolated lacunar infarction was defined as at least one focal lesion and a leukoaraiosis score of 1 (absent or mild). Ischaemic leukoaraiosis was defined as at least one focal lesion and a leukoaraiosis score of 2–3. Twenty MRI scans were also randomly selected for re-evaluation by the same observer. There was perfect repeatability ($\kappa = 1.0$) for allocation of subtype. For the MRI disease scores, the repeatability (weighted kappa) was good for the leukoaraiosis category ($\kappa = 0.85$), and moderate for both the large ($\kappa = 0.58$) and small focal lesion categories ($\kappa = 0.43$).

Statistical analysis

Natural logarithmic transformation was used to normalize distributions prior to analysis by parametric tests. Otherwise non-parametric tests were used. Homocysteine levels are expressed as geometric means with the 95% confidence interval (CI) for the mean. Separate analyses were performed in those with MRI to determine differences between SVD subtypes and association with extent of disease. The χ^2 test was used to compare proportions and the unpaired *t* test or analysis of variance to compare normally distributed data between two or more groups. *Post hoc* tests were performed using Bonferroni's method. Logistic regression analysis was performed to determine the relationship between homocysteine and SVD presence. Linear regression was used to determine the association between homocysteine and SVD grade. Association between MTHFR genotype and presence of SVD was tested according to additive (TT versus CT versus CC), dominant (TT + CT versus CC) and recessive (TT versus CC + CT) models in univariate and multivariate analyses. The -2 log likelihood ratio was used to assess logistic regression models where SVD was the dependent variable and homocysteine a covariate, before and after inclusion of endothelial markers. Repeatability measures for phenotype assignment and MRI grading scales was based on κ scores (Altman, 1991).

Results

Characteristics of cases and controls

SVD patients and controls were well matched for age and gender, but hypertension, smoking and diabetes were more common in the SVD group (Table 1). Of the 142 cases with MRI, 52 were subtyped as isolated lacunar infarction (36.6%) and 90 as ischaemic leukoaraiosis (63.4%). In these 142, the severity of leukoaraiosis was scored as grade 1 in 52 patients (36.6%), grade 2 in 68 (47.9%) and grade 3 in 22 (15.5%). For large focal lesions (6–14 mm) the scores were as follows: grade 1, 109 cases (76.8%); grade 2, 25 cases (17.6%); grade 3, eight cases (5.6%). For small focal lesions (≤ 5 mm) the distribution was: grade 1, 17 (12.0%); grade 2, 11 (7.7%); grade 3, 114 (80.3%).

Relationships between homocysteine and conventional risk factors

In the whole study population, there were significant associations between homocysteine levels and age ($r = 0.20$, $P <$

Table 2 Association between homocysteine quartiles and risk of SVD

Homocysteine quartile (μmol/l)	OR (unadjusted)	OR (adjusted) (– MTHFR)	(+ MTHFR)
<10.3 (reference)	–	–	–
10.3–13.0	1.50 (0.80–2.82)	1.42 (0.70–2.89)	1.50 (0.72–3.14)
13.1–15.9	3.28 (1.75–6.15)*	2.02 (1.37–2.99)*	2.05 (1.38–3.04)*
>15.9	4.98 (2.59–9.57)*	2.06 (1.53–2.78)*	2.05 (1.52–2.77)*

OR (95% CI) unadjusted and adjusted for conventional risk factors and creatinine, both before and after additional adjustment for MTHFR genotype (* $P < 0.0005$).

0.0005) and serum creatinine ($r = 0.35$, $P < 0.0005$). Elevated homocysteine levels were associated with male gender (13.83 μmol/l, 95% CI 13.14–14.57 versus 12.40 μmol/l, 95% CI 11.76–13.08 in females; $P = 0.005$), hypertension (13.90 μmol/l, 95% CI 13.28–14.60 versus 12.32 μmol/l, 95% CI 11.63–13.05; $P = 0.002$), and smoking (ever smoked 13.64 μmol/l, 95% CI 13.01–14.3 versus 12.40 μmol/l, 95% CI 11.67–13.19 in never smokers; $P = 0.02$). Vitamin status was available in a subgroup of cases; there were significant negative correlations between homocysteine and both serum folate ($n = 45$, $\rho = -0.34$, $P = 0.024$) and serum B12 ($n = 35$, $\rho = -0.38$, $P = 0.025$).

Homocysteine levels in cases and controls

Mean homocysteine levels were higher in patients with SVD than in controls (14.55 μmol/l, 95% CI 13.78–15.35, versus 12.01 μmol, 95% CI 11.42–12.64; $P < 0.0005$). The difference remained significant after controlling for age, sex, hypertension, diabetes mellitus, smoking history, history of myocardial infarction, serum cholesterol, creatinine concentration and MTHFR genotype ($P < 0.0005$). The odds ratio (OR) for SVD increased with increasing quartile of homocysteine with the lowest quartile as reference ($P < 0.0005$ for trend) (Table 2). After adjustment for risk factors and creatinine, the OR associated with homocysteine was 8.34 (95% CI 3.63–19.14) per 1 μmol increase in log concentration ($P < 0.0005$). This was only slightly reduced (to 7.91, 95% CI 3.93–18.44; $P < 0.0005$) after additionally controlling for MTHFR genotype.

Homocysteine and SVD subtype

There were significant differences in homocysteine levels among the subtypes of SVD and controls (analysis of variance, $P < 0.0005$). Homocysteine levels were higher amongst patients with ischaemic leukoaraiosis (15.15 μmol/l, 95% CI 14.13–16.24) compared with both those with isolated lacunar infarction (13.14 μmol/l, 95% CI 11.99–14.39; $P = 0.04$) and controls (12.01 μmol/l, 95% CI 11.42–12.64; $P < 0.0005$). After adjustment for conventional risk factors and creatinine, and both before and after additional adjustment for MTHFR genotype, homocysteine levels remained significantly higher amongst ischaemic leukoaraiosis and isolated

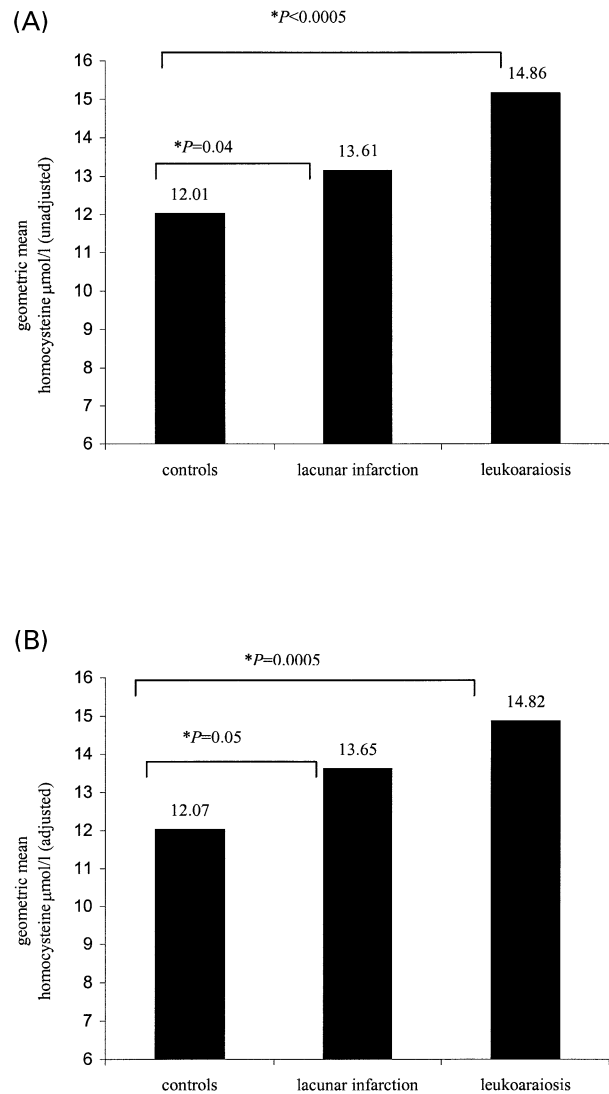


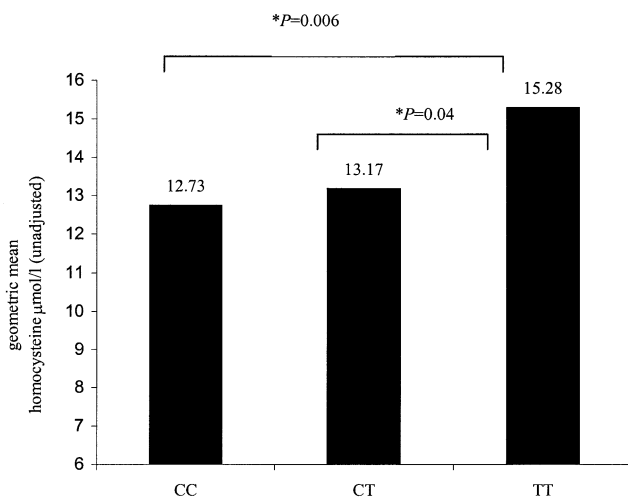
Fig. 1 Comparison of homocysteine levels between SVD subtypes and controls (A) adjusted for conventional risk factors and creatine concentration and (B) adjusted for conventional risk factors, creatinine concentration and MTHFR genotype. **Post hoc* analysis using the Bonferroni method.

lacunar infarction in comparison with controls (Fig. 1). For isolated lacunar infarction, homocysteine was associated with an OR of 4.22 (95% CI 1.29–13.73, $P = 0.02$) per 1 μmol increase in log homocysteine concentration after adjustment

Table 3 OR associated with the MTHFR genotype for different subtypes of SVD

	OR unadjusted	P	OR adjusted*	P
Ischaemic leukoaraiosis (n = 90)				
Recessive model	2.79 (1.36–5.7)	0.005	3.05 (1.32–7.07)	0.004
Additive model	1.84 (1.26–2.69)	0.002	2.02 (1.31–3.1)	0.001
Isolated lacunar infarction (n = 52)				
Recessive model	1.79 (0.72–4.5)	0.21	2.34 (0.83–6.64)	0.11
Additive model	1.02 (0.64–1.63)	0.93	1.04 (0.62–1.75)	0.87

*Adjusted for conventional risk factors and creatinine concentration. Recessive model = TT versus CT + CC; additive model = TT versus CT versus CC.

**Fig. 2** Homocysteine concentration (geometric mean) and MTHFR genotype. *Post hoc analysis using Bonferroni method.

for risk factors and creatinine, and 4.00 (95% CI 1.21–13.19, $P = 0.02$) after additional adjustment for MTHFR genotype. For ischaemic leukoaraiosis the adjusted OR was 12.92 (95% CI 4.40–37.98, $P < 0.0005$), and 11.05 (95% CI 3.66–33.38, $P < 0.0005$) after additional adjustment for MTHFR genotype.

Homocysteine and extent of SVD

Homocysteine levels correlated with the grade of leukoaraiosis ($P = 0.02$) and large focal lesions ($P = 0.04$), but not with the grade of small focal lesions ($P = 0.80$). After adjustment for risk factors and creatinine, both leukoaraiosis and large focal lesions remained associated with homocysteine levels ($P = 0.02$ and $P = 0.006$, respectively), and ($P = 0.048$ and $P = 0.008$ respectively) after additional adjustment for MTHFR genotype.

MTHFR genotype and homocysteine concentration

The MTHFR genotype was determined in 170 cases and 170 controls. In two cases and two controls DNA could not be amplified. MTHFR genotype was in Hardy–Weinberg equilibrium

amongst the entire study population ($\chi^2 = 1.00$, $P = 0.61$), cases ($\chi^2 = 1.48$, $P = 0.48$) and controls ($\chi^2 = 0.00$, $P = 1.00$). In the whole study group, there was a graded increase in homocysteine concentration across the MTHFR genotypes ($P = 0.005$ for trend) with homocysteine concentrations highest in those with the TT genotype (Fig. 2). Following adjustment for vascular risk factors and creatinine the trend remained significant ($P = 0.02$).

MTHFR genotype and SVD

There was a significant difference in MTHFR genotype distribution between cases and controls [TT 33 (19.4%), CT 71 (41.8%), CC 66 (38.8%) in cases versus TT 16 (9.41%), CT 73 (42.94%) and CC 81 (47.6%) in controls; $\chi^2 = 7.46$, $P = 0.02$]. According to an additive model, the OR for SVD associated with the 677T allele was 1.47 (95% CI 1.08–2.00, $P = 0.01$). Using a recessive model the OR was 2.32 (95% CI 1.22–4.40, $P = 0.01$). There was no association with the MTHFR 677T allele according to a dominant model (OR 1.19, 95% CI 0.95–1.59, $P = 0.14$). The MTHFR 677T allele remained associated with the presence of disease following adjustment for vascular risk factors and serum creatinine (additive model, OR 1.54, 95% CI 1.09–2.18, $P = 0.01$; recessive model, OR 2.72, 95% CI 1.32–5.60, $P = 0.007$).

MTHFR genotype and SVD subtype

The MTHFR polymorphism was associated with the ischaemic leukoaraiosis subtype, but there was no significant association with isolated lacunar infarction (Table 3). Consistent with this, the MTHFR genotype was over-represented in patients with moderate or severe white matter changes (grade 2 or above) on MRI ($\chi^2 = 11.37$, $P = 0.02$), whilst there was no difference in genotype distribution across the different grades of focal lesions scored (large lesions, $\chi^2 = 3.30$, $P = 0.51$; small lesions, $\chi^2 = 2.83$, $P = 0.59$).

Homocysteine and endothelial markers

Homocysteine levels correlated with TM ($r = 0.37$, $P < 0.0005$) and ICAM1 ($r = 0.18$, $P = 0.02$). In a logistic regression model, inclusion of endothelial markers improved

Table 4 Effect of controlling for levels of circulating endothelial markers on the relationship between homocysteine and SVD in the first 110 cases and 50 controls

Model*	Adjusted OR (95% CI)/ μmol log homocysteine	P	-2 log likelihood
Homocysteine	3.16 (1.05–9.56)	0.04	182.89
Homocysteine + TM	1.74 (0.53–5.71)	0.36	161.53
Homocysteine + ICAM1	2.60 (0.82–8.21)	0.10	167.00
Homocysteine + ICAM1 + TM	1.59 (0.46–5.54)	0.47	151.92

*All models adjusted for conventional risk factors and creatinine

the predictive model for the presence of SVD (decrease in -2 log likelihood), but homocysteine was no longer independently associated with SVD (Table 4).

Discussion

This study confirms that homocysteine is an independent risk factor for SVD as a whole, but extends previous findings to suggest that it is a much stronger risk factor for the ischaemic leukoaraiosis subtype. Furthermore, we found evidence of a dose-response relationship, with increasing homocysteine concentrations associated with greater risk of developing SVD. We also found that homocysteine levels were associated with the markers of endothelial dysfunction ICAM1 and TM. Inclusion of these markers as covariates reduced the association with homocysteine but improved the overall logistic regression model for prediction of SVD. These findings are consistent with the hypothesis that endothelial dysfunction is an important mechanism through which homocysteine mediates its effects in SVD. The effects of homocysteine could include direct endothelial damage or stimulation of an endothelial inflammatory response, which have been noted previously in SVD (Kario *et al.*, 1996; Fassbender *et al.*, 1999a).

Our findings are compatible with the known effects of homocysteine. In a number of *in vitro* and animal experiments, homocysteine has been shown to lead to endothelial cell injury and functional abnormalities in the release of endothelial nitric oxide (Wall *et al.*, 1980; Stamler *et al.*, 1993; Lentz *et al.*, 1996). In human subjects, high levels of homocysteine impair nitric oxide-mediated blood flow responses (Tawakol *et al.*, 1997; Woo *et al.*, 1997) and previous studies have confirmed the association between homocysteine and circulating markers of endothelial dysfunction (Rohde *et al.*, 1999).

In comparison with large vessel atherosclerosis (Yoo *et al.*, 1998; McQuillan *et al.*, 1999; Spence *et al.*, 1999), the role of homocysteine in SVD is less well established, but the results of our study are consistent with earlier reports (Fassbender *et al.*, 1999b; Matsui *et al.*, 2001) that homocysteine is an important risk factor for SVD. In addition, we found homocysteine to be associated with the extent of disease, correlating with grade of both large focal lesions and leukoaraiosis. There was no association with the grade of small focal lesions ≤ 5 mm. This may reflect inconsistency in

rating small T2 hyperintensities or heterogeneous aetiology, since very small lesions can equally represent perivascular spaces, gliosis and demyelination as well as ischaemic lacunar infarcts (Awad *et al.*, 1986). However, focal hyperintensities >5 mm are likely to represent true lacunar infarcts associated with cerebral SVD (Braffman *et al.*, 1988).

Cerebral SVD may represent a clinical and pathological spectrum, with different mechanisms underlying isolated lacunar infarcts and diffuse leukoaraiosis. It has been hypothesized that localized microatheroma at the origin of perforating arterioles may in part be responsible for some cases of isolated lacunar infarction, whilst diffuse arteriosclerosis is the predominant pathology in the leukoaraiosis group (de Jong *et al.*, 2002). Consistent with this heterogeneity, we found differing associations between both homocysteine and the MTHFR genotype and between the two subtypes of SVD. Homocysteine levels were highest among ischaemic leukoaraiosis patients, whilst a more modest increase was seen in those with isolated lacunar infarcts. Therefore homocysteine could be more important in the development of small vessel arteriosclerosis than microatheroma. The differences in pathogenesis may explain the controversy concerning the relative importance of homocysteine in large and small vessel diseases. Some reports have concluded that homocysteine is particularly damaging to small penetrating arteries (Evers *et al.*, 1997; Fassbender *et al.*, 1999b), whilst other studies have found lower levels of homocysteine in SVD compared with other stroke subtypes (Lindgren *et al.*, 1995; Eikelboom *et al.*, 2000). The inconsistencies could reflect different SVD case mixes and the methods used to subtype patients. Interestingly, our ischaemic leukoaraiosis group were similar to the group studied by Fassbender and colleagues (Fassbender *et al.*, 1999b). They similarly found very high levels of homocysteine among patients with subcortical arteriosclerotic encephalopathy, characterized by diffuse ischaemic white matter changes, cognitive impairment and lacunar stroke. In this group of patients, hyperhomocysteinaemia could predispose to cognitive decline and gait disturbance through direct excitotoxic and pro-apoptotic effects on white matter tracts as well as predisposing to small vessel ischaemic demyelination (Lipton *et al.*, 1997; Kruman *et al.*, 2000; Seshadri *et al.*, 2002; Vermeer *et al.*, 2002).

There is strong evidence from twin (Carmelli *et al.*, 1998) and family history (Polychronopoulos *et al.*, 2002) studies to

suggest that genetic influences are important in the pathogenesis of SVD. Given the importance of homocysteine in SVD, we tested a commonly occurring polymorphism in the MTHFR gene (C677T). As previously demonstrated (Frosst *et al.*, 1995), we found that the 677T allele influenced homocysteine levels, but it was also significantly over-represented in the ischaemic leukoaraiosis group but not in the isolated lacunar infarction group. This is consistent with our findings of a greater elevation of serum homocysteine levels in this group. The role of the MTHFR gene in stroke and vascular diseases in general remains controversial, but our study supports the notion that separation of phenotypes according to pathogenic basis is important in establishing underlying molecular genetic risk factors.

We did not specifically exclude the presence of white matter hyperintensities in controls by brain imaging but used a representative community control population. The requirement for brain imaging markedly reduces the recruitment rate and can introduce bias in control recruitment. Even if our controls did have asymptomatic cerebral SVD, this would not alter our conclusions; in contrast, their inclusion would be expected to reduce the magnitude of association reported with homocysteine and MTHFR genotype.

Random homocysteine was measured, as it was not practical to obtain fasting samples on patients who were seen in an outpatient setting. However, we were still able to replicate previously reported associations between homocysteine and a number of lifestyle and cardiovascular risk factors including blood pressure, male sex, smoking history and serum creatinine concentration (Eikelboom *et al.*, 2000; Matsui *et al.*, 2001).

Our results may have important clinical implications. Current treatment of patients with cerebral SVD is limited. Homocysteine levels can be modified using folate or B12 (Homocysteine Lowering Trialists' Collaboration, 1998), which could be particularly effective in patients with SVD for a number of reasons. First, homocysteine levels in SVD correlated with low B12 and folate, consistent with earlier findings (Fassbender *et al.*, 1999b). Secondly, the MTHFR 677TT genotype was over-represented in SVD, and it has been found that folate status particularly influences homocysteine levels in individuals with this genotype (Jacques *et al.*, 1996; Malinow *et al.*, 1997). Promisingly, lowering of homocysteine with folic acid has been shown to have beneficial effects on endothelial dysfunction, including improvement in TM levels (Constans *et al.*, 1999) and endothelium-dependent blood flow responses (Chambers *et al.*, 2000). Given our findings, if vitamin supplementation is effective it may well offer varying therapeutic benefit in different stroke subtypes and could be particularly beneficial in patients with leukoaraiosis.

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