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 µmol/l [95% confidence interval (CI) 13.78–15.35] versus 12.01 µmol/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, hyperhomocysteaemia 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...
ischaemic basis (Jones group of patients in whom leukoaraiosis is likely to have an account for the vast majority of cases. The term ‘ischaemic ischaemic pathologies, although ischaemia is believed to be a radiological definition and can also be caused by non-ischaemic injury (leukoaraiosis). Leukoaraiosis itself is a radiological definition, which 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 (Botten 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 (Bevilaqua 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 (Frostt 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
genotyping was performed as described previously (Frosst et al., 1995). DNA was extracted from leucocytes and MTHFR genotyping was performed as described previously (Frosst et al., 1995). 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 (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 has 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 (κ = 1.0) for allocation of subtype. For the MRI disease scores, the repeatability (weighted kappa) was good for the leukoaraiosis category (κ = 0.85), and moderate for both the large (κ = 0.58) and small focal lesion categories (κ = 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 χ² 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 genotype assignment and MRI grading scales was based on k 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 < 0.05) and between homocysteine levels and hypertension, smoking and diabetes (P < 0.05). For isolated lacunar infarction, homocysteine was associated with hypertension (P = 0.01) and diabetes (P = 0.03); in the leukoaraiosis group, homocysteine was associated with hypertension (P = 0.03) and smoking (P = 0.02). Between SVD subtypes, homocysteine was higher in those with leukoaraiosis than in those with isolated lacunar infarction (P < 0.0005). In addition, homocysteine was associated with the severity of leukoaraiosis (P < 0.0005) and was higher in those with grade 3 than in those with grades 1 or 2.
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 ($n = 45$, $r = -0.34$, $P = 0.024$) and serum B12 ($n = 35$, $r = -0.38$, $P = 0.025$).

### 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.

#### Table 2 Association between homocysteine quartiles and risk of SVD

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

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

### 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 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

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*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, creatine concentration and MTHFR genotype. *Post hoc analysis using the 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 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 (9.4%), CT 73 (42.9%), 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

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

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

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

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**Fig. 2** Homocysteine concentration (geometric mean) and MTHFR genotype. *Post hoc* analysis using Bonferroni method.
the predictive model for the presence of SVD (decrease in –
2 log likelihood), but homocysteine was no longer independ-
ently 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 associ-
ated 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 experi-
ments, 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 dys-
function (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 microtherma at the origin of
perforating arterioles may in part be responsible for some
cases of isolated lacunar infarction, whilst diffuse arterio-
sclerosis is the predominant pathology in the leukoaraiosis
group (de Jong et al., 2002). Consistent with this hetero-
genity, we found differing associations between both homo-
cysteine 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 microather-
oma. The differences in pathogenesis may explain the
controversy concerning the relative importance of homo-
cysteine 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 homo-
cysteine 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 predis-
spose 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

### 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

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

*All models adjusted for conventional risk factors and creatinine.
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 Trials’ 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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