Moderately Elevated Plasma Homocysteine, Methylenetetrahydrofolate Reductase Genotype, and Risk for Stroke, Vascular Dementia, and Alzheimer Disease in Northern Ireland

Stephen P. McIlroy, PhD; Kevin B. Dynan, MD; John T. Lawson, FRCR; Christopher C. Patterson, PhD; A. Peter Passmore, MD

Background and Purpose—Elevated plasma homocysteine level has been associated with increased risk for cardiovascular and cerebrovascular disease. Variation in the levels of this amino acid has been shown to be due to nutritional status and methylenetetrahydrofolate reductase (MTHFR) genotype.

Methods—Under a case-control design we compared fasting levels of homocysteine and MTHFR genotypes in groups of subjects consisting of stroke, vascular dementia (VaD), and Alzheimer disease patients and normal controls from Northern Ireland.

Results—A significant increase in plasma homocysteine was observed in all 3 disease groups compared with controls. This remained significant after allowance for confounding factors (age, sex, hypertension, cholesterol, smoking, creatinine, and nutritional measures). MTHFR genotype was not found to influence homocysteine levels, although the T allele was found to increase risk for VaD and perhaps dementia after stroke.

Conclusions—We report that moderately high plasma levels of homocysteine are associated with stroke, VaD, and Alzheimer disease. This is not due to vascular risk factors, nutritional status, or MTHFR genotype.

Key Words: cerebrovascular disorders ▪ dementia ▪ genetics ▪ homocyst(e)ine

Homocysteine is a thiol-containing amino acid that is generated during 1-carbon metabolism. Elevation of the plasma levels of this metabolite has been associated with increased risk for cardiovascular and cerebrovascular disease in 2 studies. The results of these studies have suggested that the graded risk associated with elevated homocysteine levels is causal. Furthermore, it has been suggested that even moderately elevated levels (14 μmol/L) confer increased risk of cardiovascular disease.

Many cases of moderately elevated homocysteine are associated with deficiencies of enzyme cofactors (folate, vitamin B₁₂, and vitamin B₆) required for homocysteine metabolism. However, a genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in homocysteine metabolism, has been associated with elevated plasma homocysteine. This polymorphism (also called the thermolabile variant) is a C-T transition at nucleotide 677 causing an alanine to valine substitution, leading to a reported 50% reduction in enzyme activity. The association of this polymorphism with elevated homocysteine levels is not a universal finding because individuals homozygous for the T allele and who have folic acid levels above the median are found to have normal plasma homocysteine levels. Therefore, the activity of the thermolabile variant of this enzyme must be influenced by the availability of folate.

In addition to folate, plasma levels of homocysteine are influenced by other known cardiovascular risk factors. Nygard et al found a positive association between elevated homocysteine and high blood pressure and elevated cholesterol levels. The authors also reported a linear relationship between homocysteine levels and number of cigarettes smoked. It is also known that homocysteine levels increase with age, which is independent of vitamin B status. In addition, plasma homocysteine levels are higher in males than in females. In any analysis of homocysteine levels with disease, it would therefore be necessary to allow for these possible confounding factors.

Metabolites of homocysteine, such as homocysteic acid, have been shown to have an excitotoxic effect on glutamatergic N-methyl-D-aspartate receptors. This effect is several times greater than that of glutamate and causes elevation of intracellular Ca²⁺, activation of proapoptotic proteins, and...
cell death.\textsuperscript{13} Homocysteine can also be metabolized to S-adenosyl homocysteine, which may exhibit neurotoxicity by inhibition of methylation reactions and thus monoamine neurotransmitter metabolism and protein and phospholipid methylation.\textsuperscript{14,15} Therefore, raised homocysteine levels have the potential to effect neurodegeneration.

Hypothesizing that elevated plasma homocysteine could influence the etiology and progression of stroke, vascular dementia (VaD), and/or Alzheimer disease (AD), we compared, under a case-control design, fasting levels of homocysteine, apolipoprotein E (APOE), and MTHFR genotypes in a clinically well-defined population of AD patients, VaD patients, nondemented stroke patients (NDS), and normal controls from the relatively genetically homogeneous Northern Ireland population.\textsuperscript{16}

Subjects and Methods

Ethical approval for this study was obtained from the Research Ethics Committee, Queen’s University of Belfast. Written informed consent was obtained from patients/caregivers and controls before the collection of blood samples. Patients and controls were white and were ascertained to have at least parents and grandparents born in Northern Ireland to ensure ethnicity. All subjects with a strong family history of dementia (>1 first-degree relative) were excluded.

Subjects were randomly recruited from the Memory Clinic (AD: n=83; mean age, 77.2 years; SD=8.1 years; 30% male; VaD: n=78; mean age, 77.3 years; SD=9.3 years; 33% male), Stroke Unit, and Day Hospital, Belfast City Hospital (NDS: n=64; mean age, 73.8 years; SD=8.1 years; 58% male). Healthy controls (n=71; mean age, 74.3 years; SD=7.6 years; 20% male) were recruited from the local podiatry clinic and volunteers previously known to the department. The diagnosis of probable AD was determined by 2 experienced clinicians using criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition and National Institute of Neurological Disorders and Stroke (NINDS)–Alzheimer’s Disease and Related Disorders Association (ADRA).\textsuperscript{17} A CT scan was performed to aid diagnosis in the majority of cases. Diagnosis of VaD was made with the use of strict NINDS–Association Internationale pour la Recherche et l’Enseignement en Neurosciences (AIREN) criteria, which require a temporal relationship between onset of dementia and cerebrovascular incident. Cognitive status as measured by the Mini-Mental State Examination\textsuperscript{18} was a median of 83; mean age, 77.2 years; SD=78; 1 first-degree relative) were excluded.

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All subjects and controls had a nutritional score calculated with the use of the Mini-Nutritional Assessment (MNA),\textsuperscript{23} which encompasses anthropometric measures, a dietary questionnaire, and a global assessment. Other recorded details included history of hypertension, cardiovascular disease, peripheral vascular disease, smoking, educational status, and socioeconomic status (childhood and adult). A fasting venous blood sample was collected from all subjects.

Serum levels of vitamin B\textsubscript{12}, (pyridoxal phosphate and pyridoxal) were measured by high-performance liquid chromatography; levels of vitamin B\textsubscript{6}, folate, and fasting cholesterol were measured in hospital laboratories by standard methods. Blood samples for measurement of plasma total homocysteine levels were kept on ice until separation of plasma, which was performed <1 hour after venesection for all samples. Plasma was then kept at ~20°C until measurement by high-performance liquid chromatography according to the method described by Ubbink et al.\textsuperscript{24} Subjects were also genotyped for APOE\textsuperscript{25} and MTHFR\textsuperscript{7} polymorphisms.

Statistical Analysis

Quantitative variables that were positively skewed were logarithmically transformed to approximate a normal distribution before statistical analysis. Quantitative variables were summarized as mean and SD or median and interquartile range if the distribution of the variable was skewed. Subject groups were compared on quantitative characteristics by use of 1-way ANOVA and on qualitative characteristics by use of Pearson’s χ\textsuperscript{2} test. Radiological scales were compared by the Mann-Whitney U test. Significance levels were set at 5% for these comparisons.

Multiple regression analysis was used to adjust for possible confounders (age, sex, smoking status, blood pressure, blood pressure history, cholesterol, APOE status, log creatinine, childhood and adult social status, educational status, and nutritional measures of vitamin B\textsubscript{12}, folate, pyridoxal and pyridoxal phosphate, MNA, and body mass index) in the comparison of homocysteine levels between groups. These confounding variables were added to the model individually before the most relevant were included simultaneously. Odds ratios (ORs) (95% CI) for raised homocysteine were calculated by reference to a cutoff value for homocysteine levels represented by the upper quartile of the distribution in the control group. Adjustment for the influence of confounding variables was performed with a logistic regression model.

Genotype and allele frequencies were compared with the use of Pearson’s χ\textsuperscript{2} test. Since 5 comparisons were used (disease groups versus controls, AD versus VaD, and VaD versus NDS), the level of significance was adjusted to 1%. ORs and 95% CIs were calculated.

Results

Subject group characteristics are shown in Table 1. The subject groups differed significantly in a number of measurements. There was a small but significant difference between ages, with the mean age in the AD and VaD groups being different from the mean age in the NDS and control groups (77 versus 74 years, respectively; \(P<0.05\)). A higher, although not statistically significant, percentage of controls and NDS cases had been educated to a secondary level or higher (33% [controls] and 31% [NDS] versus 26% [AD] and 21% [VaD]; \(P>0.05\)). The NDS and VaD groups had significantly smaller proportions of subjects who never smoked (45% and 50% versus 64% [AD] and 70% [controls]; \(P<0.01\)).

Mean serum folate was significantly higher in the control group than in the other subject groups. The controls also had the highest mean levels of vitamins B\textsubscript{12} and B\textsubscript{6}, although these values did not reach significance. Furthermore, this group also had the lowest mean creatinine level, a potential mediator of homocysteine levels. Controls also scored the highest on the MNA, suggesting least risk for malnutrition (\(P<0.001\)). The NDS and AD MNA scores were not significantly different (\(P=0.42\)), but the VaD score differed significantly from both the AD group (\(P=0.005\)) and the NDS group (\(P<0.001\)).

Radiological measures showed that the AD group was different from the VaD group (data not shown). The VaD (37%) and NDS (29%) groups were not significantly different in overall levels of cerebrovascular disease as depicted on CT scan (\(P=0.42\)) but were significantly different from AD cases (6%) (\(P<0.001\)). White matter lesions were significantly more prevalent in the VaD group (23%) than in the NDS group (0%) or the AD group (9%) (\(P<0.001\)).

Homocysteine levels were positively skewed and were therefore logarithmically transformed. After transformation, 1-way ANOVA showed significant differences in means.
between the groups, and Dunnett’s multiple range tests revealed that the mean level in the control group was significantly lower than the means in each of the other groups (P<0.001) (Table 1). Although the mean homocysteine levels were higher in the VaD group than in the NDS group, this just failed to reach significance. Furthermore, while mean levels in VaD and AD differed significantly (P=0.03), the levels in AD and NDS groups were similar (P=0.6). The difference in mean log-transformed homocysteine levels with associated 95% CIs were converted to anti-log values to give a ratio of geometric means with CIs on the original (untransformed) scale (Table 2). Multiple regression analysis was used to adjust for possible confounding by other variables. While adjustment for these factors individually had very little effect, adjustment for the most influential factors simultaneously reduced the difference between the control group and other groups. However, the difference remained significant (Table 2).

The upper quartile of the homocysteine distribution in the control group was 13.3 μmol/L. ORs were calculated with the use of logistic regression to estimate the risk of having homocysteine levels >13.3 μmol/L in AD, VaD, or NDS groups compared with controls. The ORs for all 3 patient groups were significant (Table 2). Possible confounding variables, both individually and simultaneously, were included as independent variables in the logistic regression

<table>
<thead>
<tr>
<th>TABLE 1. Group Characteristics</th>
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<tbody>
<tr>
<td>AD (n = 83) (25M, 58F) &amp; VaD (n = 78) (26M, 52F) &amp; NDS (n = 64) (37M, 27F) &amp; Control (n = 71) (14M, 57F) &amp; P</td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
</tr>
<tr>
<td>Body mass index, mean (SD)</td>
</tr>
<tr>
<td>Systolic BP, mean (SD), mm Hg*</td>
</tr>
<tr>
<td>Diastolic BP, mean (SD), mm Hg*</td>
</tr>
<tr>
<td>Cholesterol, mean (SD), mmol/L</td>
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<tr>
<td>Triglyceride, mean (SD), mmol/L</td>
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<tr>
<td>B12 level, median (IQR), ng/L</td>
</tr>
<tr>
<td>Serum folate, median (IQR), μg/L</td>
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<tr>
<td>Homocysteine, geometric mean (IQR), μmol/L</td>
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<tr>
<td>MNA score, mean (SD)</td>
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<tr>
<td>Creatinine, median (IQR), μmol/L</td>
</tr>
<tr>
<td>Pyridoxal phosphate, median (IQR), μg/L</td>
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<td>Pyridoxal, median (IQR), μg/L</td>
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</table>

*History of hypertension in 27%, 51%, 56%, and 1%, respectively (P<0.001).
IQR indicates interquartile range.

<table>
<thead>
<tr>
<th>TABLE 2. Ratio of Geometric Mean Homocysteine Levels Between the 3 Disease Groups and the Control Group and OR for Homocysteine Levels &gt;13.3 μmol/L in the 3 Disease Groups Relative to the Control Group, Before and After Adjustment for Potential Confounders and Nutritional Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD vs Control &amp; VaD vs Control &amp; NDS vs Control</td>
</tr>
<tr>
<td>Ratio of geometric mean homocysteine levels (95% CI)</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted for vascular risk factors and creatinine*</td>
</tr>
<tr>
<td>Adjustment for nutritional measures†</td>
</tr>
<tr>
<td>Adjusted for vascular risk factors, creatinine, and nutritional measures*†</td>
</tr>
<tr>
<td>OR (95% CI) for homocysteine level &gt;13.3 μmol/L</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted for vascular risk factors and creatinine*</td>
</tr>
<tr>
<td>Adjusted for nutritional measurements†</td>
</tr>
<tr>
<td>Adjusted for vascular risk factors, creatinine, and nutritional measures*†</td>
</tr>
</tbody>
</table>

*Age, sex, hypertension, cholesterol, adult and childhood social status, educational status, smoking, and log creatinine.
†B12, folate, pyridoxal phosphate, pyridoxal (all log transformed), body mass index, and MNA.
‡P<0.001.
§P<0.01.
‖P<0.05.
Mean homocysteine levels increased nonsignificantly from the CC genotype (15.4 \(\mu\)mol/L; SD 8.0) through the CT genotype (15.9 \(\mu\)mol/L; SD 6.9) to the TT genotype (19.5 \(\mu\)mol/L; SD 13.1). However, there was no association between MTHFR genotype and homocysteine levels in any subject group. Similarly, no association was detected between the MTHFR T allele and homocysteine levels even after adjustment for a possible interaction between T allele carriage and folate levels (\(P=0.35\)).

**Discussion**

The main finding in this study was the significant increase in plasma homocysteine levels in all disease groups compared with controls. This difference remained significant after adjustment for conventional vascular risk factors and other possible confounders. Although the groups differed in their MNA scores and folate and vitamin \(B_6\) levels, the increase in plasma homocysteine remained significant even when these factors were included in the logistic regression model. This indicates that mildly elevated homocysteine levels may therefore significantly increase risk for VaD, AD, or stroke.

These results agree with other recent studies that have assessed the involvement of elevated homocysteine and dementia. The mean level in the AD patients assessed in this study agreed exactly with the histologically confirmed patient in the study of Clarke et al\(^{26}\) at 16.3 \(\mu\)mol/L. The 2 studies also agree that the VaD groups had the highest levels;
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TABLE 4. Distribution of MTHFR Genotype and Allele Frequencies Between Groups

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>AD</th>
<th>%</th>
<th>VaD</th>
<th>%</th>
<th>NDS</th>
<th>%</th>
<th>Controls</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>59</td>
<td>71.1</td>
<td>31</td>
<td>40.8</td>
<td>39</td>
<td>61.9</td>
<td>50</td>
<td>70.4</td>
</tr>
<tr>
<td>CT</td>
<td>20</td>
<td>24.1</td>
<td>37</td>
<td>48.7</td>
<td>23</td>
<td>36.5</td>
<td>19</td>
<td>26.8</td>
</tr>
<tr>
<td>TT</td>
<td>4</td>
<td>4.8</td>
<td>8</td>
<td>10.5</td>
<td>1</td>
<td>1.6</td>
<td>2</td>
<td>2.8</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>138</td>
<td>83.1</td>
<td>99</td>
<td>65.1</td>
<td>101</td>
<td>80.2</td>
<td>119</td>
<td>83.8</td>
</tr>
<tr>
<td>T</td>
<td>28</td>
<td>16.9</td>
<td>53</td>
<td>34.9</td>
<td>25</td>
<td>19.8</td>
<td>23</td>
<td>16.2</td>
</tr>
</tbody>
</table>

The effect of the APOE polymorphism on the risk for AD was as expected. However, as we previously reported,27 there was a nonsignificant trend for increased representation of the APOE 4 allele in VaD cases compared with controls or NDS cases. The role of APOE 4 in vascular dementia is less clear than in AD.28–31 This has led to the claim that the APOE 4 increased risk for VaD in some populations may be due to the presence of coexisting AD.32 However, it has been reported that the presence of APOE 4 increases risk for all dementias and that this is not mediated by vascular factors.33 The present report indicates that possession of the APOE 4 allele may increase risk for VaD in those patients who suffer stroke. In accordance with the conclusions of Prince et al,33 the APOE 4 allele did not appear to influence risk for stroke itself since the distribution of genotypes and alleles in cases and controls was similar.

The presence of known vascular risk factors in all 3 patient groups appears to confirm a definite vascular component to AD. However, the gross pathological features, as assessed by CT, are very different. All measures used in this study showed evidence of significantly greater cerebrovascular disease in the VaD group than in the AD group. Interestingly, although the overall level of cerebrovascular disease was similar in the VaD and NDS groups, the presence of white matter ischemia was significantly more prevalent in the VaD group.

In conclusion, we report that moderately high plasma levels of homocysteine significantly increase the risk for dementia and stroke. The increased risk for dementia was independent of APOE, the only widely acknowledged genetic risk factor for AD, and was also not due to disease having an

however, the levels of homocysteine reported for the controls in that study were slightly higher than those reported here. This discrepancy may be accounted for by the fact that the samples taken in that study were not fasting, unlike those in the present study. In addition, the study of Clarke et al did not appear to adjust for vitamin B status or other measures of nutritional status, nor did they include potential confounders of the analysis such as blood pressure, cholesterol, or creatinine levels.26 Additionally, although the levels of homocysteine did not statistically differ between VaD and NDS cases when previous history of coronary heart disease and peripheral vascular disease was included in the analysis, the difference between VaD and controls remained significant, whereas that between NDS and controls did not.

The results from this study also indicate that possession of the T allele of the MTHFR C677T polymorphism significantly increases risk for VaD. When the VaD group was compared with the NDS group, the T allele was significantly overrepresented in the former, leading to the possibility that this allele confers increased risk for dementia after stroke. It is feasible that this increased risk could be mediated by the effect of the reduction in activity of the enzyme7 associated with the substitution of valine for an alanine residue,8 leading to an increase in homocysteine levels. However, although the VaD subject group had the highest mean homocysteine levels, an association between the T allele of the MTHFR polymorphism and plasma homocysteine levels was not detected. In addition, the distribution of genotypes and alleles in the other 3 subject groups was similar even though homocysteine levels were increased significantly in the AD and NDS groups compared with controls.
adverse effect on nutritional intake, since we adjusted for these factors. The higher homocysteine levels also could not be accounted for by a common polymorphism in the MTHFR gene, a gene coding for an enzyme involved in homocysteine metabolism, although we found the T allele of this polymorphism to increase risk for VaD and perhaps dementia after stroke. The aforementioned factors, if replicated, may argue for a randomized trial of the efficacy of folate, vitamin B12, and/or vitamin B6 in subjects who are designated as being at high risk for stroke or dementia.

Acknowledgment
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References
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