Folate, Vitamin B\textsubscript{12}, and Risk of Ischemic and Hemorrhagic Stroke
A Prospective, Nested Case-Referent Study of Plasma Concentrations and Dietary Intake

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**Background and Purpose**—Folate metabolism has been implicated in stroke. However, the possibility of a role for folate and vitamin B\textsubscript{12}, independent of their effects on homocysteine status, remains to be explored. The aim of this prospective, nested case-referent study was to relate plasma and dietary intake levels of folate and vitamin B\textsubscript{12} to risk of stroke, taking into consideration plasma homocysteine concentrations and methylenetetrahydrofolate reductase polymorphisms.

**Methods**—Subjects were 334 ischemic and 62 hemorrhagic stroke cases and matched double referents from the population-based Northern Sweden Health and Disease Cohort.

**Results**—Plasma folate was statistically significantly associated with risk of hemorrhagic stroke in an inverse linear manner, both in univariate analysis and after adjustment for conventional risk factors including hypertension (odds ratio [OR] for highest versus lowest quartile 0.21 (95% confidence interval [CI], 0.06 to 0.71; \(P\) for trend=0.008)). Risk estimates were attenuated by inclusion of homocysteine in the model (OR, 0.34; 95% CI, 0.08 to 1.40; \(P\) for trend=0.088). A similar pattern was observed for increasing folate intake (multivariate OR, 0.07; 95% CI, 0.01 to 0.55; \(P\) for trend=0.031 without homocysteine, and OR, 0.16, 95% CI, 0.02 to 1.23; \(P\) for trend=0.118 with homocysteine in the analysis). We found little evidence of an association between plasma or dietary folate and risk of ischemic stroke. Neither plasma nor dietary vitamin B\textsubscript{12} was associated with risk of either stroke subtype.

**Conclusions**—The results of this study suggest a protective role for folate, possibly in addition to its effects on homocysteine status, in hemorrhagic but not ischemic stroke. ([Stroke. 2005;36:1426-1431.])

Key Words: cerebral infarct ■ intracerebral hemorrhage ■ folate ■ vitamin B\textsubscript{12} ■ risk factors ■ Sweden

The importance of folate metabolism in cardiovascular disease is well established, with meta-analyses demonstrating a modest but consistent positive association between homocysteine concentrations and risk.\textsuperscript{1-3} Folate provides one-carbon groups for the methylation of homocysteine to form methionine, with vitamin B\textsubscript{12} acting as a cofactor, and studies have tended to focus on the role of these vitamins primarily as determinants of homocysteine levels.

Some evidence supports an independent protective effect for folate in vascular diseases, with suggested mechanisms including antioxidant effects and effects on blood pressure via interactions with nitric oxide synthase.\textsuperscript{4} Yet whereas inverse associations between folate and cerebrovascular outcomes have been reported in prospective studies,\textsuperscript{5-8} in no study has homocysteine been considered in the analyses. Furthermore, only 2 of these reports addressed circulating concentrations of folate, one with 98 ischemic stroke cases,\textsuperscript{7} and one with 62 adverse cerebrovascular events, including 31 fatal stroke cases.\textsuperscript{8} Thus, the putative role for folate in stroke has yet to be established, and whether it might be independent of homocysteine remains to be explored.

The aim of this prospective population-based study was to relate plasma and dietary intake levels of folate and vitamin B\textsubscript{12} to risk of first-ever ischemic and hemorrhagic stroke, taking into consideration plasma homocysteine concentrations and polymorphisms in the methylenetetrahydrofolate reductase (\textit{MTHFR}) gene, 677C\textsuperscript{T} and 1298A\textsuperscript{C}, which are detrimental to folate and homocysteine status.\textsuperscript{9,10}

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Subjects and Methods

Study Cohorts and Diagnostic Criteria

This nested case-referent study was based on the Northern Sweden Health and Disease Cohort, comprising 2 subcohorts, the Northern Sweden MONICA Study and the Västerbotten Intervention Project (VIP). In the MONICA cohort, 2000 to 2500 randomly selected 25- to 74-year-old subjects living in the 2 northernmost counties in Sweden have been invited to participate in a health survey every 4 or 5 years since 1986, and mean participation rate was 77%. In VIP, all residents of Västerbotten County are invited to a similar health survey at their primary health care center on turning 30, 40, 50, and 60 years old, and mean participation rate is 59%. Comparisons of social characteristics between participants and nonparticipants have shown little evidence of selection bias.13 As part of both programs, participants are invited to donate a fasting blood sample to the Northern Sweden Medical Biobank for use in future research and by 2000, 74 000 individuals had done so.

For the MONICA registry, all cases of first-ever acute stroke between 1985 and 2000 were identified through screening of hospital discharge records, general practitioners’ reports, and death certificates, and classified according to strict WHO and MONICA criteria,12 with ICD-9 codes 430 to 438 corresponding to ICD-10 codes I60 to I69. Of the 14 030 stroke cases, 473 (excluding subarachnoid hemorrhages) had donated data and/or samples to the Northern Sweden Medical Biobank and met the inclusion criteria of no previous acute myocardial infarction and no cancer diagnosis within the 5 years before recruitment; 458 cases (97%) had been examined with either a computed tomography/magnetic resonance scan or, if the stroke was fatal, autopsy. Ischemic stroke accounted for 387 cases, 71 were hemorrhagic, and the remaining 15 unclassified stroke were excluded from this study.

Five potential referents were matched to each case by sex, age (±2 years), cohort (MONICA or VIP), date of health survey (±1 year), and geographical area, and were sent a questionnaire with items concerning cardiovascular events since the baseline survey. The first 2 referents for whom samples were available and who had consented to genotyping were used in the analyses. For nonresponders, the third (or occasionally the fourth) referent was selected instead.

The study protocol was approved by the Research Ethics Committee of Umeå University, Umeå, Sweden, and the data handling procedures were approved by the National Computer Data Inspection Board.

Blood Sampling and Laboratory Procedures

Venous blood samples were drawn after a minimum of 4 hours of fasting (66% after minimum 8 hours) and without stasis into evacuated glass tubes. Heparinized plasma or serum was obtained by centrifugation at 1500g for 15 minutes, aliquoted, and stored at −80°C. Folate and vitamin B12 were analyzed by Quantaphase II radioassay (BioRad Diagnostic Group). Coefficients of variation were <7.5%. Plasma specimens were analyzed in triplets of 1 case and 2 referents, with the position of the cases varied at random within each case-referent triplet to avoid systemic bias and interassay variability. The investigators and laboratory staff were blinded to case and referent status.

Dietary Assessment

Dietary data, assessed by food frequency questionnaire (FFQ), were available for approximately half of the subjects. Of these, 85% had completed an 84-item and 15% a 65-item FFQ. Daily intakes of folate and vitamin B12 were calculated with the aid of specific portion questions in the FFQ, standard portion sizes, the Swedish Food Tables, and the internet-based Swedish Food Database.13–15 The folate content of various foods was updated according to recent analyses. For multivitamin users, 200 µg of folate and 2.5 µg of vitamin B12 (conservative estimates based on supplements available in Sweden during the relevant time period) were added to the dietary intakes from foods to estimate total intake. Exclusion criteria for the dietary data have been described previously and were, in brief, missing values for >10% of the FFQ items or for the portion size questions.16

Statistical Analysis

Baseline characteristics and study variables for cases and referents were compared by Mann–Whitney and χ2 tests. Associations between variables were assessed using Spearman rank correlations. Odds ratios (ORs) for disease and 95% confidence intervals (CIs) were calculated by conditional logistic regression for quartiles based on the variable distributions of the referent subjects. Quartile cutoffs were calculated separately by sex and analysis occasion for plasma folate, vitamin B12, and homocysteine, and by sex and version of the FFQ for the dietary intake variables. Tests for trend were performed by χ2 linear trend test and by including quartiles as continuous variables in the regression analyses. In multivariate analyses, missing values were treated as a separate category (categorical variables) or given the median value of the referent subjects (continuous variables) so as to ensure a conservative effect on risk estimates. Statistical tests and corresponding probability values were 2-sided, and SPSS version 12.0 was used for all statistical analyses.

Results

Of the 458 stroke cases identified, assessment of plasma folate, plasma vitamin B12, dietary intake, plasma homocysteine, and/or the MTHFR polymorphisms was possible for 334 ischemic and 62 hemorrhagic stroke cases. Exclusion was most commonly caused by lack of blood samples or FFQ, although 17 case and 32 referent subjects were excluded from analysis of dietary intake variables because of inadequate data. Mean baseline ages and follow-up times were 55.1 (standard deviation [SD] 8.0) and 4.2 (SD 2.7) years for ischemic stroke and 54.8 (SD 8.1) and 4.1 (SD 2.7) years for hemorrhagic stroke, respectively. Baseline characteristics of the study subjects are presented in Table 1 (available online only at http://www.strokeaha.org), and plasma concentrations and dietary intake levels of folate and vitamin B12 are given in Table 1.

For the full study group, plasma folate and vitamin B12 were negatively correlated to homocysteine (Spearman correlation coefficient −0.427; P<0.001 for folate and −0.217; P<0.001 for vitamin B12) and positively correlated to each other (0.182; P<0.001). Plasma concentrations and dietary intake were correlated for folate (0.244; P<0.001) but not vitamin B12 (−0.010; P=0.831), and alcohol intake was positively correlated to plasma folate (0.162; P<0.001). None of the correlations was affected materially by adjusting for the MTHFR polymorphisms (data not shown).

In subjects with both plasma and dietary data available, multivitamin use (21%) was associated with statistically significantly higher plasma folate (median and 25 to 75 percentiles 10.9 [8.1 to 16.9] versus 7.8 [6.1 to 10.3] nmol/L; P<0.001) and lower plasma homocysteine concentrations (9.5 [8.2 to 11.5] versus 11.4 [9.6 to 13.0] µmol/L; P<0.001). Plasma vitamin B12 did not differ significantly by multivitamin use.

Plasma folate demonstrated a U-shaped association with risk of ischemic stroke, the third quartile having a statistically significant OR both in univariate analysis and after adjustment for other risk factors, including hypertension and homocysteine (OR, 0.58; 95% CI, 0.36 to 0.92) (Table 2). For hemorrhagic stroke, plasma folate was statistically significantly associated with risk in an inverse linear manner in
univariate analysis and after adjustment for conventional risk factors (OR for highest versus lowest quartile, 0.21; 95% CI, 0.06 to 0.71; \( P \) for trend \( < 0.008 \)). When homocysteine was included in the model, the OR was attenuated and was no longer statistically significant (OR, 0.34; 95% CI, 0.08 to 1.40; \( P \) for trend \( = 0.088 \)). Plasma vitamin B12 was not associated with either stroke subtype (Table 3).

Dietary folate was not related to risk of ischemic stroke but demonstrated an inverse association with hemorrhagic stroke risk (Table 2). All ORs were <0.2 and statistically significant in univariate analysis and after adjustment for conventional risk factors including hypertension (OR, 0.07; 95% CI, 0.01 to 0.55; \( P \) for trend \( = 0.031 \), but not after further adjustment for homocysteine (OR, 0.16; 95% CI, 0.02 to 1.23; \( P \) for trend \( = 0.118 \)). Dietary intake of vitamin B12 was not statistically significantly related to either stroke subtype, although there was a nonsignificant positive association between increasing intake and risk of ischemic stroke (Table 3).

Adjusting for other dietary variables, including vitamin E, fiber, saturated fats, and alcohol, in risk analyses did not materially affect main findings (data not shown). Analysis of sex subgroups yielded results consistent with those for the full study group (data not shown).

**Discussion**

In this prospective population-based study, increasing plasma concentrations and dietary intakes of folate were associated with reduced risk of hemorrhagic, but not ischemic, stroke. These findings were independent of conventional risk factors, including hypertension. Although risk estimates were not statistically significant after adjustment for homocysteine, they were attenuated only slightly in magnitude, suggesting that although determination of homocysteine status is likely to be an important component of the putative role for folate in hemorrhagic stroke prevention, other mechanisms may also be involved. To our knowledge, this is the first prospective report relating circulating levels of folate to risk of hemorrhagic stroke, the first for either stroke subtype to incorporate both plasma and dietary data, and the first to account for homocysteine and/or MTHFR polymorphisms in risk assessment.

The main strength of this study is its prospective population-based design. Our results were based on a single measurement of plasma folate per subject, but this has been found to be an appropriate measure of folate status in large epidemiological studies. Although fasting duration was not considered in the matching of referents to incident cases, excluding subjects with fasting times <8 hours did not materially affect risk estimates (data not shown). We were unable to control for use of aspirin and other nonsteroidal anti-inflammatory drugs, which was a limitation of the study.

Whereas a greater sample size would have been desirable, especially with respect to the dietary intake data, the consis-
Excluding folate obtained from multivitamin supplements resulted in similar ORs, suggesting that confounding by other vitamins was not likely to have been a problem (data not shown). The quality of the dietary data might also be called into question, but the FFQ used has been found to have good reproducibility and an estimated validity similar to that of FFQs in other prospective cohort studies, although folate and vitamin B12 were not assessed in that report.

The lack of a clear association for either plasma or dietary folate with risk of ischemic stroke was not expected, given the wealth of data implicating homocysteine and possibly also folate. A true U-shaped association between plasma folate and risk seems unlikely, suggesting that the significant risk reduction observed for the third quartile may be a chance finding or the result of confounding by unidentified factors. In Sweden, foods are not fortified with folate, and consumption of fruits and vegetables is relatively low. Thus, folate levels may not have been high enough or had sufficient range in the present study to detect a role in ischemic stroke. If effects of folate on risk are apparent at lower levels in hemorrhagic compared with ischemic stroke, then this might speculatively support an involvement via mechanisms common to both outcomes but to different degrees, for example, hypertension.

The lack of an inverse relationship between folate intake and risk of hemorrhagic stroke was somewhat unexpected, given the role of folate in methylation and the association of folate and homocysteine levels with risk of cardiovascular disease and stroke. However, in this study, the association between a high intake of folate and risk of hemorrhagic stroke was not confirmed, even after adjustment for homocysteine levels.

### Table 2: Odds Ratios and 95% Confidence Intervals for Ischemic and Hemorrhagic Stroke by Quartiles of Folate

<table>
<thead>
<tr>
<th></th>
<th>Quartiles of Folate</th>
<th>1 (ref)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>P for Trend*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic stroke</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma†</td>
<td></td>
<td>87/154</td>
<td>72/151</td>
<td>55/157</td>
<td>81/131</td>
<td>0.938</td>
</tr>
<tr>
<td>Unadjusted OR</td>
<td>1.00</td>
<td>0.82 (0.56–1.21)</td>
<td>0.61 (0.40–0.93)</td>
<td>1.01 (0.68–1.52)</td>
<td>0.746</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR1‡</td>
<td>1.00</td>
<td>0.82 (0.54–1.25)</td>
<td>0.60 (0.38–0.95)</td>
<td>1.13 (0.74–1.74)</td>
<td>0.874</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR2§</td>
<td>1.00</td>
<td>0.81 (0.53–1.23)</td>
<td>0.58 (0.36–0.92)</td>
<td>1.07 (0.66–1.71)</td>
<td>0.631</td>
<td></td>
</tr>
<tr>
<td>Intake¶</td>
<td></td>
<td>34/75</td>
<td>41/75</td>
<td>27/68</td>
<td>37/68</td>
<td>0.790</td>
</tr>
<tr>
<td>Unadjusted OR</td>
<td>1.00</td>
<td>1.16 (0.64–2.09)</td>
<td>0.94 (0.49–1.81)</td>
<td>1.14 (0.61–2.09)</td>
<td>0.843</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR1‡</td>
<td>1.00</td>
<td>1.13 (0.63–2.01)</td>
<td>0.98 (0.50–1.89)</td>
<td>1.21 (0.66–2.22)</td>
<td>0.331</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR2§</td>
<td>1.00</td>
<td>1.14 (0.64–2.03)</td>
<td>0.94 (0.48–1.83)</td>
<td>1.19 (0.64–2.20)</td>
<td>0.419</td>
<td></td>
</tr>
<tr>
<td>Hemorrhagic stroke</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma†</td>
<td></td>
<td>18/25</td>
<td>19/28</td>
<td>12/19</td>
<td>8/38</td>
<td>0.014</td>
</tr>
<tr>
<td>Unadjusted OR</td>
<td>1.00</td>
<td>1.04 (0.44–2.46)</td>
<td>0.63 (0.22–1.77)</td>
<td>0.22 (0.08–0.66)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR1‡</td>
<td>1.00</td>
<td>0.72 (0.24–2.17)</td>
<td>0.74 (0.23–2.39)</td>
<td>0.21 (0.06–0.71)</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR2§</td>
<td>1.00</td>
<td>1.21 (0.35–4.14)</td>
<td>1.09 (0.28–4.17)</td>
<td>0.34 (0.08–1.40)</td>
<td>0.088</td>
<td></td>
</tr>
<tr>
<td>Intake¶</td>
<td></td>
<td>10/8</td>
<td>4/12</td>
<td>6/17</td>
<td>5/15</td>
<td>0.060</td>
</tr>
<tr>
<td>Unadjusted OR</td>
<td>1.00</td>
<td>0.15 (0.02–1.00)</td>
<td>0.15 (0.02–0.95)</td>
<td>0.17 (0.03–0.90)</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR1‡</td>
<td>1.00</td>
<td>0.10 (0.01–0.93)</td>
<td>0.18 (0.02–1.43)</td>
<td>0.07 (0.01–0.55)</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td>Adjusted OR2§</td>
<td>1.00</td>
<td>0.13 (0.01–1.26)</td>
<td>0.16 (0.02–1.21)</td>
<td>0.16 (0.02–1.23)</td>
<td>0.118</td>
<td></td>
</tr>
</tbody>
</table>

*χ² test for linear trend for cases/referents and by analyzing quartiles as continuous variables in conditional logistic regression.
†Quartile cutoffs (nmol/L): Men, analysis occasion 1: 3.3, 4.2, 5.8; analysis occasion 2: 5.8, 7.6, 9.9; Women, analysis occasion 1: 5.8, 7.6, 9.9; analysis occasion 2: 6.8, 8.9, 12.2.
‡Adjusted for body mass index, current smoking, cholesterol, diabetes, and hypertension.
§Further adjusted for plasma homocysteine.
¶Quartile cutoffs (µg/1000 kcal/day) for total intake including supplements: Men, FFQ1: 105, 134, 173; FFQ2: 112, 132, 162; Women, FFQ1: 132, 166, 274; FFQ2: 132, 164, 223.
significant manner (due at least partly to multivitamin use, data not shown), and adjusting for the MTHFR polymorphisms in the multivariate risk analyses did not alter risk estimates appreciably (data not shown). Thus, effect modification by the MTHFR polymorphisms was not likely to have been a problem in this study.

The observed lack of effect for vitamin B12 intake and hemorrhagic stroke (and possible detrimental association with ischemic stroke risk) may reflect the poor correlation with plasma concentrations. However, null results were also obtained for plasma vitamin B12. This apparent discrepancy in findings likely reflects the relatively stronger effect of folate on homocysteine levels.

In conclusion, our results suggest that folate may have a protective role in hemorrhagic, but not ischemic, stroke, possibly in addition to its effects on homocysteine status.

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References


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