Elevated plasma total homocysteine levels are a strong, graded independent risk factor for stroke and myocardial infarction. Mechanisms by which homocysteine may cause vascular disease include a propensity for thrombosis and impaired thrombolysis, increased production of hydrogen peroxide, endothelial dysfunction and increased oxidation of low-density lipoproteins and Lp(a) lipoproteins.

Folic acid fortification of enriched cereal grain products began in North America in March 1996 and was made mandatory in 1998. Fortification has reduced the number of neural tube defects by half, which is clearly a beneficial outcome, but so far it has had little impact on cardiovascular mortality.

Carotid plaque area is a strong predictor of cardiovascular events. High homocysteine levels, which are associated with plaque formation, can result from inadequate intake of folate and vitamin B\textsubscript{12}. Now that folic acid fortification is widespread, vitamin B\textsubscript{12} has become an important determinant of homocysteine levels. We sought to determine the prevalence of low serum levels of vitamin B\textsubscript{12}, and their relation to homocysteine levels and carotid plaque area among patients referred for treatment of vascular disease since folic acid fortification of enriched grain products.

Methods

We evaluated 421 consecutive new patients with complete data whom we saw in our vascular disease prevention clinics between January 1998 and January 2002. We measured total carotid plaque area by ultrasound and determined homocysteine and serum vitamin B\textsubscript{12} levels in all patients.

Results: The patients, 215 men and 206 women, ranged in age from 37 to 90 years (mean 66 years). Most were taking medications for hypertension (67%) and dyslipidemia (62%). Seventy-three patients (17%) had vitamin B\textsubscript{12} deficiency (vitamin B\textsubscript{12} level < 258 pmol/L with homocysteine level > 14 µmol/L or methylmalonic acid level > 271 nmol/L). The mean area of carotid plaque was significantly larger among the group of patients whose vitamin B\textsubscript{12} level was below the median of 253 pmol/L than among those whose vitamin B\textsubscript{12} level was above the median: 1.36 (standard deviation [SD] 1.27) cm\textsuperscript{2} v. 1.09 (SD 1.0) cm\textsuperscript{2}; \( p = 0.016 \).

Conclusions: Vitamin B\textsubscript{12} deficiency is surprisingly common among patients with vascular disease, and, in the setting of folic acid fortification, low serum vitamin B\textsubscript{12} levels are a major determinant of elevated homocysteine levels and increased carotid plaque area.

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Serum methylmalonic acid, the specific metabolic indicator of vitamin B12 deficiency, was measured in the last 50 patients included in the study, using stable isotope dilution gas chromatography–mass spectrometry. In this subgroup, there were no patients with severe renal impairment; the lowest serum creatinine level was 47 µmol/L; 90% had creatinine levels greater than 60 µmol/L, and the mean was 86.7 (standard deviation [SD] 23) µmol/L. Creatinine levels were measured in 224 patients, including all those in whom methylmalonic acid levels were measured.

Carotid plaque area was measured with high resolution duplex ultrasonography, using an ATL 5000 HDI (Advanced Technology Laboratories, Seattle, Wash.). Plaque was defined as a local thickening of the intima exceeding 1 mm in thickness. Measurements were made in magnified longitudinal views of each plaque seen in the right and left common, internal and external carotid arteries. The plane in which each plaque was measured was chosen by scanning around the artery until the view showing the largest extent of that plaque was obtained. The image was then frozen and magnified, and the plaque was measured by tracing around the perimeter with a cursor on the screen. The microprocessor in the scanner then displayed the cross-sectional area of the plaque. The operator repeated the process for every plaque seen, until all visible plaque were measured. The sum of cross-sectional areas of all plaques seen between the clavicle and the angle of the jaw was taken as total plaque area. Intraobserver reliability (intraclass correlation) was 0.94 for repeat measurements; interobserver reliability was 0.85.

All 50 patients whose methylmalonic acid levels were measured attended the Stroke Prevention Clinic and consented to a protocol approved by the University of Western Ontario ethics review board. The results from clinical practice were exempted by the ethics board because they involved only a chart review.

We analyzed the relation of serum vitamin B12 and homocysteine levels to carotid plaque area. We defined vitamin B12 deficiency as a serum vitamin B12 level of less than 258 pmol/L with homocysteine levels of 14 µmol/L or more or methylmalonic acid levels of 271 nmol/L or more. The median vitamin B12 level in our population was 253 pmol/L, which is very close to the level indicating deficiency; we therefore used analysis of variance (ANOVA) to compare means of baseline characteristics of the study population to determine differences between patients whose vitamin B12 level was above the median and those whose level was below. The study population was large enough to permit analysis of relations between carotid plaque area, vitamin B12 levels and homocysteine levels by quartiles, so this was done to explore the range of values of these parameters. A 2-sample t test was used to examine sex differences in mean vitamin B12 levels, and multiple regression was used to adjust for age and sex because both are determinants of homocysteine levels.

We used multiple regression analysis to evaluate adjusted effects on carotid plaque, controlling for age, sex, homocysteine levels, vitamin B12 levels and, where this was measured, serum creatinine levels.

Multiple regression with forced entry of age, sex, creatinine levels, homocysteine levels and vitamin B12 was carried out to evaluate the relative contribution of creatinine and homocysteine to carotid plaque area. The distribution of the dependent variable, plaque area, was normalized by a cubic root transformation.

### Results

The study subjects included 421 vascular patients whose ages ranged from 37 to 90 years (mean 65.63 [SD 10.76] years) and with serum vitamin B12 levels ranging from 24 to 954 pmol/L (mean 103 pmol/L). The levels of homocysteine were between 5 and 149 µmol/L (mean 34 µmol/L), and methylmalonic acid levels were between 5 and 125 nmol/L (mean 35 nmol/L). Serum creatinine levels ranged from 0.5 to 204 µmol/L (mean 86 µmol/L), and both HDL and LDL cholesterol levels were between 0.5 and 5.6 mmol/L (mean 2.3 mmol/L).

The relation of serum vitamin B12 and homocysteine to carotid plaque area was examined by quartiles of serum vitamin B12 levels, with vitamin B12 deficiency defined as a serum vitamin B12 level of less than 258 pmol/L with homocysteine levels of 14 µmol/L or more or methylmalonic acid levels of 271 nmol/L or more. The median serum vitamin B12 level was 253 pmol/L, which is very close to the level indicating deficiency; we therefore used analysis of variance (ANOVA) to compare means of baseline characteristics of the study population to determine differences between patients whose vitamin B12 level was above the median and those whose level was below. The study population was large enough to permit analysis of relations between carotid plaque area, vitamin B12 levels and homocysteine levels by quartiles, so this was done to explore the range of values of these parameters. A 2-sample t test was used to examine sex differences in mean vitamin B12 levels, and multiple regression was used to adjust for age and sex because both are determinants of homocysteine levels.

We used multiple regression analysis to evaluate adjusted effects on carotid plaque, controlling for age, sex, homocysteine levels, vitamin B12 levels and, where this was measured, serum creatinine levels.

Multiple regression with forced entry of age, sex, creatinine levels, homocysteine levels and vitamin B12 was carried out to evaluate the relative contribution of creatinine and homocysteine to carotid plaque area. The distribution of the dependent variable, plaque area, was normalized by a cubic root transformation.

![Figure 1: Total homocysteine by quartiles of serum vitamin B₁₂ levels. Error bars represent 95% confidence intervals.](image-url)
years); 49% were female; 67.1% were taking medication for hypertension and 61.5% for dyslipidemia.

Table 1 shows important study population characteristics by median vitamin B12 level. Vitamin B12 deficiency was found in 73 cases (17.3%). Among those 50 patients whose methylmalonic acid levels were measured, 39% exceeded 2 standard deviations for the reference range and 20% exceeded 3 standard deviations, which confirmed metabolic vitamin B12 deficiency.

Plasma homocysteine levels were higher at lower levels of vitamin B12 for all age groups ($p < 0.001$). There was no difference in levels of vitamin B12 ($p = 0.43$) associated with sex; patients whose vitamin B12 levels were in the lower stratum were slightly but not significantly older (66.66 [SD 10.91] v. 64.64 [SD 10.56] years; $p = 0.055$).

Age and vitamin B12 were found to be significant predictors of homocysteine levels (both $p < 0.001$), but sex was not; the R$^2$ was 0.14; $\beta$ coefficients were 0.26 for age and $-0.22$ for vitamin B12, which indicated that, independent of age and sex, vitamin B12 accounted for an additional 22% of the explained variance of homocysteine levels.

Analysis of the relations between carotid plaque area, vitamin B12 levels and homocysteine levels by quartiles showed that homocysteine levels fell significantly as vitamin B12 levels rose (Fig. 1) and that, above the median vitamin B12 level of 253 pmol/L, there was significantly more carotid plaque (Fig. 2). Carotid plaque area increased markedly above levels of homocysteine that would usually be regarded as low (7.7 µmol/L), but there was a ceiling effect at a levels that would usually be regarded as normal (9.6 µmol/L) (Fig. 3).

Among the 224 patients whose serum creatinine levels were measured, homocysteine accounted for a higher proportion of the explained variance in plaque area than either creatinine or vitamin B12 (Table 2).

The effect of vitamin B12 levels and creatinine levels on carotid plaque appeared to be related to their effect on homocysteine levels. In a simple Pearson correlation, creatinine ($r = 0.331$, $p < 0.001$) and vitamin B12 ($r = 0.297$, $p < 0.001$) both correlated with homocysteine, and homocysteine ($r = 0.147$, $p = 0.028$) correlated with plaque area, but creatinine ($r = 0.054$, $p = 0.43$) and vitamin B12 ($r = 0.03$, $p = 0.65$) did not.

**Interpretation**

We found that 17.3% of our patients had vitamin B12 deficiency; this may be an underestimate, since only 10% of the study group had methylmalonic acid levels measured. Half of our patients had vitamin B12 levels below 258 pmol/L, a cut-off point below which levels have been shown to be associated with a high prevalence of vitamin B12 deficiency, defined by elevated homocysteine or methylmalonic acid levels.11–13

Carotid plaque area was associated with elevated homocysteine levels and low vitamin B12 levels. The strong relation between quartiles of vitamin B12 and homocysteine shown in Fig. 1 supports the finding by Quinlivan and associates7 that, since folic acid fortification, vitamin B12 has become the main nutritional determinant of homocysteine levels. The striking reciprocity of vitamin B12, homocysteine and plaque area shown in Fig. 2 and Fig. 3 suggests a key role for vitamin B12 in the treatment of elevated homocysteine levels for vascular disease. Our findings also suggest that “folate therapy,” the term commonly used for vitamin therapy for lowering homocysteine levels, is no longer appropriate.

Inspection of Fig. 3 raises the possibility of a threshold...
or ceiling effect of homocysteine on carotid plaque, which may indicate that target levels for homocysteine should be lower. It is possible that the null effect of the VISP trial may be explained in part by the failure to sufficiently reduce homocysteine levels. It may be necessary in future to individualize treatment using multiple daily doses of B vitamins or other therapies such as betaine or thiols to achieve target homocysteine levels.

The results of the VISP trial showed that a modest reduction of total homocysteine levels (by 1.5 to 2.0 \( \mu \text{mol/L} \)) using folic acid, vitamin B\(_6\), and vitamin B\(_12\) after non-disabling cerebral infarction had no effect on vascular outcomes. Possible reasons for this result include the improvement in outcomes in the low-dose arm due to folic acid fortification, which negated the effect of high-dose folic acid, and problems with the handling of vitamin B\(_12\): the high-dose group received only 400 mcg daily, whereas Rajan and associates have shown that 1000 mcg daily is required for adequate absorption among elderly patients; furthermore, the low-dose group received the recommended daily intake of vitamin B\(_6\) (6 mcg), and in both treatment arms those with low vitamin B\(_6\) levels received injections of vitamin B\(_12\). Differences in results between the coronary angioplasty trials reported by Schneider and associates (in which patients benefitted from vitamin therapy for homocysteine levels) and that of Lange and associates (in which they did not) may be because of the difference in vitamin B\(_6\) doses (400 mcg daily vs. 40 mcg daily respectively). A number of trials are underway to determine whether treatment of vascular disease with folic acid, vitamin B\(_6\), and vitamin B\(_12\) reduces stroke.

An important weakness of our study was that we did not have data on serum folate levels. Folate was not measured because the study was based on measurements performed as part of patient care, and folate deficiency has essentially been eliminated by folic acid fortification: Selhub and associates have shown that 1000 mcg daily v. 40 mcg daily respectively). A number of trials are underway to determine whether treatment of vascular disease with folic acid, vitamin B\(_6\), and vitamin B\(_12\) reduces stroke.

Among the 224 patients whose creatinine levels were measured, creatinine levels were found to be as strong a predictor of homocysteine levels as were vitamin B\(_12\) levels. Among the 10% of patients whose methylmalonic acid levels were measured, creatinine levels were also as strong a predictor of homocysteine levels than were vitamin B\(_12\) levels, and vitamin B\(_12\) levels were a stronger predictor of methylmalonic acid levels than were creatinine levels. These findings suggest that creatinine may be a stronger predictor than or as strong a predictor as vitamin B\(_12\) of homocysteine levels and are worthy of further investigation.

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Table 2: Determinants of plaque area among the 224 patients with measured creatinine levels*

| Variable     | \( \beta \) coefficient | 95% confidence interval |
|--------------|--------------------------|-------------------------|
| Age          | 0.29†                    | 0.0 to 4.4              |
| Sex          | 0.31                     | 0.0 to 4.9              |
| Homocysteine | 0.13                     | 0.1 to 1.9              |
| Vitamin B\(_12\) | 0.05                    | 0.5 to 0.7              |
| Creatinine   | -0.08                    | -1.3 to 0.2             |

*Adjusted for age, sex, homocysteine levels, vitamin B\(_12\) levels and creatinine levels.
†To read this table, age, for example, accounts for 29% of the explained variance in plaque area.
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