Vitamin B$_{12}$, homocysteine and carotid plaque in the era of folic acid fortification of enriched cereal grain products

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Abstract

Background: 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$_{12}$. Now that folic acid fortification is widespread in North America, vitamin B$_{12}$ has become an important determinant of homocysteine levels. We sought to determine the prevalence of low serum levels of vitamin B$_{12}$, 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$_{12}$ 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$_{12}$ deficiency (vitamin B$_{12}$ 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$_{12}$ level was below the median of 253 pmol/L than among those whose vitamin B$_{12}$ level was above the median: 1.36 (standard deviation [SD] 1.27) cm^2 v. 1.09 (SD 1.0) cm^2; $p = 0.016$.

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

levated plasma total homocysteine levels are a strong, graded independent risk factor for stroke and myocardial infarction.[1,2] 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.[3]

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,[4] which is clearly a beneficial outcome, but so far it has had little impact on cardiovascular mortality.[5]

Carotid plaque area is a strong predictor of cardiovascular events.[6] High homocysteine levels, which are associated with plaque formation, can result from inadequate intake of folate and vitamin B$_{12}$. Now that folic acid fortification is widespread, vitamin B$_{12}$ has become an important determinant of homocysteine levels.[7] We sought to determine the prevalence of low serum levels of vitamin B$_{12}$, 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

The subjects of this study were all consecutive new patients referred to 1 of us (JDS) for secondary vascular prevention in either the Premature Atherosclerosis Clinic or the Stroke Prevention Clinic of the University Campus of the London Health Science Centre in London, Ont., between January 1998 (after folic acid fortification had begun) and January 2002 (coinciding with JR's time in our research unit). During that time, 520 patients were referred. A chart review showed that 421 patients had complete data, including measurement of carotid plaque areas, serum vitamin B$_{12}$ levels, plasma total homocysteine levels, blood pressure, smoking (pack-years), serum cholesterol levels, triglyceride levels, high-density lipoprotein cholesterol levels and calculated low-density lipoprotein cholesterol levels. The data used for analysis were abstracted from the clinical records; except for the measurement of serum methylmalonic acid levels in a subgroup from the Stroke Prevention Clinic, all investigations were done as part of patient care.

Vitamin B$_{12}$ levels were measured on the Advia Centaur Analyzer (Bayer Diagnostics, Walpole, Mass.) by competitive immunoassay using direct chemiluminescent technology, in which vitamin B$_{12}$ from the patient sample competes with labelled vitamin B$_{12}$ for a limited amount of purified intrinsic factor. Plasma total homocysteine was measured by high-performance liquid chromatography with electrochemical detection, which had been

standardized against reference methods in both Oregon and Cleveland.[8,9]

Serum methylmalonic acid, the specific metabolic indicator of vitamin B_{12} deficiency, was measured in the last 50 patients included in the study, using stable isotope dilution gas chromatography–mass spectrometry.[10] 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.).[6] 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 B_{12} and homocysteine levels to carotid plaque area. We defined vitamin B_{12} deficiency as a serum vitamin B_{12} 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.[11] The median vitamin B_{12} 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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12} levels and, where this was measured, serum creatinine levels.

Multiple regression with forced entry of age, sex, creatinine levels, homocysteine levels and vitamin B_{12} 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]

Table 1: Baseline characteristics of the study population by median serum vitamin B_{12} level*

Characteristic	Mean value (standard deviation)		
	Below median vitamin B_{12} level $n = 221$	Above median vitamin B_{12} level $n = 220$	p value†
Age, yr	66.66 (10.91)	64.64 (10.56)	0.055
Total cholesterol level, mmol/L	5.20 (1.02)	5.35 (1.35)	0.65
Triglyceride level, mmol/L	2.07 (1.47)	2.23 (2.62)	0.43
HDL cholesterol level, mmol/L	1.32 (0.39)	1.30 (0.41)	0.62
LDL cholesterol level, mmol/L	3.06 (1.10)	3.28 (1.71)	0.11
Systolic BP, mm Hg	148.50 (22.09)	147.13 (22.11)	0.53
Diastolic BP, mm Hg	80.42 (13.08)	77.93 (13.84)	0.56
Smoking, pack-years	16.51 (23.15)	15.63 (19.50)	0.69
Serum vitamin B_{12} level, pmol/L	198.27 (35.50)	359.46 (116.53)	< 0.0001
Total homocysteine level, µmol/L	11.40 (4.64)	9.29 (3.68)	< 0.0001
Carotid plaque area, cm^2	1.36 (1.27)	1.09 (1.0)	0.016
Serum creatinine level, µmol/L	$n = 113$ 93.67 (32.34)	$n = 111$ 91.29 (25.29)	0.54
Methylmalonic acid level, nmol/L	$n = 36$ 396.67 (462.36)	$n = 14$ 266.0 (112.33)	0.30

Note: HDL = high-density lipoprotein, LDL = low-density lipoprotein, BP = blood pressure.
*The median serum vitamin B_{12} level was 253 pmol/L.
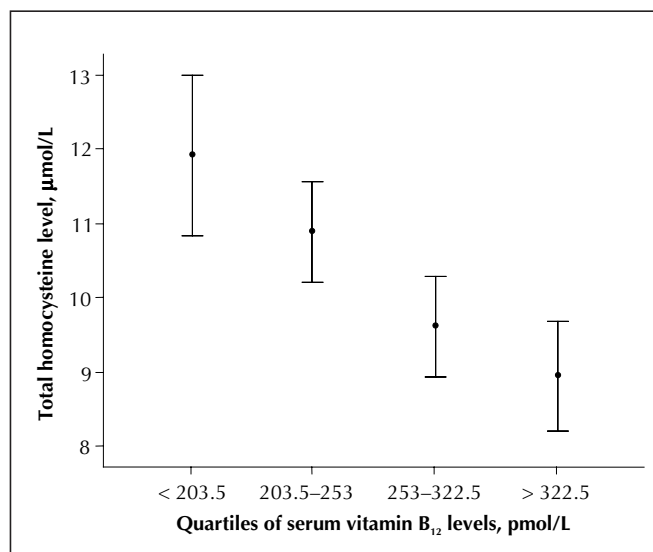†P values are from analysis of variance.

Fig. 1: Total homocysteine by quartiles of serum vitamin B_{12} levels. Error bars represent 95% confidence intervals.

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 B$_{12}$ level. Vitamin B$_{12}$ 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 B$_{12}$ deficiency.

Plasma homocysteine levels were higher at lower levels of vitamin B$_{12}$ for all age groups ($p < 0.001$). There was no difference in levels of vitamin B$_{12}$ ($p = 0.43$) associated with sex; patients whose vitamin B$_{12}$ 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 B$_{12}$ were found to be significant predictors of homocysteine levels (both $p < 0.001$), but sex was not; the R^2 was 0.14; β coefficients were 0.26 for age and –0.22 for vitamin B$_{12}$, which indicated that, independent of age and sex, vitamin B$_{12}$ accounted for an additional 22% of the explained variance of homocysteine levels.

Analysis of the relations between carotid plaque area, vitamin B$_{12}$ levels and homocysteine levels by quartiles showed that homocysteine levels fell significantly as vitamin B$_{12}$ levels rose (Fig. 1) and that, above the median vitamin B$_{12}$ 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 pro-portion of the explained variance in plaque area than either creatinine or vitamin B$_{12}$ (Table 2).

The effect of vitamin B$_{12}$ 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 B$_{12}$ ($r = 0.297$, $p < 0.001$) 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 B$_{12}$ ($r = 0.03$, $p = 0.65$) did not.

Interpretation

We found that 17.3% of our patients had vitamin B$_{12}$ deficiency; this may be an underestimate, since only 10% of the study group had methylmalonic acid levels measured. Half of our patients had vitamin B$_{12}$ levels below 258 pmol/L, a cut-off point below which levels have been shown to be associated with a high prevalence of vitamin B$_{12}$ deficiency, defined by elevated homocysteine or methylmalonic acid levels.[11–13]

Carotid plaque area was associated with elevated homocysteine levels and low vitamin B$_{12}$ levels. The strong relation between quartiles of vitamin B$_{12}$ and homocysteine shown in Fig. 1 supports the finding by Quinlivan and associates[7] that, since folic acid fortification, vitamin B$_{12}$ has become the main nutritional determinant of homocysteine levels. The striking reciprocity of vitamin B$_{12}$, homocysteine and plaque area shown in Fig. 2 and Fig. 3 suggests a key role for vitamin B$_{12}$ 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.

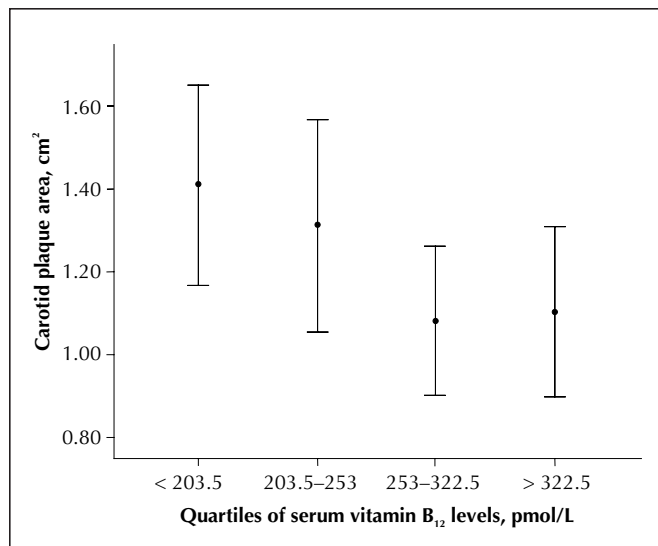Inspection of Fig. 3 raises the possibility of a threshold

Fig. 2: Carotid plaque area by quartiles of serum vitamin B$_{12}$ levels. Error bars represent 95% confidence intervals. Carotid plaque area is significantly related to a median split of vitamin B$_{12}$ ($p = 0.012$).
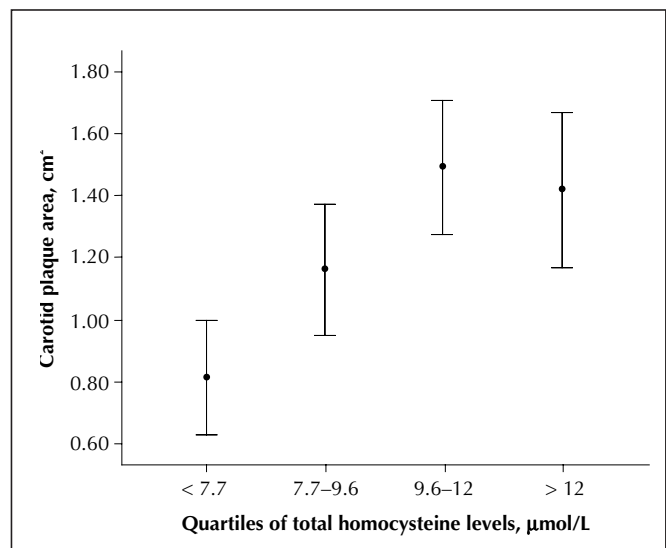

Fig. 3: Carotid plaque area by quartiles of plasma total homocysteine levels. Error bars represent 95% confidence intervals.

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[14] or thiols[15,16] to achieve target homocysteine levels.

The results of the VISP trial[3] showed that a modest reduction of total homocysteine levels (by 1.5 to 2.0 μmol/L) using folic acid, vitamin B_6 and vitamin B_{12} after nondisabling 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[17] 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_{12} (6 mcg), and in both treatment arms those with low vitamin B_{12} levels received injections of vitamin B_{12}. Differences in results between the coronary angioplasty trials reported by Schnyder and associates[18] (in which patients benefitted from vitamin therapy for homocysteine levels) and that of Lange and associates[19] (in which they did not) may be because of the difference in vitamin B_{12} doses (400 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.[20,21]

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[22] reported that, since fortification, the incidence of serum folate levels of less than 3 nmol/L has been reduced from 22% to less than 2% in the Framingham population, and in the VISP trial, less than 1% had levels below 6.7 nmol/L (unpublished data).

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	β 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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