Results of B-Vitamin Supplementation Study Used in a Prediction Model to Define a Reference Range for Plasma Homocysteine

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Because high plasma concentrations of homocysteine constitute an enhanced risk for premature coronary heart disease, it is necessary to establish a reference range for normal concentrations of plasma homocysteine. The frequency distribution of plasma homocysteine concentrations tails to the right, and the nonparametric approach is unsatisfactory for defining a normal plasma homocysteine reference range. By using subjects’ responses to appropriate vitamin supplementation, we developed a mathematical prediction model to calculate the plasma homocysteine concentration that could be expected for each individual treated with a vitamin supplement. With this model, we can predict that plasma homocysteine concentrations will approach a normal frequency distribution with a 95% reference range (mean ± 2 SD) of 4.9–11.7 μmol/L, provided the vitamin status of the study population is improved.

Indexing Terms: coronary heart disease/nutritional status/risk factors/folate/vitamin B12

Coronary heart disease (CHD) is a multifactorial condition that is one of the primary causes of premature death in Western societies. Although established risk factors for CHD, such as an above-normal concentration of low-density lipoprotein cholesterol in serum, hypertension, and smoking, have been studied exhaustively, these factors explain only ~50% of all CHD cases (1). Obviously, other risk factors are also involved in the etiology of atherosclerosis and CHD. Hyperhomocysteinemia may be an additional risk factor predisposing individuals to premature CHD. Patients homozygous for cystathionine-β-synthase deficiency, or who have inherited disorders of cobalamin metabolism (e.g., Cbl C syndrome), have very high plasma homocysteine concentrations and are usually subject to severe, premature atherosclerosis (2, 3). Recently, laboratory techniques to measure plasma homocysteine concentrations have become more readily available (4–6), and the observations made in cystathionine-β-synthase-deficient patients have been extended to the general population, where milder forms of hyperhomocysteinemia may be common (7–9). Circulating homocysteine concentrations are increased in CHD (10–14) and cerebral vascular disease (15–18) as well as peripheral vascular disease (4, 17). A recent prospective study among participants from the Physicians Health Study confirms that a moderately increased plasma concentration of homocysteine is an independent risk factor for CHD (19). This study showed a 3.4-fold greater risk of myocardial infarction among men with plasma homocysteine concentrations >15.8 μmol/L than in those with concentrations <14.1 μmol/L. Further evidence supporting a role for homocysteine in atherogenesis comes from the observation that vascular disease progression was accelerated in subjects with hyperhomocysteinemia compared with patients with normal concentrations of plasma homocysteine (20). These observations were also extended to asymptomatic adults; thickening of the carotid artery wall (which is thought to reflect atherosclerosis) was associated with significantly higher plasma homocysteine concentrations (21). Observations that hyperhomocysteinemia may be atherogenic are not limited to epidemiological studies only. Results from animal and cell culture studies indicate that increased homocysteine concentrations may accelerate CHD by various mechanisms, including direct damage to the vascular endothelium (22), stimulation of smooth muscle cell proliferation (23), and enhanced LDL peroxidation (24). Furthermore, homocysteine may interfere with hemostasis by various mechanisms, thus contributing to a biochemical environment conducive to thrombus formation (25–29).

Given the accumulating evidence that homocysteine is involved in atherogenesis, it has become essential to define normal values of plasma homocysteine concentrations. Such values are usually expressed by defining the distribution of values around the mean for an apparently healthy population. Conventionally, the reference range is calculated as the mean ± 2 SD from the mean. However, this statistical approach may be inappropriate in a high-risk CHD population, because an unknown number of subjects from the reference population may have subclinical CHD. Experience with other CHD risk factors has shown that other approaches are required. For example, serum cholesterol concentrations that confer a higher-than-basal risk for premature CHD start well below the upper limit of the statistical reference range calculated in a “healthy” population with a high CHD incidence (30, 31). Therefore, optimal values for serum cholesterol associated with good health have been derived from cholesterol concentrations in populations with a low CHD incidence and from serum cholesterol concentrations in the lowest-risk segment of populations with a high CHD incidence (30). We do not yet have the wealth of epidemiological data needed to define “safe” plasma concentrations of homocysteine.
homocysteine concentrations that are available for cholesterol. Until such epidemiological trials have been completed, nutritional considerations may guide us to establish a preliminary homocysteine reference range. Given that the plasma homocysteine concentration is a function of folate, vitamin B12, and possibly vitamin B6 status (7,8), we used data from vitamin supplementation trials and a mathematical prediction model to propose a suitable plasma homocysteine reference range.

Materials and Methods

High-CHD-Risk Population and Homocysteine Assay

Since July 1991 we have measured plasma homocysteine concentrations in 1437 adult white men, ages 18–65 years. All apparently healthy and employed by major employers in the Pretoria area, they adhered to a typical Western life-style. The prevalence of CHD in this group is among the highest in the world (32).

Venous blood samples with EDTA as anticoagulant were obtained for homocysteine analyses; the blood samples were chilled on ice and plasma was separated from blood cells within 1 h. Plasma homocysteine concentrations were determined by HPLC according to a modification of the method of Araki and Sako (5,6). This method entails complete reduction of homocysteine and the mixed disulfide (cysteine–homocysteine), and the release of protein-bound homocysteine. The method therefore measures total (free + protein-bound) plasma homocysteine concentrations.

Vitamin Supplementation Study

From the group of 1437 men, 72 who had relatively high (>16.3 μmol/L) plasma homocysteine concentrations and did not use vitamin supplements participated in three clinical trials to study the effect of vitamin supplementation on circulating homocysteine concentrations. Full details of these trials have been published (7,33,34) and the results are summarized in Table 1.

In each study, different vitamin supplements (Table 1) were used to lower the plasma homocysteine concentrations over a 6-week period. Plasma homocysteine concentrations were measured at the start of each study and after 6 weeks of vitamin supplementation. The vitamin concentrations in plasma were monitored after 3 and 6 weeks to assess compliance.

These trials, however, provided information only on the effect of vitamin therapy on plasma homocysteine concentrations >16.3 μmol/L. To establish the effect of vitamin supplementation on lower plasma homocysteine concentrations, we studied 19 white men [mean (SD) age: 20 (1.0) years] who did not consume vitamin supplements before the study and who had relatively low plasma homocysteine concentrations (<16.3 μmol/L). They were supplied with a vitamin combination (1.0 mg of folic acid, 0.4 mg of vitamin B12, 10 mg of pyridoxine) and were requested to take one tablet per day for 6 weeks. Plasma homocysteine concentrations were measured at the start of the study and again after the 6-week supplementation period. Plasma vitamin concentrations were monitored as indicated above to assess compliance.

Mathematical Model for Estimating Probable Effect of Vitamin Supplementation on Plasma Homocysteine

In all, data from 91 subjects who had been treated for 6 weeks with various vitamin supplements were available to determine the relationship between plasma homocysteine concentrations before and after vitamin supplementation. Although different supplements were used in separate trials (Table 1), we were able to combine the data for the following reasons:

1. The designs of the trials were similar; each studied the effect of the vitamin supplement on plasma homocysteine concentrations for 6 weeks.

2. The various vitamin supplements used were equally effective and did not differ significantly in their respective effects on plasma homocysteine concentrations. For example, supplementation with a vitamin combination (folic acid, vitamin B12, and pyridoxine) did not differ significantly from folic acid supplementation alone with respect to lowering plasma homocysteine concentrations (34). The data from the 91 vitamin-treated subjects were carefully screened, and those from 16 subjects were omitted from further data analyses. Excluded subjects either had an unsatisfactory compliance record according to blood vitamin analyses (n = 6), or were severely vitamin B12 deficient and did not respond to oral vitamin supplementation (n = 4). Six individuals who had basal plasma homocysteine concentrations >50 μmol/L were also excluded; the number of those data points was considered too few to allow accurate estimation of the general effect of vitamin supplementation on plasma homocysteine concentrations >50 μmol/L, and we wanted not to bias the response to vitamin supple-
mentation by the few patients with very high basal plasma homocysteine values.

Using data from the remaining subjects (n = 75), we constructed a scatter plot of pretreatment plasma homocysteine concentrations vs the ratio of post- and pretreatment plasma homocysteine concentrations. Then, by the method of least squares, a piecewise linear regression (36) line with one node at a pretreatment homocysteine concentration of 26 μmol/L was fitted to the data (Fig. 1).

The node at 26 μmol/L corresponds to the piecewise linear regression line with the least mean square error. The general notation of the piecewise linear regression model is:

\[ y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \epsilon \]

where \( y \) = ratio of posttreatment to pretreatment homocysteine concentrations; \( x_1 \) = pretreatment homocysteine concentration; \( x_2 = 0 \) if pretreatment homocysteine \( \leq 26.0 \) μmol/L, or the pretreatment homocysteine concentration \( > 26 \) if the pretreatment homocysteine is \( > 26.0 \) μmol/L; \( \beta_0, \beta_1, \) and \( \beta_2 \) are the regression coefficients; and \( \epsilon \) is the residual.

Four influential points [identified by Cook's method (36), i.e., points with \( P < 0.05 \) according to the t-statistic for the outlier test] were omitted in the calculation of the piecewise linear regression line and are identified in Fig. 1. The piecewise linear regression line was eventually fitted to 71 data points, and the regression equation was used to calculate the estimated plasma homocysteine concentration for each individual in the total population group, as if he had been treated with a suitable vitamin formulation for 6 weeks.

**Results**

The mean (SD) plasma homocysteine concentration of the study population was 12.0 (6.7) μmol/L (Table 2), but the frequency distribution was positively skewed near the upper limit of the normal range. The mean adjusted plasma homocysteine concentration in these subjects was 8.3 (1.7) μmol/L (Table 2).

**Table 2. Measured and adjusted plasma homocysteine concentrations in white South African men at high risk for CHD.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured*</td>
<td>1437</td>
<td>12.0</td>
<td>6.7</td>
<td>4.2</td>
<td>112.0</td>
</tr>
<tr>
<td>Adjusted*</td>
<td>1428</td>
<td>8.3</td>
<td>1.7</td>
<td>3.9</td>
<td>13.2</td>
</tr>
</tbody>
</table>

* Wilk-Shapiro statistic for normal distribution = 0.64.
* See text; Wilk-Shapiro statistic = 0.99.

[Wilk-Shapiro test statistic (37, 38) for normality = 0.64 (Fig. 2)].

We reported previously that a high plasma homocysteine concentration is significantly reduced after vitamin supplementation (7, 33, 34). In the present study, we found that treating a young adult population with a mean (SD) plasma homocysteine concentration of 10.0 (3.7) μmol/L with a vitamin combination also reduced the mean plasma homocysteine concentration significantly (\( P < 0.001 \), Student's paired t-test), to 7.3 (1.6) μmol/L. Therefore, we combined these data with the previously reported results obtained on vitamin-treated subjects with higher plasma homocysteine concentrations (Table 1) and fitted a piecewise linear regression line to the combined sets of data to describe the relation between the ratio of post- to pre- and the pretreatment plasma homocysteine concentrations. The regression line is described by the equation

\[ y = 1.0089 - 0.022 x_1 + 0.0144 x_2 \]

and the coefficient of determination [defined as the square of the multiple correlation coefficient (R) and expressed as a percentage] is 72.2%. Therefore, 72.2% of the variation in the plasma homocysteine concentration ratio described above can be explained by the variation in pretreatment plasma homocysteine concentrations. Using the above-mentioned equation, we calculated the plasma homocysteine concentration that
could be expected for each individual if he had been treated with an appropriate vitamin supplement, i.e., adjusted homocysteine concentrations. The mean (SD) adjusted homocysteine concentration was 8.3 (1.7) μmol/L, and the frequency distribution was normal (Fig. 2; Wilk–Shapiro test statistic for normality = 0.99).

Discussion

The skew (tiled) frequency distribution of plasma homocysteine concentrations in the studied population group may be typical of CHD-prone Western populations and has been reported previously (7, 19, 21). According to the Physicians Health Study, the risk for myocardial infarction increased 3.4-fold in cases with plasma homocysteine concentrations in the tailed part of the homocysteine frequency distribution curve (19). It may therefore be inappropriate to include the tail of the plasma homocysteine distribution curve in a definition of plasma homocysteine concentrations associated with good health. Therefore, even the nonparametric determination of 95% normal limits will be unsuitable to define a reference range for plasma homocysteine. According to the nonparametric approach, the 97.5th percentile represents the upper normal limit of the variable. In our study, the 97.5th percentile corresponds to a plasma homocysteine concentration of 30.1 μmol/L, and the 95% reference range will still include a substantial proportion of the tail of the frequency distribution.

Because the nonparametric approach is unsatisfactory, we used a mathematical prediction model to calculate a possibly more appropriate plasma homocysteine reference range. Using the regression equation that describes the relation between pre- and postvitamin supplementation homocysteine concentrations, we calculated the effect that vitamin therapy could be expected to have on each individual (with plasma homocysteine <50 μmol/L) from the study population. The results show that vitamin therapy would probably reduce the mean (SD) plasma homocysteine concentration from 12.0 (6.7) to 8.3 (1.7) μmol/L. Furthermore, the distribution of adjusted plasma homocysteine concentrations is gaussian (Fig. 2), implying that the plasma homocysteine reference range will then be 4.9–11.7 μmol/L (mean ± 2 SD). This calculated upper normal limit is considerably lower than the nonparametrically defined upper normal limit. Our mathematical model and calculated reference range are based on nutritional considerations, given that an adequate vitamin status was obtained in a relatively small study population through vitamin supplementation. The proposed reference range was then derived by extrapolation from the change in plasma homocysteine concentrations in the vitamin-supplemented individuals to the general population.

It may be argued that the derived reference range is invalid because it is a function of the daily vitamin dose in supplemented individuals. However, we have shown that the vitamin supplements used in our previous studies elicited a maximum response with respect to lowering plasma homocysteine concentrations (7), implying that calculations based on higher vitamin doses are unlikely to shift the projected distribution curve further to the left. It may also be argued that this reference range is artificially low; however, at least three recent studies support that this approach may be appropriate to define a plasma homocysteine range compatible with good health (14, 21, 39). Malinow et al. recently found that the risk for having a thickened carotid artery wall was significantly increased when the plasma homocysteine concentrations were >10.5 μmol/L (21). Selhub et al. also studied the association between carotid artery stenosis and plasma homocysteine concentrations and found that the risk of stenosis was greater in subjects with homocysteine concentrations >11.4 μmol/L (39). Pancharuniti et al. studied patients with angiographically demonstrated CHD and found that CHD risk started to increase from plasma homocysteine concentrations ≥11.7 μmol/L (14).

This approach to determining a plasma homocysteine reference range based on vitamin-supplementation trials is novel and will require confirmation from clinical and epidemiological studies. It is not yet clear whether there is a threshold below which the homocysteine concentration does not pose a CHD risk. Large epidemiological studies are required to assess at which plasma homocysteine concentrations a higher-than-basal risk for premature CHD is conferred to the patient. Such studies will be essential for evaluating the normal reference range for plasma homocysteine as predicted by our model. Furthermore, epidemiological studies on population groups with a low CHD incidence will also be useful to gain a better perspective on the preferred plasma homocysteine concentration range. Finally, it may also be possible to refine the model by taking into account other factors (i.e., age) that might influence plasma homocysteine concentrations, and women may require a separate reference range.

In summary, our results indicate that the plasma homocysteine frequency distribution in a CHD-prone population tails to the right, but mathematical modeling suggests that the tail of the frequency distribution reflects an inadequate vitamin status, which will disappear when the general vitamin status is improved. Based on the mathematical prediction model, a plasma homocysteine reference range between 4.9 and 11.7 μmol/L is suggested. This study again emphasizes that a substantial proportion of an affluent society may have high plasma homocysteine concentrations due to an imprudent diet, which may put them at increased risk for premature vascular disease. Because hyperhomocysteinemia may be easily corrected by simple dietary measures, it seems appropriate to screen for, and treat, this disorder on a routine basis.

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References


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