Age- and gender-specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation

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We present reference intervals for total homocysteine and methylmalonic acid in plasma based on samples from 126 women (ages 20–85 years, median 49 years) and 109 men (ages 20–84 years, median 50 years). The central 0.95 interval for methylmalonic acid was 0.08–0.28 μmol/L. Supplementation with cyanocobalamin caused a nonsignificant decrease in methylmalonic acid. Supplementation with folic acid caused a decrease in homocysteine concentrations, with data analysis identifying two significantly different clusters: 182 subjects with the lowest initial concentrations (7.76 ± 1.54 μmol/L, mean ± SD) and the smallest decrease (1.26 ± 0.96 μmol/L), and 53 subjects with the highest initial concentrations (12.33 ± 2.04 μmol/L) and greatest decrease (4.14 ± 1.32 μmol/L). We argue in favor of the age- and gender-specific central 0.95 intervals obtained for the 182 subjects before being supplemented with folic acid: 4.6–8.1 μmol/L for subjects at <30 years; 4.5–7.9 μmol/L for women, ages 30–59 years; 6.3–11.2 μmol/L for men, ages 30–59 years; and 5.8–11.9 μmol/L for subjects at >60 years.

INDEXING TERMS: nutritional status • folic acid • folate • cardiovascular disease • atherosclerotic disease • risk factors

Determination of total homocysteine in plasma is becoming an important diagnostic procedure in clinical chemistry because of rapidly accumulating evidence that a slightly increased concentration of total homocysteine in plasma is a significant independent risk factor for atherosclerotic diseases [1–3]. Furthermore, measurements of total homocysteine and methylmalonic acid combined improve the rate of detecting cobalamin deficiency over that of methylmalonic acid measurements alone [4].

So far, several useful methods, e.g., detection by HPLC or stable-isotope-dilution [5] or immunoassay techniques [6] have been developed for the routine determination of homocysteine in plasma. A reliable stable-isotope-dilution technique is widely used for routine determinations of methylmalonic acid in plasma [7]. Recently, a capillary electrophoresis assay for analysis of methylmalonic acid in plasma has been described [8]. Furthermore, adding fluoride to blood samples has recently solved the drawback that, after sampling, blood cells still produce and release homocysteine into the plasma, causing an artificial increase in measured concentrations of 10%/h [9]. Nonetheless, reliable reference intervals for concentrations of homocysteine in plasma have not yet been established. Normal upper reference limits (mean ± 2 SD) for homocysteine differ greatly from one laboratory to another; e.g., since 1992, values at 12.3 μmol/L [9], 16.1 μmol/L [10], 21.2 μmol/L [11], 13.9 μmol/L [12], and 30.1 μmol/L [13] have been reported. The variability may be related not only to different methodologies or differences in sample collection but also, perhaps more importantly, to the selection of reference individuals. Methyltetrahydrofolate and cobalamin being coenzymes in homocysteine metabolism, an adequate status for folate and vitamin B₁₂ is necessary to prevent accumulation of homocysteine in the blood. Recent studies, however, suggest that a large proportion of the population—perhaps 40%—is not consuming enough folate to keep the concentrations of homocysteine in plasma low [14, 15]. How then should investigators establish appropriate reference values? Recently, Stampfer and Malinow [16] introduced the term “a stable low level” to denote the plasma homocysteine concentrations reached in a population with high folate intakes, and Ubbink et al. [13] have published a reference interval based on a mathematical prediction model extrapolating results from four vitamin-supplemented groups to an unsupplemented general population.

In the present work, we report the results of a vitamin supplementation trial. Our goal was to define the upper reference limit for plasma homocysteine in non-cobalamin- and non-folate-deficient subjects. We also examined possible age-
and gender-related differences. We included measurements of methylmalonic acid in plasma in the present investigation to make probable the absence of cobalamin deficiency in our reference population and to obtain the same reference population for homocysteine and methylmalonic acid in plasma for future use in the diagnosis of cobalamin deficiency.

Materials and Methods

Subjects. Our intention was to recruit ~40 subjects in each decade of life from age 20 years to >70 years, evenly distributed between the two sexes. During a period of 4 months, 249 apparently healthy volunteers participated in and completed the study. Of these, 241 participants were recruited from among the employees of the hospital and their acquaintances; 8 of the elderly were recruited from the patient register in a rural general practice.

The volunteers agreed to participate after informed consent, in accordance with the Helsinki Declaration. The study was approved by the local committee of medical ethics.

Study design. Three blood samples were taken at intervals of between 1 and 3 weeks. After the initial blood collection (sample 0), the subjects received one tablet containing 1 mg of cyanocobalamin (Betolvex®; Dumed, Copenhagen, Denmark) two times a day for 1 week; in a few cases, for practical reasons, this was followed by one tablet daily for as long as 14 days until the second blood collection (sample 1) could take place. Likewise, one tablet containing 5 mg of folic acid (Nycomed DAK, Copenhagen, Denmark) was subsequently taken two times a day for 1 week—in a few cases, followed by one tablet daily for as many as 14 days until the last blood collection (sample 2).

Homocysteine was assayed in all three samples, methylmalonic acid in the first two samples. To exclude kidney insufficiency, we measured the creatinine content of sample 0.

A questionnaire was filled out by the participants regarding whether daily vitamin supplementation containing folic acid was taken on a regular basis, or whether antiepileptic drugs were taken, or whether the participant had psoriasis.

Specimens. Blood samples were taken from an antecubital vein. Plasma samples for measurements of homocysteine and methylmalonic acid were collected in tubes that contained heparin as an anticoagulant and with sodium fluoride added to a final concentration of 4 g/L blood, according to the procedure described previously [9]. Plasma was separated by centrifugation within 2 h and stored at −20 °C until analyzed.

Assays. Total homocysteine and methylmalonic acid in plasma were measured by stable isotope dilution with solid-phase extraction of the sample [7, 9]. Creatinine in plasma was determined by using an Ektachem 700 × RC analyzer.

Statistical analysis. We used the program package Solo Statistical System (BMDP Statistical Software, Los Angeles, CA). Data were tested for normality with the Martinez and Iglewicz test. Differences between group means were tested by using analysis of variance, t-tests, or the Wilcoxon tests. Duncan’s posthoc test was used in multiple comparison of means. Testing for outliers was performed according to the criteria discussed by Strike [17]. To separate the subjects into two groups according to their response to supplementation with folic acid, we performed cluster analysis, using the K-means algorithm from the Solo Statistical System. P <0.05 was considered significant unless otherwise stated.

Results

Analytical Variation
The internal quality-control system for the analysis of methylmalonic acid in plasma for the study period of 4 months yielded SDs of 0.03 and 0.04 μmol/L at mean concentrations of 0.17 and 0.61 μmol/L, respectively. For homocysteine in plasma the corresponding results were 0.36 and 1.28 μmol/L at mean concentrations of 7.1 and 23.2 μmol/L, respectively.

Selection of the Study Group
Of the 249 participants, 3 had a high probability of cobalamin deficiency in the tissues according to the criteria of Nexø et al. [18], i.e., methylmalonic acid in plasma >0.45 μmol/L and a subsequent decrease of >20% after cobalamin supplementation. Five had a statistically outlying concentration of methylmalonic acid in plasma initially (>0.36 μmol/L), compared with the entire set of results but also within their age and gender groups. Furthermore, six subjects had deviant concentrations of homocysteine in the first plasma sample (>19.4 μmol/L for women and >20.8 μmol/L for men); these data were also classified as outliers within their age and gender groups. The data from all 14 subjects were excluded from the subsequent analysis. None of the 14 had an abnormal kidney function estimated by the concentration of creatinine in plasma. Details are summarized in Table 1.

Of the remaining 235 participants, 126 were women, ages 20–85 years (median 49 years), and 109 were men, ages 20–84 years (median 50 years). Distribution by age and gender groups is shown in Table 2.

All except one subject had concentrations of creatinine in plasma within the reference interval for healthy subjects, 55–120 μmol/L. One man, age 60 years, had a concentration of creatinine in plasma at 141 μmol/L. In agreement with previous studies [19], this moderate increase in creatinine did not correlate with any increase in methylmalonic acid in the plasma, and his concentration of homocysteine in plasma was not significantly different from the concentrations of the other men in his age group.

Effect of Vitamin Supplementation

Interindividual Variation before Vitamins. Fig. 1 shows that the initial concentrations of methylmalonic acid in plasma for the 235 subjects were approximately normally distributed with a slight positive skewness. The mean value was 0.154 μmol/L (SD 0.050 μmol/L). The central 0.95 interval was 0.08–0.28 μmol/L, as calculated nonparametrically. Women older than 60 years had a significantly higher concentration of methylmalonic acid in plasma, by an average of 0.037 μmol/L, than did the younger women. In contrast, the concentration of methylma-
MMA₀ and MMA₁, concentrations of methylmalonic acid in plasma before and after cyanocobalamin supplementation; HCY₀ and HCY₁, concentrations of total homocysteine in plasma before and after supplementation with cyanocobalamin; HCY₂, concentration after supplementation with folic acid.

Data excluded because the initial concentrations of methylmalonic acid in plasma were consistent with cobalamin deficiency (see text).

Data excluded because the initial concentrations of homocysteine in plasma were outliers in the statistical context.

Data excluded because the initial concentrations of homocysteine in plasma were outliers.

This concentration declined to 10.2 μmol/L after further supplementation with folic acid for 1 week.

Homocysteine in men exhibited no significant dependence on age. When age was not considered, however, there was no significant difference between the concentrations in men and women. The mean and SD concentrations for the age and sex groups are shown in Table 2.

Figure 2 shows that the initial concentrations of homocysteine for all 235 subjects did not follow a normal distribution curve but exhibited a marked positive skewness. Fig. 3 shows significant differences in homocysteine concentrations between age groups and between the sexes. The mean and SD concentrations for the age and sex groups are shown in Table 2. Women between ages 30 and 59 years had lower concentrations than men at those ages, and for both sexes the homocysteine concentrations increased significantly with age. For all 235 subjects the mean (±SD) of the initial total homocysteine in plasma was 9.23 ± 2.64 μmol/L (8.65 ± 2.54 μmol/L for women and 9.90 ± 2.45 μmol/L for men). The central 0.95 interval, calculated nonparametrically, was 5.5–15.7 μmol/L (4.9–14.9 μmol/L for women and 6.3–15.7 μmol/L for men).

**Effect of cobalamin supplementation.** Supplementation with cyanocobalamin did cause a nonsignificant decrease (0.005 ± 0.056 μmol/L, mean ± SD) in the concentration of methylmalonic acid in plasma (Fig. 1). However, there was a significant tendency of larger decreases in methylmalonic acid when initial concentrations were >0.24 μmol/L.

Supplementation with cyanocobalamin also caused a small but statistically significant decrease (0.44 ± 1.32 μmol/L, mean ± SD) in the concentrations of homocysteine in plasma (Fig. 2). The decrease correlated positively with the initial concentration of homocysteine and with the decrease in methylmalonic acid (P = 0.02). The distribution of homocysteine in plasma after cyanocobalamin administration was positively skewed, like the initial concentrations, and the mean (SD) concentration then was 8.79 ± 2.54 μmol/L.

**Effect of folic acid supplementation.** Fig. 2 shows that the subsequent supplementation with folic acid caused a larger decrease in homocysteine concentrations in men and women, and this decrease was statistically significant.
(1.91 ± 1.60 μmol/L, mean ± SD) in homocysteine concentrations than cyanocobalamin did. This decrease correlated positively with the initial concentration of homocysteine. The mean (SD) concentration after folic acid was 6.88 ± 1.64 μmol/L, with a distribution more symmetrical than before but still positively skewed. The central 0.95 interval was 4.4–10.9 μmol/L, calculated nonparametrically (4.4–9.8 μmol/L for women and 5.0–12.1 μmol/L for men). As with the initial concentrations, women between ages 30 and 59 years had lower concentrations than men of those ages; for both sexes, the groups >60 years had the highest concentrations (see Fig. 3).

Cluster analysis. Using as variables (a) the concentration of homocysteine in plasma before supplementation with folic acid and (b) the decrease with folic acid, cluster analysis demarcated two significantly different clusters of results. The first cluster included the samples with the lowest concentrations and the smallest decreases; the second contained the highest concentrations and the greatest decreases. The first cluster, with the weak responders to folic acid, contained data from 182 participants; their mean (SD) concentration of homocysteine before supplementation with folic acid was 7.76 ± 1.54 μmol/L, and the nonparametrically calculated central 0.95 interval was 4.9–11.4 μmol/L. The average decrease in the first cluster after folic acid was 1.26 ± 0.96 μmol/L. The second cluster, with the strong responders, comprised the data from the remaining 53 subjects, in whom the mean (SD) homocysteine concentration before folic acid was 12.33 ± 2.04 μmol/L and the central nonparametrically calculated 0.95 interval was 9.5–16.0 μmol/L. The average decrease after folic acid in the second cluster was 4.14 ± 1.32 μmol/L.

Table 3 lists the corresponding results of cluster analysis for each individual sex and age group.

Effect of previous regular vitamin intake. A comparison between the initial and final concentrations of homocysteine in participants taking regular supplementation of vitamins or not is shown in Table 4. The group of participants taking vitamin supplementation containing folic acid had significantly lower concentrations of homocysteine in plasma than did subjects taking no supplements, as had a group taking various food supplements containing an indeterminate amount of folic acid.

Only three of the participants stated they took antiepileptic drugs, and no significant effect from this was evident. Eleven reported that they had psoriasis or closely related skin diseases; however, no significant difference between their results and the results of the 202 who stated that they were unaffected with such conditions could be found.

Discussion

A prerequisite for the interpretation of homocysteine and methylmalonic acid measurements is knowledge of analytical variation, biological variation, and the influence of vitamin intake. The variation of both analytical methods during the study period was in reasonable agreement with those originally reported from our laboratory [7, 9]. Although the present study provides no data on intraindividual variations, it is well documented that the intraindividual variation of the concentration of methylmalonic acid in plasma is negligible [20]. In contrast, data on intraindividual variations of the concentrations of homocysteine in plasma are still sparse and inconsistent [5].
Table 3. Effect of vitamin supplementation on concentrations of homocysteine in plasma.

<table>
<thead>
<tr>
<th>Group*</th>
<th>n</th>
<th>Before folic acid</th>
<th>After folic acid</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women and men, &lt;30 years</td>
<td>39</td>
<td>7.31 ± 1.66</td>
<td>5.75 ± 1.08</td>
<td>1.57 ± 1.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.7 - 10.2)</td>
<td>(3.9 - 7.7)</td>
<td>(—0.6 to 4.7)</td>
</tr>
<tr>
<td>Weak responders</td>
<td>30</td>
<td>6.65 ± 0.95</td>
<td>5.62 ± 1.11</td>
<td>1.03 ± 0.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.6 - 8.1)</td>
<td>(3.8 - 7.9)</td>
<td>(—1.0 to 2.6)</td>
</tr>
<tr>
<td>Strong responders</td>
<td>9</td>
<td>9.52 ± 1.66</td>
<td>6.16 ± 0.89</td>
<td>3.37 ± 1.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7.7 - 13.5)</td>
<td>(4.6 - 7.1)</td>
<td>(2.1 to 6.6)</td>
</tr>
<tr>
<td>Women, 30–59 years</td>
<td>68</td>
<td>7.38 ± 1.71</td>
<td>5.96 ± 1.13</td>
<td>1.42 ± 1.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.6 - 11.2)</td>
<td>(4.3 - 8.4)</td>
<td>(—0.8 to 4.0)</td>
</tr>
<tr>
<td>Weak responders</td>
<td>45</td>
<td>6.45 ± 0.96</td>
<td>5.68 ± 1.06</td>
<td>0.78 ± 0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.5 - 7.9)</td>
<td>(4.3 - 8.1)</td>
<td>(—0.9 to 2.0)</td>
</tr>
<tr>
<td>Strong responders</td>
<td>23</td>
<td>9.18 ± 1.37</td>
<td>6.50 ± 1.08</td>
<td>2.67 ± 1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7.2 - 11.8)</td>
<td>(4.8 - 8.4)</td>
<td>(0.8 to 4.8)</td>
</tr>
<tr>
<td>Men, 30–59 years</td>
<td>55</td>
<td>9.66 ± 2.23</td>
<td>7.45 ± 1.32</td>
<td>2.21 ± 1.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.4 - 15.2)</td>
<td>(5.4 - 10.0)</td>
<td>(—0.3 to 5.8)</td>
</tr>
<tr>
<td>Weak responders</td>
<td>43</td>
<td>8.76 ± 1.36</td>
<td>7.27 ± 1.21</td>
<td>1.50 ± 0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.3 - 11.2)</td>
<td>(5.4 - 10.0)</td>
<td>(—0.2 to 3.0)</td>
</tr>
<tr>
<td>Strong responders</td>
<td>12</td>
<td>12.88 ± 1.74</td>
<td>8.12 ± 1.51</td>
<td>4.76 ± 1.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10.3 - 15.2)</td>
<td>(6.2 - 9.2)</td>
<td>(3.1 to 6.7)</td>
</tr>
<tr>
<td>Women and men, &gt;60 years</td>
<td>73</td>
<td>10.24 ± 2.67</td>
<td>7.92 ± 1.65</td>
<td>2.32 ± 1.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.1 - 16.3)</td>
<td>(5.7 - 11.4)</td>
<td>(—0.6 to 5.7)</td>
</tr>
<tr>
<td>Weak responders</td>
<td>51</td>
<td>8.92 ± 1.60</td>
<td>7.53 ± 1.56</td>
<td>1.39 ± 1.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.8 - 11.9)</td>
<td>(5.4 - 10.6)</td>
<td>(—0.5 to 2.9)</td>
</tr>
<tr>
<td>Strong responders</td>
<td>22</td>
<td>13.30 ± 2.08</td>
<td>8.85 ± 1.51</td>
<td>4.46 ± 1.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10.7 - 16.2)</td>
<td>(6.3 - 11.2)</td>
<td>(3.0 to 7.2)</td>
</tr>
</tbody>
</table>

* The 235 participants are divided into four comparable groups according to sex and age (analysis of variance, Duncan’s test). By cluster analysis, each group is further divided into a group with a weak response and a group with strong response to folic acid.

* Results are shown as mean ± SD (and nonparametrically calculated central 0.95 interval).

Table 4. Effect of regular vitamin supplementation on concentrations of homocysteine in plasma.

<table>
<thead>
<tr>
<th>Supplementation status</th>
<th>n</th>
<th>Before vitamin supplementation</th>
<th>After vitamin supplementation</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>No information</td>
<td>22</td>
<td>9.33 ± 1.79</td>
<td>7.09 ± 1.79</td>
<td>2.24 ± 1.20</td>
</tr>
<tr>
<td>No regular supplementation</td>
<td>110</td>
<td>9.69 ± 2.92</td>
<td>6.94 ± 1.65</td>
<td>2.75 ± 2.08</td>
</tr>
<tr>
<td>Regular supplementation without folic acid</td>
<td>14</td>
<td>9.61 ± 2.27</td>
<td>7.42 ± 1.81</td>
<td>2.19 ± 1.28</td>
</tr>
<tr>
<td>Regular supplementation with folic acid</td>
<td>75</td>
<td>8.61 ± 2.39*</td>
<td>6.79 ± 1.59*</td>
<td>1.82 ± 1.36</td>
</tr>
<tr>
<td><em>Alternative</em> supplementation</td>
<td>14</td>
<td>8.44 ± 2.41*</td>
<td>6.09 ± 1.21*</td>
<td>2.35 ± 1.60</td>
</tr>
</tbody>
</table>

* Significantly different from homocysteine concentrations in the groups not taking regular vitamin supplementations with folic acid (P <0.05).

* Indeterminate content of folic acid.

REFERENCE INTERVALS BEFORE VITAMIN INTAKE
The 0.95 reference interval for methylnalonic acid concentrations before intake of vitamins (0.08–0.28 μmol/L) was narrower than the 0.05–0.37 μmol/L we reported previously [20], but the selections of the study group in the two studies were not completely comparable. Our present finding is closer to the interval 0.06–0.25 μmol/L reported by Naurath et al. [12]. Our results provide the first evidence that women >60 years have higher concentrations of methylnalonic acid in plasma than do younger women.

The 0.95 reference interval for homocysteine concentrations before intake of vitamins (5.5–15.7 μmol/L) was broader and had a higher mean value than the 4.6–12.3 μmol/L interval we observed in an earlier study [9]. The difference is due to a different selection of subjects, the earlier results being based on a much younger study group. Our finding that, in general, men have higher concentrations than women (Fig. 3) is in agreement with an earlier report from our laboratory [21]. The finding that women >60 years have higher concentrations than younger women (see Fig. 3) has been reported previously by Andersson et al. [22] and may be related to menopausal status. In general, the concentration of total homocysteine is dependent on both age and gender. Nutritional status is another important determinant of homocysteine concentrations, as illustrated by our
findings that subjects taking regular vitamin supplementation, either traditional or alternative forms, have lower concentrations of homocysteine.

**EFFECT OF SUPPLEMENTATION WITH COBALAMIN**

For convenience, cobalamin was given orally. About 1.2% of an oral dose is absorbed without the mediation of intrinsic factor [23]; i.e., the subjects absorbed at least 160 μg of cobalamin daily. The present results are in accordance with our previous findings [20] that concentrations of methylnalonic acid <~0.30 μmol/L cannot be suppressed with administration of cobalamin. These findings indicate the existence of a stable, low reference interval for plasma methylnalonic acid (0.08–0.28 μmol/L), this terminology (stable, low reference interval) denoting the reference interval observed in a population consuming enough vitamin B₁₂ to keep the analyte concentrations low and stable. Since we completed our study, Naurath et al. [12] have reported that the maximum decline in plasma methylnalonic acid is achieved after 5–12 days of cobalamin supplementation. Thus, administration of cobalamin for longer than 1 week could arguably have had a slightly greater effect in our subjects.

In contrast, cobalamin supplementation was followed by a small but significant average decrease in homocysteine concentrations. What this phenomenon might indicate is not clear, but it might reflect a subtle intracellular cobalamin deficiency, given the significant correlation between the individual changes in concentrations of homocysteine and methylnalonic acid. The decrease in homocysteine concentrations may thus reflect the use of different derivatives of cobalamin in the intracellular metabolism of homocysteine and methylnalonic acid: Methylcobalamin is an essential cofactor for the enzyme methionine synthase (EC 2.1.1.12), and 5'-deoxyadenosylcobalamin is an essential cofactor for the enzyme methylnalonyl-CoA mutase (EC 5.4.99.2).

**EFFECT OF SUPPLEMENTATION WITH FOLIC ACID**

The reduction in homocysteine concentrations with folic acid was much larger than with cobalamin; even low-normal concentrations were significantly reduced after intake of folic acid. Our results are close to those predicted by Ubbink et al. [13], who studied the effects of various vitamin supplements on homocysteine concentrations in 72 men with high concentrations (>16.3 μmol/L) and 19 men with concentrations <16.3 μmol/L. From the results for 75 of these subjects they extrapolated the effect of vitamin supplementation to a large group of unsupplemented men. This extrapolated reference interval of 4.9–11.7 μmol/L (mean ± 2SD) corresponds to the nonparametrically calculated central 0.95 reference interval for the vitamin-supplemented men in the present study group (5.0–12.1 μmol/L). The agreement between these two intervals supports the statement of Ubbink et al. that a higher dose than they used was unlikely to shift the interval further toward lower concentrations. However, the fact that subjects taking regular vitamin supplementation had lower concentrations of homocysteine, even after the high-dose supplementation we added, remains unexplained.

Again, since we completed our study, a Norwegian population screening study reporting concentrations of homocysteine in plasma from >16 000 subjects has been published [24]. The data accord well with the findings of the present study except that their concentrations of homocysteine generally were 1–3 μmol/L higher, a difference probably related to differences in selection of subjects and sample collection. In their subjects, plasma homocysteine was higher in men than in women and increased with age in both sexes. This adds further support to our own findings. Because the Norwegian study demonstrated a significant increase with cigarette smoking, possibly even narrower reference intervals could be achieved by distinguishing between smokers and nonsmokers.

The distribution curve for the homocysteine concentrations of the entire study group in our investigation became more symmetrical after supplementation with folic acid (Fig. 2) but, in contrast to the prediction of Ubbink et al., a positive skewness was still present. The data shown in Fig. 3 suggested a heterogeneity in the population, and the subsequent analysis of variance combined with Duncan's test identified the four significantly different subgroups of different age and gender specified in Table 3.

The cluster analysis identified both in our study group as a whole and in each subgroup a cluster of subjects who responded strongly to folic acid, indicating that, in general agreement with the findings of Ubbink et al. [13], one-quarter of our participants was actually deficient in folic acid.

The group taking regular vitamin supplementation all used vitamin supplements of the recommended daily dose of 0.1 mg of folic acid, which in average reduced the homocysteine concentration ~1 μmol/L. In contrast, our short high-dose supplementation reduced the concentrations twice as much, indicating that the "recommended" dose may be inadequate.

In our opinion, concentrations of homocysteine in plasma above the reference interval for weak responders before supplementation with folic acid should be regarded as higher than optimal. Accordingly, the upper reference limits of age- and gender-specific central 0.95 intervals should be 8.1 μmol/L for subjects <30 years, 7.9 μmol/L for women ages 30–59 years, 11.2 μmol/L for men ages 30–59 years, and 11.9 μmol/L for subjects >60 years. These upper limits are considerably lower than the current accepted limit of ~15–16 μmol/L [2, 5], but one cannot argue that these limits are artificially low, because they are based on findings in individuals before the folic acid supplementation. In general, the task is to identify subjects who will benefit from additional supplementation with folic acid. Even if it is not yet clear whether there is a threshold below which the homocysteine concentration does not pose a risk for cardiovascular disease [13], implementation of the above upper reference limits may well prevent large numbers of cases of cardiovascular disease, given that the risk for cardiovascular disease starts to increase with homocysteine concentrations at 10.5–11.7 μmol/L [1, 25–27].

Alternatively, the reference intervals obtained for each group after vitamin supplementation could be used. The upper reference limits, however, will be almost identical. The results of our study do not support the hypothesis that "stable, low reference intervals" exist for plasma homocysteine, in contrast to plasma methylnalonic acid; thus, such upper limits obtained after
supplementation with folic acid may be questionable because they are functions of the amounts of daily vitamin supplementation.

In conclusion, until prospective randomized clinical trials have shown that further lowering the proposed upper reference limits will further reduce the risk for cardiovascular disease, we recommend the use of the age- and gender-specific reference intervals we listed above for homocysteine in plasma, which are based on the concentrations observed in weak responders before supplementation with folic acid.

This work was in part supported by a grant from The Institute of Experimental Clinical Research, Århus University, Denmark. We also thank Dumex Ltd., Denmark, and Nycomed DAK, Denmark, for the generous donations of the Betolvex tablets and folic acid tablets, respectively, used in the study.

References


