We established the cutoff value for plasma folic acid, using plasma homocyst(e)ine as the functional marker. To do this, we investigated the relationship of the plasma folic acid of 103 apparently healthy adults with their fasting plasma homocyst(e)ine and with their plasma homocyst(e)ine 6 h after oral methionine challenge (100 mg/kg). We also studied the relationship of their plasma folic acid with the decline of fasting plasma homocyst(e)ine after 7 days of folic acid supplementation (5 mg/day). The three approaches suggested a cutoff value of 10 nmol/L. The chances of individuals to significantly ($P < 0.05$) lower their plasma homocyst(e)ine after folic acid supplementation proved significantly higher at plasma folic acid concentrations $\leq 10$ nmol/L, as compared with folic acid concentrations above this value (odds ratio, 5.02; 95% confidence interval, 1.87–13.73). We suggest adopting a 10 nmol/L plasma folic acid cutoff value on functional grounds.

The diagnosis of subclinical vitamin deficiencies is important because it is becoming increasingly clear that such conditions may contribute to development of disease in the long run (1–3). The use of reference values is not appropriate for the diagnosis of subclinical vitamin deficiencies. Reference values are usually determined by calculation of the 95% confidence interval of data from apparently healthy subjects (4). Individuals with subclinical vitamin deficiencies cannot be excluded from the reference population on clinical grounds because they do not exhibit symptoms that can be directly related to their suboptimal vitamin status. A solution to the above vicious cycle is to define suboptimal vitamin status on biochemical grounds, i.e., by the measurement of a vitamin-dependent function (functional marker). Functional markers may in this way serve as criteria to define adequate vitamin status or, alternatively, to establish the cutoff value of static markers such as circulating vitamin concentrations (5).

Folic acid plays a role in the transfer of methyl groups and is, therefore, important in DNA and RNA synthesis and amino acid metabolism. Homocyst(e)ine derives from the essential amino acid methionine. It may be retroconverted to methionine through vitamin B$_{12}$ and folic acid-dependent pathways, or degraded via a vitamin B$_{6}$-dependent route (6). Folic acid status appears to be the main determinant of the plasma homocyst(e)ine concentration (7, 8); in addition, many authors have shown that plasma homocyst(e)ine can be lowered substantially by folic acid supplementation of both healthy subjects (9, 10) and patients with hyperhomocyst(e)inemia (11–13). Hyperhomocyst(e)inemia is a laboratory diagnosis that is based on increased fasting plasma homocyst(e)ine or increased plasma homocyst(e)ine 6 h after the ingestion of 100 mg methionine/kg body weight (the oral methionine tolerance test; OMTT).4

Currently, on the basis of epidemiological (1, 14) and case-control studies (15–17), hyperhomocyst(e)inemia is considered to be an independent risk factor for atherosclerotic disease, and this risk is likely to increase with increasing plasma homocyst(e)ine without a clear threshold (15, 16). In addition, recent data from a prospective study among patients with angiographically determined atherosclerosis showed that plasma homocyst(e)ine concentrations are inversely related with survival (18).

It is clear that hyperhomocyst(e)inemia is an independent atherosclerosis risk factor and that homocyst(e)ine can be lowered substantially by folic acid supplementation. Adequate folic acid status may be defined as the magnitude of the folic acid body pool size that causes the…
lowest possible homocyst(e)ine concentration. The plasma homocyst(e)ine concentration serves in this way as a functional marker to establish the cutoff value of the plasma folic acid concentration (5, 19). It should be noted, however, that plasma homocyst(e)ine is not a specific indicator of folic acid status, because circulating homocyst(e)ine concentrations are also determined by vitamin B₆ and vitamin B₁₂ status (5, 19), although to a lesser extent (10).

We estimated the cutoff value for the plasma folic acid concentration in apparently healthy adults. To do this, we established at baseline the relationship of folic acid with fasting plasma homocyst(e)ine and plasma homocyst(e)ine at 6 h during OMTT, and also its relationship with the decline of fasting homocyst(e)ine after 7 days of supplementation with a pharmacological dose of folic acid. The finally selected plasma folic acid cutoff value was defined as the concentration at or below which individuals have a substantially higher chance to lower their plasma homocyst(e)ine after folic acid supplementation, compared with the chance to exhibit such a decrease when the plasma folic acid concentration exceeds this value.

Subjects and Methods

STUDY DESIGN AND STUDY GROUP

Apparently healthy subjects 20–80 years of age were eligible to participate in this study. The exclusion criteria were as follows: (suspect) premature atherosclerosis, premature atherosclerosis in first degree family members, stroke, thrombosis, renal disease (serum creatinine >120 μmol/L), liver disease [defined as alkaline phosphatase (AP), glutamic-oxaloacetic transaminase (GOT; aspartate 2-oxoglutarate aminotransferase), glutamic-pyruvic transaminase (GPT; alanine 2-oxoglutarate aminotransferase) and/or γ-glutamyltransferase (γ-GT) above the respective reference intervals], pregnancy, psoriasis, seizures, and use of methotrexate and phenytoin. Participants were recruited by advertisement from hospital employees and students of the Department of Pharmacy of the Groningen University. Participants 65 years of age and older were recruited at local swimming pool facilities and social activity centers. In addition, we asked participating students to motivate their parents and grandparents to take part in this study. Written informed consent was obtained from all participants. The study protocol was approved by the medical ethical committee of the Groningen University Hospital and was in agreement with local ethical standards and the Helsinki declaration of 1975, as revised in 1989.

Folic acid (5 mg/tablet; folic acid purity > 96%) and vitamin B₆ (20 mg/tablet; pyridoxine-HCl purity > 99%) were produced by the Hospital pharmacy; L-methionine (powder; purity > 99%) was obtained from Bufa. Vitamin B₆ (dose 1 mg per kg of body weight per day, rounded to the closest number of 20-mg tablets) was taken after supper from day 0 up to and including day 13. Blood and EDTA-anticoagulated blood were collected in the fasting state (t₀) before supplementation (day 0), after vitamin B₆ supplementation (day 7), and after 7 days of folic acid supplementation (day 14). OMTTs were carried out on days 0 and 14. A dose of 100 mg methionine/kg body weight (methionine rounded to grams), dissolved in orange juice, was taken after blood sampling in the fasting state. The succeeding breakfast included protein-poor bread, low fat margarine, jam, and sweet strands. EDTA-anticoagulated blood samples were collected 6 h after methionine intake (t₆). All participants were instructed to consume a protein-poor diet from at least 24 h before blood sampling on days 0, 7, and 14, and during the OMTT. The consumption of meat, fish, eggs, dairy products, beans, and alcohol (beer because of protein) was to be avoided.

The final study group consisted of 45 men (43 ± 15 years; range, 20–73) and 58 women (44 ± 16 years; range, 20–75). Two participants were not able to attend on day 7. Two other participants reported late for blood sampling at 6 h after methionine intake on day 14.

SAMPLE PROCESSING AND ANALYSES

EDTA-anticoagulated blood for the analysis of homocyst(e)ine was immediately centrifuged (1700g for 10 min at 4 °C) for the preparation of plasma. Plasma was stored at −20 °C until analysis within 4 weeks. Analysis of plasma homocyst(e)ine was done with the high-performance liquid chromatographic method of Araki and Sako (20). Intra- and interassay CVs were 0.85% (29.2 μmol/L) and 4.3% (28.9 μmol/L), respectively. EDTA blood for the analysis of vitamin B₆, vitamin B₁₂, and folic acid was stored in the refrigerator at 4 °C for 30 min at most. Part of the EDTA blood was centrifuged (1700g for 10 min at 4 °C) for the preparation of plasma and was stored at −20 °C for the analyses of vitamin B₁₂ and folic acid within 4 weeks. Analyses were done with radio-assays that are based on competitive protein binding (Becton Dickinson). The other part of the EDTA blood was stored in the refrigerator at 4 °C for the analysis of vitamin B₆ within 1 week. Analyses were done with a high-performance liquid chromatographic method based on those described by Schrijver et al. (21) and Ubbink et al. (22). Blood was collected on day 0 for the analysis of standard clinical chemical indices. It was allowed to coagulate for 10 min at room temperature for the subsequent preparation of serum by centrifugation at 1700g and 4 °C during 10 min. Creatinine, AP, GOT, GPT, and γ-GT were immediately analyzed with a MEGA automated analyzer (Merck).

DATA EVALUATION AND STATISTICS

For the establishment of hyperhomocyst(e)inemia at baseline, we used the local cutoff values for plasma homocyst(e)ine concentrations, i.e., 15 μmol/L (premenopausal women), 19 μmol/L (postmenopausal women), and 18
μmol/L (men) for fasting plasma homocyst(e)ine, and 50 μmol/L for plasma homocyst(e)ine at 6 h during OMTT. For the calculation of vitamin status, we used our local reference ranges, i.e., whole-blood vitamin B₉ (55–110 nmol/L), plasma vitamin B₁₂ (170–700 pmol/L), and plasma folic acid (4–30 nmol/L). Local reference values for the routine clinical chemical tests were as follows: serum GOT (0–40 U/L), GPT (0–120 U/L), γ-GT (0–65 U/L), and creatinine (62–106 μmol/L).

Time-dependent changes of fasting homocyst(e)ine and vitamins (day 7 vs day 0; day 14 vs day 7; day 14 vs day 0) and plasma homocyst(e)ine at 6 h during OMTT (day 14 vs day 0) were analyzed with Student paired t-tests with Bonferroni adjustment for type 1 errors at P < 0.05. A longitudinal change of an individual’s fasting plasma homocyst(e)ine concentration was considered significant (P < 0.05) when the proportional difference (in percentage) amounted to >2.8 times the combined analytical and intraindividual biological CVs (2.8 × CVanal,biol; 23). Regarding the high-performance liquid chromatographic method that was used, this combined CV has been estimated at 8.25% (24). The interassay CV of this method has been estimated at 4.3%. Relationships between plasma folic acid and plasma homocyst(e)ine and between plasma folic acid and changes in plasma homocyst(e)ine were analyzed with the Spearman rank correlation analysis at P < 0.05. A longitudinal change of an individual’s fasting plasma homocyst(e)ine concentration was considered significant (P < 0.05) when the proportional difference (in percentage) amounted to >2.8 times the combined analytical and intraindividual biological CVs (2.8 × CVanal,biol; 23). Regarding the high-performance liquid chromatographic method that was used, this combined CV has been estimated at 8.25% (24). The interassay CV of this method has been estimated at 4.3%. Relationships between plasma folic acid and plasma homocyst(e)ine and between plasma folic acid and changes in plasma homocyst(e)ine were analyzed with the Spearman rank correlation analysis at P < 0.05 (25). For computerized exponential curve fitting, we divided the folic acid range into 10 parts that each contained the data of 10 or 11 subjects. The corresponding homocyst(e)ine data were calculated. The means of the deciles were fitted to monoeponential curves, if possible, and their asymptotic values were recorded.

The plasma folic acid concentration cutoff value was defined as the concentration at or below which individuals have a significantly higher chance to lower their plasma homocyst(e)ine after folic acid supplementation, compared with the chance to exhibit such a decrease when the plasma folic acid concentration exceeds this value. To establish this value, we calculated, at various plasma folic acid cutoff values, the odds ratios and their 95% confidence intervals. Plasma folic acid concentrations at baseline were used in these calculations. Whether an individual exhibited a homocyst(e)ine decline was derived from the 2.8 × CVanal,biol criterion (see above).

Results

Baseline values

Serum GOT, GPT, AP, γ-GT, and creatinine concentrations of all participants were within the respective reference ranges (data not shown). Table 1 shows the baseline concentrations (i.e., data of day 0) for fasting plasma homocyst(e)ine (indicated as t0), plasma homocyst(e)ine at 6 h during OMTT (t6), whole-blood vitamin B₉, plasma vitamin B₁₂, and plasma folic acid. Six participants had fasting homocyst(e)ine concentrations above the respective cutoff values for fasting homocyst(e)ine, and 10 had plasma homocyst(e)ine concentrations above the cutoff value for homocyst(e)ine at 6 h during OMTT. Taken together, we found a total number of 12 subjects (11.6%) with hyperhomocyst(e)inemia at baseline. The number of participants with circulating vitamin concentrations below the lower limit of the respective reference ranges were eight (vitamin B₉), and zero (folic acid). Low values of plasma vitamin B₁₂ were found in 19 subjects.

EFFECT OF VITAMIN SUPPLEMENTATION ON HOMOCYST(E)INE

Vitamin B₉ and folic acid tablets were taken during a period of 6–8 days. We considered the participants compliant, because they returned empty pillboxes at the study end and because each of them showed increases of the circulating vitamin concentrations at the appropriate sampling times.

Apart from baseline values, Table 1 also shows plasma homocyst(e)ine and circulating vitamin concentrations on day 7 (i.e., after vitamin B₉) and on day 14 (i.e., after folic acid). As compared with day 0, whole-blood vitamin B₉ was higher on both day 7 and day 14 (P < 0.0001). Plasma folic acid decreased slightly from day 0 to day 7 (P < 0.0001), but increased from day 7 to day 14 (P < 0.0001). There were no significant changes in plasma vitamin B₁₂ concentrations. Fasting plasma homocyst(e)ine concentrations (indicated as t0) did not change

Table 1. Plasma homocysteine, folic acid, and vitamin B₁₂, and whole-blood vitamin B₉ on days 0, 7, and 14.a,b

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Day 0 (n = 103)</th>
<th>Day 7 (n = 101)</th>
<th>Day 14 (n = 103)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine t₀ (μmol/L)</td>
<td>10.5 (6.1–43.3)</td>
<td>10.3 (6.2–39.6)</td>
<td>8.3 (5.0–24.1)</td>
</tr>
<tr>
<td>Homocysteine t₆ (μmol/L)</td>
<td>33.0 (12.9–79.4)</td>
<td>744 (315–2392)</td>
<td>154 (94–629)</td>
</tr>
<tr>
<td>Vitamin B₉ (nmol/L)</td>
<td>244 (111–487)</td>
<td>237 (105–521)</td>
<td>233 (96–529)</td>
</tr>
<tr>
<td>Folic acid (nmol/L)</td>
<td>11.9 (5.3–67.0)</td>
<td>9.7 (4.0–67.0)</td>
<td>70 (14.2–304.0)</td>
</tr>
</tbody>
</table>

a Apparently healthy subjects (ages, 20–75 years) took vitamin B₉ (1 mg · kg⁻¹ · day⁻¹ for 7 days), and subsequently folic acid (5 mg/day for 7 days). Blood samples were taken in the fasting state on day 0 (before supplementation), day 7 (after vitamin B₉), and day 14 (after folic acid), and at 6 h during an OMTT (300 mg methionine/kg) on days 0 and 14 (indicated as t6).
b Data represent median (range).
c Significantly different from day 0 (P < 0.0001).
d Significantly different from day 7 (P < 0.0001).
after vitamin B₆ administration (day 7 vs day 0), but decreased after folic acid administration (day 14 vs day 7; \( P < 0.0001 \)). After vitamin B₆ supplementation, one participant showed a decrease of fasting plasma homocyst(e)ine concentration beyond 2.8 \( \times \) CV_

al.,biol. Using the same criterion, we found that the fasting plasma homocyst(e)ine concentrations of 40 subjects (40%) decreased after folic acid supplementation. Plasma homocyst(e)ine concentrations at 6 h during OMTT (t6) were significantly lower on day 14, as compared with day 0 (\( P < 0.001 \)).

**RELATIONSHIP BETWEEN FOLIC ACID AND HOMOCYST(E)INE AT BASELINE**

The plasma folic acid concentration range on day 0 was divided into 10 parts that each contained the data of 10 or 11 subjects. For each of the ensuing deciles, we calculated the means ± SE for the plasma folic acid and the corresponding homocyst(e)ine concentrations at t0 and at 6 h during OMTT. Fig. 1 shows the relationship between the calculated means of the plasma folic acid concentration and the fasting plasma homocyst(e)ine (top), and between plasma folic acid and plasma homocyst(e)ine at 6 h during oral methionine tolerance test (bottom). The 103 plasma folic acid data points were divided into 10 parts that each contained the data of 10 or 11 subjects. All data were collected at baseline (i.e., d0) and indicate the mean ± SE. Exponential curve fitting revealed the following equations: fasting homocyst(e)ine: \( y = 9.97 + 6.91(7.27 - x)/1.68 \) and homocyst(e)ine at 6 h during OMTT: \( y = 32.36 + 14.64(6.89 - x)/1.83 \) in which \( x \) is plasma folic acid (in nmol/L) and \( y \) is plasma homocyst(e)ine (in \( \mu \)mol/L).

**RELATIONSHIP BETWEEN FOLIC ACID AND HOMOCYST(E)INE DECREASE AFTER FOLIC ACID SUPPLEMENTATION**

We calculated the absolute (in \( \mu \)mol/L) decreases of the fasting plasma homocyst(e)ine concentrations from day 7 to day 14. These data were correlated with the folic acid concentrations at baseline, as divided in deciles. The relationship between the calculated means ± SE of the folic acid concentrations at baseline and the absolute decreases of the fasting homocyst(e)ine concentrations from day 7 to day 14 are shown in Fig. 2. The resulting relationship proved significant at \( P < 0.0001 \). The data in Fig. 2 were not found to comply with exponential curve fitting, but showed that plasma homocyst(e)ine changes after folic acid supplementation reached a stable value of about -1.5 \( \mu \)mol/L from a baseline plasma folic acid concentration of \( \sim 10 \) nmol/L.

![Fig. 1. Relationship between plasma folic acid and fasting plasma homocyst(e)ine (top), and between plasma folic acid and plasma homocyst(e)ine at 6 h during oral methionine tolerance test (bottom).](https://academic.oup.com/clinchem/article-abstract/44/7/1545/5642779)

![Fig. 2. Relationship between plasma folic acid at baseline and the absolute change of fasting plasma homocyst(e)ine after folic acid supplementation.](https://academic.oup.com/clinchem/article-abstract/44/7/1545/5642779)
PLASMA FOLIC ACID AS PREDICTOR OF A SIGNIFICANT HOMOCYST(E)INE DECREASE AFTER FOLIC ACID SUPPLEMENTATION

The 10 nmol/L plasma folic acid cutoff value, as suggested from the above data, was further evaluated on its capacity to predict a significant decrease of plasma homocyst(e)ine after folic acid supplementation. The investigated cutoff values were 9, 10, and 11 nmol/L. The corresponding calculated odds ratios were 2.84 (95% confidence interval, 0.93–8.94) at a cutoff value of 9 nmol/L, 5.02 (1.87–13.73) at 10 nmol/L, and 6.04 (2.32–16.03) at 11 nmol/L. In other words, the chance of a significant individual homocyst(e)ine decrease was significantly higher at folic acid concentrations ≥ 10 nmol/L, compared with folic acid concentrations >10 nmol/L.

Discussion

We established the cutoff value for the plasma folic acid concentration by the use of plasma homocyst(e)ine as a functional marker. To do this, we investigated the relationship of the plasma folic acid of 103 apparently healthy adults with their fasting plasma homocyst(e)ine (Fig. 1, top), and with their plasma homocyst(e)ine at 6 h during OMTT (Fig. 1, bottom). We also studied the relationship of their plasma folic acid with the decline of their fasting plasma homocyst(e)ine after 7 days of folic acid supplementation (Fig. 2). The subjects received vitamin B6 for 7 days before folic acid supplementation to eliminate the possible confounding influence of low vitamin B6 status on plasma homocyst(e)ine concentrations. To our surprise, we found 19 subjects to have low vitamin B12, for which we have no explanation. Low vitamin B12 in these subjects cannot, however, explain a lack of response to folic acid supplementation, because 12 of them (63%) exhibited responses to supplementation.

The results of the three approaches suggested a cutoff value of 10 nmol/L, the concentration from which the subjects reached their lowest fasting plasma homocyst(e)ine, their lowest plasma homocyst(e)ine 6 h during OMTT, and the smallest decline in fasting plasma homocyst(e)ine after folic acid supplementation. This value was subsequently confirmed by defining the plasma folic acid cutoff value as the concentration at or below which individuals have a significantly higher chance to lower their plasma homocyst(e)ine after folic acid supplementation, compared with the chance to exhibit such a decrease when the plasma folic acid concentration exceeds this value.

A decrease of plasma homocyst(e)ine after folic acid supplementation has previously been established for populations in many Western countries. The investigated subjects included both patients with hyperhomocyst(e)inemia (11–13) and apparently healthy subjects (9, 10). It seems clear, therefore, that the Western diet does not provide sufficient folic acid to reach the lowest possible plasma homocyst(e)ine concentrations. Because the possibly resulting subclinical deficiency cannot be derived from the study of the plasma folic acid concentrations of the general apparently healthy population, it seems imperative to estimate its cutoff value using functional markers. There have been previous attempts to define folic acid cutoff values on such grounds (19). Pietrzik et al. (26) suggested a cutoff value of 10 nmol/L, based on neutrophilic granulocyte hypersegmentation, whereas Lewis et al. (27) suggested a 15 nmol/L cutoff value, derived from the relationship between plasma folic acid and plasma homocyst(e)ine in a combined group of 108 healthy males and 101 males with angiographically demonstrated coronary artery disease.

The presently suggested plasma folic acid cutoff value contrasts clearly with our locally used reference range of 4–30 nmol/L. This reference range reflects the 95% confidence interval of apparently healthy subjects, and it was consequently not surprising that the newly selected population had values within this range. We now find that 32 subjects (31%) have values below the new cutoff value of 10 nmol/L. From those who had values <10 nmol/L, 66% significantly decreased their fasting plasma homocyst(e)ine upon folic acid supplementation, whereas this was the case for 28% with values >10 nmol/L. Values >10 nmol/L, therefore, do not necessarily imply that the lowest homocyst(e)ine concentrations are always reached.

The acceptance of the new cutoff value is much dependent on the existence of a causal relation of low plasma folic acid and high plasma homocyst(e)ine with increased atherosclerosis risk. The present evidence is, however, based on epidemiological (1, 14) and case-control (15–17) studies, and not on the results of randomized prospective intervention trials showing that lowering homocyst(e)ine by folic acid supplementation reduces atherosclerosis risk. On the other hand, augmentation of folic acid status either by increased consumption from foodstuffs such as green leafy vegetables, beans, and fruit, or alternatively, from vitamin supplements or vitamin-fortified food, does not seem harmful either. In addition, in many countries it is advised to augment folic acid status for the reduction of neural tube defects (28, 29).

There is compelling, although circumstantial, evidence that low folic acid and high homocyst(e)ine are associated with atherosclerosis risk. We therefore advice to adopt plasma folic acid cutoff values that are based on functional grounds, not on the 95% confidence interval of the folic acid concentration of an apparently healthy population. The relationship between plasma folic acid and plasma homocyst(e)ine suggest that the cutoff value on functional grounds is 10 nmol/L. This cutoff value became confirmed by the capacity of this value to predict on a statistically significant basis a decrease of plasma homocyst(e)ine after folic acid supplementation.

We thank P.J.F. de Vries (Friesland Dairy Foods, Leeuwarden) for advice, Ingrid Martini, Leid de Ruyter-Buitenhuis, Elly de Hoog, Marian Snieders, Pim Modder-
man, and Hermie Kingma for technical assistance and Marcel Volmer for the statistical analyses.

References