Folic acid improves vascular reactivity in humans: a meta-analysis of randomized controlled trials¹,²

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ABSTRACT
Background: The effect of folic acid on endothelial function, a prognostic factor for cardiovascular diseases, is not well established. We calculated this effect in a meta-analysis of randomized, double-blind, placebo-controlled trials in humans.

Objective: The objective of the study was to quantify the effect of folic acid on endothelial function, as measured with the use of flow-mediated dilatation (FMD).

Design: We conducted a meta-analysis of randomized, double-blind, placebo-controlled folic acid trials evaluating endothelial function. Trials were identified through MEDLINE (1966–15 Sept 2005), by hand-searching of references, and by contact with investigators for unpublished results. Two of us (AdB and RD) independently extracted trial data. A pooled estimate was calculated by using random-effects meta-analysis. Previously defined stratified analyses were conducted to explore the influence of study characteristics.

Results: Of 163 identified studies, 14 met inclusion criteria and provided data on 732 persons. Evidence for publication bias was not obvious. In the overall pooled estimate, folic acid improved FMD by 1.08 (95% CI: 0.57, 1.59; P = 0.0005) percentage points over placebo. Of the study characteristics, only folic acid dose significantly influenced the outcome. Post hoc analysis, which should be interpreted with caution, seemed to indicate a dose-response effect: the change in FMD was −0.07 (95% CI: −0.37, 0.22) percentage points at doses between 400 and 800 μg/d, 1.37 (95% CI: 1.12, 1.54) percentage points at doses of 5000 μg/d, and 2.04 (95% CI: 1.43, 2.65) percentage points at doses of 10 000 μg/d.

Conclusion: This study indicates that high doses of folic acid improve endothelial function, which could potentially reduce the risk of cardiovascular disease. Am J Clin Nutr 2007;86:610–7.

KEY WORDS Folic acid, homocysteine, flow-mediated dilatation, FMD, endothelial function, randomized trial, meta-analysis

INTRODUCTION
A large amount of epidemiologic evidence links elevated homocysteine concentrations to an increased risk of cardiovascular disease (CVD) (1, 2). This linkage has initiated the execution of secondary prevention trials testing whether homocysteine-lowering therapy reduces the risk of recurrent CVD events (3). Because the B vitamin folic acid and, to a lesser extent, vitamin B-6 and vitamin B-12 lower homocysteine concentrations (4), they are used in such trials. Many of these trials are ongoing, and data from 4 of them have been published, showing disappointing results (5–8). A combined analysis of these 4 trials lacked the power to detect significant differences. However, the CIs around the summary risk estimates of these 4 trials were compatible with a 10% lower risk of ischemic heart disease and a 20% lower risk of stroke associated with a 25% lower homocysteine concentration (9). Furthermore, we should also bear in mind that these secondary prevention trials typically look at risk reduction after short-term treatment in high-risk subjects, and thus these results should not be generalized to the overall population. Indeed, a beneficial effect of folic acid fortification on stroke mortality in the United States and Canada was recently reported (10).

Although folic acid may not be able to reverse advanced atherosclerosis in CVD patients, it may affect the early stages of the CVD process, such as endothelial dysfunction (11). This possibility has not been investigated in a systematic way. Endothelial function can be measured by the degree of flow-mediated dilatation (FMD) (12). FMD represents the ability of the brachial artery to dilate in response to ischemia-induced hyperemia in the forearm, and as such it reflects the bioavailability of the endogenous vasodilator nitric oxide (NO). In the present study, we systematically evaluated the effect of folic acid (with or without vitamin B-6, vitamin B-12, or both) on FMD in humans by performing a meta-analysis of randomized, placebo-controlled clinical trials.

STUDY METHODS
Strategy to search randomized trials
The Quality of Reporting of Meta-analyses standards (13) were followed during all phases of the design and implementation of the present analysis. Included studies were randomized clinical trials that measured vascular reactivity by using the percentage of FMD (%FMD) after folic acid supplementation without a vascular challenge (such as a methionine or fat load). Trials were identified by searching the MEDLINE database from 1966 to 15 September 2005 with the use of the search terms homocysteine OR folate OR folic acid OR vitamin B-12 OR cobalamin OR vitamin B-6 OR B-6 and flow mediated OR flow-mediated OR endothelium-dependent OR vasomotor OR vasoacti* OR “blood flow” OR brachial* OR intima OR vasodilat* OR dilat* OR circulation OR endothel* OR distensibility OR microcirculat* OR...
micro-circulation OR vascular resistance OR wave OR plethysmography OR “blood supply” OR claudication OR cold hands.

We limited the search to clinical trials conducted in humans aged >19 y and published in the English language. Furthermore, we hand-searched the reference lists of the articles obtained through MEDLINE and of conference abstract books for additional studies.

Trial review

A flow chart of the selection of the included trials is given in Figure 1. The MEDLINE search identified 158 studies. The abstracts of these studies were screened independently by 2 of us (AbD and RD). After the exclusion of 127 studies, the remaining 31 studies were assessed more specifically, including a review of their reference lists. This resulted in the identification of 4 additional studies for inclusion (14–17). Finally, 1 other trial was identified in a conference abstract book, for a total of 36 studies. Two of us (AbD and RD) independently evaluated these studies in detail—reviewing the complete publication, if available—which left 13 published trials (18–30) and 1 then-unpublished trial (M Olthof, personal communication, 22 November 2005; now: 31) that met the inclusion criteria. The inclusion and exclusion decisions were unanimous.

Data extraction

For each of the 14 studies meeting the inclusion criteria, data on study design, population, sample size and number of dropouts, intervention type, dose, and duration were independently extracted (by AbD and RD). Standard forms in EXCEL were used to calculate the net change in %FMD after folic acid supplementation compared with that after administration of placebo and also to calculate the SE of this change (see Statistical analysis). Study quality was also independently assessed (by AbD and RD) according to the criteria for quality assessment of randomized clinical trials developed by Delphi consensus (32). The 9 criteria were treatment allocation (randomized = 1 point), similarity of groups at baseline with respect to the most important prognostic indicators (1 point), eligibility criteria (specified = 1 point), blinding (treatment allocation blinded = 1 point, outcome assessment blinded = 1 point, care provided blinded = 1 point, and patients blinded = 1 point), measures of variability presented for FMD measurement (yes = 1 point), and intention-to-treat analysis (yes = 1 point; studies with no drop-outs received 1 point for this criterion). Thus, the highest score a trial could get was 9 points.

We assessed the validity of data extraction by comparing the independently abstracted results for concordance. Discussion and review of the original manuscript resolved any discrepancies between the results abstracted by the 2 independent investigators.

Exposure and outcome variables

Supplementation with folic acid, vitamin B-6, and vitamin B-12

As exposure variable, we used the dose of folic acid and vitamin B-6 or vitamin B-12 (or both) rather than the plasma concentration of these B vitamins or of homocysteine. We did this because the absence of a gold standard method makes it very difficult to compare plasma concentrations between studies, and thus there are great variations between different laboratories (33, 34). In addition, circulating concentrations of B vitamins are known to vary and therefore are not very stable markers for actual intake (35–37).

Flow-mediated dilatation

As outcome variable, we used the net change in FMD. In most of the articles, FMD was presented as the %FMD, which is calculated by the following equation:

$$\%\text{FMD} = \left(\frac{\text{maximum diameter} - \text{baseline diameter}}{\text{baseline diameter}}\right) \times 100\% \quad (I)$$

For consistency, we have used that formulation throughout as the unit of FMD measurement.
Statistical analysis

Net change in flow-mediated dilatation

Our primary outcome was the net change in FMD due to folic acid (with or without vitamin B-6 and vitamin B-12) treatment —ie, %FMD after folic acid − %FMD after placebo. For the 6 included crossover trials, %FMD at the end of the control period was subtracted from vascular reactivity at the end of the treatment period. For parallel design trials, the %FMD change from baseline to study end in the control group was subtracted from the %FMD change from baseline to study end in the treatment group.

Standard error of the net change in flow-mediated dilatation

For the crossover trials, the SE of the net change in %FMD was derived from the P value (18, 19), from the SD (20), or directly from the author of one of the studies (31). Two crossover trials did not report %FMD but reported the baseline diameter (in mm) and the increase (in μm) after occlusion (24, 26). For these studies we estimated the %FMD for the end of the control period and the end of the treatment period. Then we calculated the variance in these %FMD values by using the following equation:

\[
\text{Variance}_{\text{FMD}} = \left[ \text{variance}_{\text{increase}} + \text{FMD}^2 \times \text{variance}_{\text{baseline diameter}} - 2 \right] \times \text{FMD} \times \text{covariance}_{\text{baseline diameter increase}} / (\text{baseline diameter}^2) \]

(2)

An estimate of the covariance between the baseline diameter and the increase after occlusion for these calculations was obtained from unpublished FMD data (ie − 16, based on 316 data points; R Draijer, personal communication, 16 November 2005). The SE at the end of the control period \( (SE_c) \) and the SE at the end of the treatment period \( (SE_T) \) were calculated by taking the square root of the estimated variance in %FMD at the end of the control period and at the end of the treatment period. The pooled SE of the net difference was then calculated according to the equation of Follmann et al (38):

\[
\text{SE of the net change} = \sqrt{[(SE_{Tg}^2 + SE_{CG}^2 - 2r(SE_{Tg})(SE_{CG})]}
\]

(3)

where \( r \) is the within-subject correlation in %FMD between the treatment and control period, which is estimated to be 0.5.

For the 8 included parallel designs, the SE of the net change in %FMD was estimated with the P value for 2 studies (23, 30). Six of the parallel trials provided either the SE (27) or the SD (17, 21, 22, 29, 30) at baseline and at the end of the study for the control and treatment groups. Respective SEs were calculated by using the following equation (17, 21, 22, 29, 30):

\[
\text{SE} = \text{SD} / \sqrt{n}
\]

(4)

The SEs were used to calculate the SE for the change within the treatment group \( (SE_{Tg}) \) and within the control group \( (SE_{CG}) \), again with the method of Follmann et al (38). For example, for the treatment group, we used the following equation:

\[
\text{SE}_{Tg} = \sqrt{[(SE_{baseline}^2 + SE_{end of study}^2) - 2r(SE_{baseline})(SE_{end of study})]}
\]

(5)

where \( r \) was estimated to be 0.5. Finally, the SE of the net change was calculated by using the following equation:

\[
\text{SE of the net change} = \sqrt{[(SE_{Tg}^2 + SE_{CG}^2]}
\]

(6)

One parallel trial (28) did not report %FMD values, but reported the baseline diameter (in mm) and the increase (in μm) after occlusion. The %FMD values and respective SEs were calculated as described above for the crossover trials. We used these values and equations 5 and 6 to calculate the SE of the net change. Data for the calculation of the change in %FMD and the SE of this change were not missing from any trial.

Because this meta-analysis brings together studies that are diverse both clinically (eg, dose and type of subjects) and methodologically (eg, design and quality), heterogeneity in their results is expected. We calculated that the proportion of total variation between studies due to heterogeneity rather than to chance was 26% (39). Although a value > 50% is considered to represent substantial heterogeneity, we used a random-effects model (SAS PROC MIXED) with inverse-variance weighting for each trial (40). In this way, we addressed the question “What is the average effect of folic acid supplementation on %FMD?” rather than using a fixed-effects model that addresses the question “What is the best estimate of the effect of folic acid on %FMD?” In addition, a random-effects model results in a more conservative estimate of statistical significance than does a fixed-effects model.

We performed defined stratified meta-analyses to roughly explore the potential effect of study design (crossover or parallel), mean population age (≤ 55 or > 55 y old), general health (healthy or at greater risk of CVD), folic acid dose (400–800, 5000, or 10 000 μg/d), duration of treatment (≤ 8 or > 8 wk), additional vitamin B-6 or vitamin B-12 or both (no or yes), and study quality (≤ 7 or ≥ 7 Delphi criteria).

To assess publication bias, a funnel plot of the treatment effect versus 1/SE was visually inspected as described earlier (41). In addition, the symmetry of the funnel plot was judged by regressing the standard normal deviate (ie, effect of folic acid supplementation on %FMD/SE of this effect) against the estimate precision (1/SE) (standard normal deviate = α + β × precision). A symmetrical funnel plot should give a regression equation in which α is close to 0 and β indicates the size and direction of effect (41).

We used SAS software (version 8.2; SAS Institute, Cary, NC) for the statistical analyses. The effect of folic acid on %FMD was reported with the use of 95% CIs. Two-sided P values < 0.05 were considered significant.

RESULTS

Trial characteristics

Of the 14 trials meeting inclusion criteria, 1 trial (29) had 2 separate intervention groups, and thus 15 intervention groups are
presented in Table 1. Six of these trials had a crossover design, and 8 had a parallel design. In total, this meta-analysis was based on 732 persons treated with folic acid (with or without vitamin B-6 or vitamin B-12 or both) or placebo for a median of 8 wk, with a median study size of 34 participants. Most trials included middle-aged male subjects: the mean age in the individual studies ranged from 29.3 to 69.1 y (overall median: 55.8 y), and the median percentage of males was 86%. Seven intervention groups were composed of populations with CVD or high cholesterol concentrations, 5 groups were generally healthy, and 3 were healthy but with slightly elevated plasma homocysteine concentrations. The median folic acid dose was 5000 µg/d (range: 400–10 000 µg/d). In 5 studies, additional vitamin B-6 and vitamin B-12 were supplied. All studies that supplied information on the plasma homocysteine concentration showed a drop in this concentration after intervention. The lowest Delphi score was 7 points; 5 trials had that score. Three trials fulfilled all 9 Delphi criteria.

**Effect of folic acid on flow-mediated dilatation**

The individual trial results and the pooled estimate are shown in Figure 2. In the overall pooled estimate, compared with placebo, folic acid improved FMD with 1.59%FMD (95% CI: 0.57, 1.60; P = 0.0005). There was no effect of design, mean age of the study population, study duration, or the addition of vitamin B-6 or vitamin B-12 on the estimated change in %FMD due to folic acid (Table 2). However, there was a tendency that subjects at greater risk of CVD had a larger improvement in %FMD, and studies that met a higher number of Delphi criteria had a smaller improvement in %FMD. The dose of folic acid was clearly important. The trials using a lower dose—ie, <5000 µg—did not show a beneficial effect of folic acid on FMD (−0.07%FMD; 95% CI: −0.37, 0.22%FMD), whereas the studies with a dose ≥5000 µg/d did (1.42%FMD; 95% CI: 1.25, 1.58%FMD). In a post hoc analysis, a dose-response effect became apparent when we created 3 strata: a low-dose stratum with folic acid doses ≤500 µg/d, a medium-dose stratum with doses ≥500 µg/d but <5000 µg/d, and a high-dose stratum with doses ≥5000 µg/d (9 intervention groups), and a high-dose stratum with doses using a folic acid dose of 10 000 µg/d (2 intervention groups). At folic acid intakes ≤800 µg/d, FMD did not change [−0.07%FMD (95% CI: −0.37, 0.22%FMD); at 5000 µg/d, it improved [1.37%FMD (1.12, 1.54%FMD)]; and, at 10 000 µg/d, it improved further [2.04%FMD (1.43, 2.65%FMD)].

Evaluation of the funnel plot showed little evidence for publication bias (Figure 3). In addition, the funnel plot was quite symmetric, as α = −0.05 and β = 1.09 (42).

**DISCUSSION**

This meta-analysis of randomized, double-blind, placebo-controlled clinical trials showed that supplementation with high doses of folic acid for ≥4 wk improves FMD assessment of endothelial function. The result of a meta-analysis depends on the studies included. In the present review, we used a broad specified search and also contacted investigators for unpublished studies.
results to prevent any possible publication bias. In addition, we avoided the inclusion of studies on the basis of their outcome by defining inclusion and exclusion criteria. The use of these criteria led to 14 eligible trials. The number of studies is not large, but our methods ensured that the trials had a high internal validity and were reasonably comparable. To account for any heterogeneity, we used a random-effects model, and we assessed characteristics, as described in Table 1. To ensure comparability of the trials, we had to exclude some trials (42–51) even though they were randomized, double-blind, placebo-controlled trials; were not conducted in a specific patient population; and assessed the effect of folic acid on endothelial function. All but one (50) of these studies support the findings of our meta-analysis.

Although several review articles have indicated that folic acid could beneficially affect endothelial function as measured with FMD (11, 52), a comprehensive meta-analysis that takes into account both within- and between-study variability was lacking. Thus, our analysis is the first that provides a quantitative estimate of the improvement in FMD after folic acid supplementation, in which the overall effect showed a favorable change of 1.08% FMD. A (statistically nonsignificant) larger improvement was seen in subjects at a higher risk of CVD (1.34% FMD) than in healthy subjects (0.84% FMD). Because the average FMD value in populations at greater risk of CVD was ≈3.6% FMD and that in healthy populations was ≈5.6% FMD, our findings indicate potential significant improvements of ≈37% in subjects at CVD risk and ≈9% in healthy populations.

An important question is the extent to which FMD can be used as a predictor of long-term CVD risk? It is clear that FMD is a predictor of this risk in a selected group of patients, such as those with coronary heart disease, heart failure, and hypertension (53–57). However, the extent to which FMD can be used to predict the risk of CVD in the general population is less clear. Nevertheless, preliminary epidemiologic data showed a modest, positive correlation between FMD and the Framingham Study risk score in a general population sample of 1016 elderly persons (58).

The FMD value indicates the bioavailability of endothelium-derived NO, which is essential to cardiovascular health (59). High homocysteine concentrations are postulated to reduce NO availability in several ways. Indeed, homocysteine may induce the formation of free radicals, as shown by in vitro studies (60, 61). A certain proportion of these free radicals can be neutralized by NO, but other free radicals may directly damage endothelial cells (60), and both processes would lead to a smaller amount of available NO. Oxidative stress may also increase as homocysteine inhibits glutathione peroxidase (61, 62), a potent cellular defense mechanism against free radicals. Homocysteine can also reduce NO availability by forming S-nitrosohomocysteine complexes (63, 64). Finally, homocysteine may induce the formation of asymmetric dymethyl arginine, which is a competitive inhibitor of enzymatic nitric oxide synthase (eNOS) (65, 66). Therefore, we postulate that folic acid administration beneficially affects FMD by lowering the plasma homocysteine concentration.

It is conceivable that folic acid could also improve the FMD value independently of homocysteine lowering. This possibility is supported by 3 mechanisms that would result in a greater availability of NO: 1) folic acid may act as an antioxidant (48); 2) it may regenerate the cofactor for eNOS (49, 54); and 3) it may...
directly stimulate eNOS (67). Two indications from the present study support an independent effect of folic acid. First, post hoc analysis hinted toward a favorable effect with higher doses of folic acid (≥5000 μg/d). A folic acid dose of 400 to 800 μg/d is typically required to achieve an almost-maximal homocysteine-lowering effect over an 8-wk period (4), but the present analysis showed that such large homocysteine reductions apparently do not result in enhanced %FMD (24, 29, 31). In contrast, this observation is based on only 3 studies involving a small number (ie, 185) of subjects. In addition, one of these studies may have been performed against the background of folic acid fortification (29), although the amount of folic acid provided by the supplements will have been much higher than that provided by the fortification program. Finally, all 3 studies involved healthy young (mean age: ≈31 y) persons, who probably have the least to gain from supplementation with folic acid. Thus, before we discard a beneficial effect of low doses of folic acid on FMD, we would like to see large studies in healthy older persons or subjects with (reversible) vascular dysfunction (eg, overweight or smoking). Second, the combination of folic acid supplementation with vitamin B-12 results in an additional 7% reduction in homocysteine (4), yet our analysis indicates no additional benefit with vitamin B-12 and vitamin B-6. In addition, because the study duration had no significant effect on our study outcome, our result would point to an acute effect of folic acid. Taken together, the findings of the studies considered in this meta-analysis suggest that the effect of folic acid is largely independent of a homocysteine-lowering effect. Yet, we must be careful with this interpretation because of the small number of studies in the low- and high-dose strata. Therefore, it would be worthwhile to investigate acute effects of several doses (low and high) of folic acid or dietary folate or both on FMD values in subjects with suboptimal folate status.

In conclusion, this meta-analysis indicates that a high dose of folic acid can improve endothelial function as measured with FMD after 4 wk of supplementation, and this effect would seem to be independent of a reduction in homocysteine. Restored endothelial function in subjects with CVD may, in the short term, not prevent another CVD event, as can be deduced from published secondary prevention trials (5–8). However, an optimized FMD may be crucial to prevent a first-ever CVD event.

![FIGURE 3. Funnel plot of trial weight against change in percentage of flow-mediated dilatation (%FMD) in the 14 intervention trials included in the meta-analysis. The vertical line indicates a mean change in all studies.](https://academic.oup.com/ajcn/article-abstract/86/3/610/4649513)
We thank Margreet Olthof for sharing unpublished results for this analysis. The authors’ responsibilities were as follows—all authors: joint conduct of the analysis and synthesis of the findings for discussion. The authors work for Unilever, which sells products enriched with folic acid, vitamin B-6, and vitamin B-12.

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