Background: Elevated concentrations of homocysteine and low concentrations of folate may lead to a pro-inflammatory state that could explain their relation to vascular disease risk. We investigated the effect of lowering homocysteine concentrations by means of folic acid supplementation on markers of inflammation.

Methods: In a double-blind, randomized, placebo-controlled trial among 530 men and postmenopausal women with homocysteine concentrations of 1.8 mg/L or higher (≥13 µmol/L) at screening, we investigated the effect of folic acid supplementation (0.8 mg/d) vs placebo for 1 year on serum concentrations of C-reactive protein, soluble intercellular adhesion molecule-1, oxidized low-density lipoprotein, and autoantibodies against oxidized low-density lipoprotein.

Results: After 1 year of supplementation, concentrations of serum folate increased by 400% (95% confidence interval [CI], 362%-436%), and those of homocysteine decreased by 28% (95% CI, 24%-36%) in the folic acid group compared with the placebo group. However, no changes in plasma concentrations of the inflammatory markers were observed.

Conclusions: Although homocysteine is associated with vascular disease risk in the general population, marked lowering of slightly elevated homocysteine concentrations by means of 1-year folic acid supplementation does not influence inflammatory responses involving C-reactive protein, soluble intercellular adhesion molecule-1, oxidized low-density lipoprotein, and autoantibodies against oxidized low-density lipoprotein.

Arch Intern Med. 2005;165:1388-1394
METHODS

SUBJECTS
Data come from men and postmenopausal women aged 50 to 70 years from the Gelderland region in the Netherlands participating in the Folic Acid and Carotid Intima-Media Thickness Study, a trial investigating whether folic acid supplementation can halt the progression of atherosclerosis. Participants were recruited using community electoral rolls and local blood bank registries. Data were collected from the last 530 of the 819 subjects of the Folic Acid and Carotid Intima-Media Thickness Study from February 2001 through December 2002. Major exclusion criteria were homocysteine levels lower than 1.8 mg/L (<13 µmol/L), vitamin B12 concentrations lower than 271 pg/mL (<200 pmol/L), renal or thyroid diseases, use of vitamin B supplements or medications that influence folate metabolism or atherosclerotic progression (eg, therapies for lowering lipid concentrations, and for hormone replacement), and less than 80% self-reported compliance during a 6-week run-in period. The Medical Ethics Committee of Wageningen University, Wageningen, the Netherlands, approved the study and subjects gave written informed consent.

DESIGN
The sequence of entry into the study was randomly allocated to treatment using permuted blocks with block sizes of 4 and 6 (the computer-generated randomization list was kindly provided by Huub P. J. Willems, MD, PhD, Department of Haematology, Leyenburg Hospital, The Hague, the Netherlands). The sequence number served also as the participant’s allocation code; thus each participant had a unique code so as to decrease the chance of unmasking by the investigator. After the measurement sessions, subjects were allocated to treatment with folic acid or placebo in capsule form. The capsules were specially produced by Swiss Caps Benelux (Roosendaal, the Netherlands). Capsules were individually packaged in foil pill strips containing 28 pills per strip, and the days of the week were printed on the back of the strips to aid compliance and registration. The capsules were indistinguishable in appearance (yellow coating and content) and taste. Members of the same household received the same intervention to avoid contamination or comparisons between pills. Blinding of the participants appeared successful; at the completion of the 3-year trial in a sample of 260 participants, 60% did not know which treatment they had had, 10% suspected folic acid treatment, and 30% suspected placebo treatment. These suspicions were equally distributed across the folic acid and placebo groups. Research assistants who allocated and distributed treatment and collected blood samples were blinded to group assignment. During the trial, compliance (defined as >80% of capsules taken) was judged by pill return counts and a calendar that registered missed pills, both of which were assessed every 12 weeks. Fasting venous blood was collected in K3-EDTA (1 mg/mL)–treated Vacutainer tubes (Becton Dickinson, Mountain View, Calif) for determination of inflammatory markers for this subtrial in the last 530 subjects at the time of randomization and after 1 year (+6 weeks). Plasma was isolated immediately by means of centrifugation and, after addition of saccharose (final concentration, 0.6%) to prevent denaturation of lipoproteins during freezing, was stored in aliquots at −80°C until analyses were performed on it.

BLOOD MEASUREMENTS
We measured plasma C-reactive protein concentrations by means of a commercially available high-sensitivity enzyme-linked immunosorbent assay according to the instructions of the manufacturer (Dakopatts, Denmark). The intra-assay coefficient of variation was 6.0%, and interassay coefficient of variation was 9.7%. The plasma-oxydized LDL level was measured with a sandwich enzyme-linked immunosorbent assay described elsewhere. Monoclonal antibody HM2 was used as the capture antibody, and the biotin-labeled monoclonal antibody HM1 was used as the detection antibody. The lower detection limit of the assay was 400 pg/mL. The intra-assay coefficient of variation was 2.7%, and interassay coefficient of variation was 9.1%. The plasma-oxydized LDL level was measured with a sandwich enzyme-linked immunosorbent assay, using the monoclonal antibody 4E6 as the capture antibody and antihuman apolipoprotein B-100 polyclonal antibody as the detection antibody. Monoclonal antibody 4E6 attaches to a conformational epitope in the apolipoprotein B-100 moiety of LDL that is generated as a consequence of aldehyde substitution of lysine residues of apolipoprotein B-100 and is considered specific for oxidatively modified LDL. Autoantibodies against oxidized LDL were measured as described previously.

Plasma total homocysteine concentrations were determined with high-performance liquid chromatography and fluorimetric detection. Serum folate and vitamin B12 levels were measured using a chemiluminescent immunoassay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, Calif). Erythrocyte folate concentration was determined in duplicate, and the average was taken to reduce measurement error. Serum creatinine and lipid levels were determined using the Hitachi 747 analyzer (Roche Diagnostics, Basel, Switzerland). We defined hypercholesterolemia as a total cholesterol concentration higher than 251 mg/dL (>6.5 mmol/L), a high-density lipoprotein cholesterol concentration lower than 35 mg/dL (<0.9 mmol/L), or use of medication to lower lipid concentrations. The 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C→T polymorphism was determined by means of polymerase chain reaction of DNA and restriction enzyme digestion with HindII.

OTHER MEASUREMENTS
A self-reported medical history, including current drug use, family history of premature vascular disease (onset at <60 years of age in a first-degree family member), and smoking were attained by questionnaire and reviewed by a research assistant. A participant was considered to have prevalent vascular disease if he or she had received a diagnosis of angina pectoris, myocardial infarction, arrhythmia, stroke, or peripheral arterial disease or had undergone certain procedures (ie, balloon angioplasty, coronary bypass surgery, or aortic aneurysm surgery). Height and weight were measured and body mass index was calculated. Blood pressure was measured using an automated meter (Dinamap Compact Pro 100; General Electric, Milwaukee, Wis) in the supine position, while the participant underwent the ultrasound examination. The average of 8 measurements was taken. We defined hypertension as a systolic blood pressure of at least 160 mm Hg, a diastolic blood pressure of at least 95 mm Hg, or use of antihypertensive medication. A food frequency questionnaire estimated folate and alcohol intake in the past 3 months.

STATISTICAL ANALYSIS
Statistical analysis was performed using SPSS 11.0 for Windows (SPSS Inc, Chicago, Ill). For each participant, we averaged the homocysteine concentrations measured at screening and at base-
line and used this average in the analyses. Descriptive data are shown as mean±SD or median (interquartile range), depending on the distribution of the data. Skewed data were (natural logarithm) transformed. We used Pearson product moment correlations to examine the relation between folate and homocysteine concentrations on the one hand with markers of inflammation on the other hand in the total population at baseline. Differences in concentrations of the markers of inflammation after 1-year supplementation between treatment groups were tested by means of a 2-tailed t test. We followed the intent-to-treat principle and used baseline concentrations for posttreatment values of the inflammatory markers when a subject prematurely discontinued the study. Analysis of variance was used to investigate whether the MTHFR 677C→T polymorphism influenced treatment effect. Statistical significance was defined as P<.05 (2-tailed). Data analysis was conducted without knowledge of the treatment code, and to retain blinding, data were assigned fake participant numbers. Researchers were denied access to the original database of study values and participant numbers.

### RESULTS

Of the 530 subjects enrolled, 5 and 4 subjects allocated to the folic acid and placebo groups, respectively, did not return for the measurements at 1 year. All subjects except 2 reported consumption of more than 90% of the capsules, as judged from self-reported compliance or from the capsule strip return. The lowest compliance reported during a 3-month period was 52%. Randomization was successful, as concentrations of homocysteine, folate, inflammatory markers, and their determinants were similarly distributed across the folic acid (n=264) and placebo (n=266) groups (Table 1). The MTHFR 677C→T allelic distribution did not significantly differ from the calculated expected distribution, assuming a Hardy-Weinberg equilibrium (Table 1).

At baseline, homocysteine and erythrocyte folate concentrations were weakly correlated with soluble intercellular adhesion molecule-1 (r = 0.10 [P = 0.03] and r = −0.10 [P = 0.03], respectively), and erythrocyte folate concentration was also weakly correlated with oxidized LDL (r = −0.10 [P = 0.04]) in crude analyses. As shown in Table 2, 1-year supplementation with 0.8 mg/d of folic acid was associated with a 400% increase in serum folate concentration (95% confidence interval [CI], 362%-436%) and a 28% decrease in homocysteine concentrations (95% CI, 24%-36%). However, folic acid treatment did not affect concentrations of the inflammatory markers. Our results did not change when we adjusted for concentrations of in-

### Table 1. Characteristics of the Folic Acid and Placebo Groups at the Start of the Study*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Folic Acid (n = 264)</th>
<th>Placebo (n = 266)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, y</td>
<td>60 ± 5</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Male, %</td>
<td>70.1</td>
<td>73.7</td>
</tr>
<tr>
<td>Homocysteine, mg/L</td>
<td>1.9 (1.7-2.0)</td>
<td>1.9 (1.7-2.1)</td>
</tr>
<tr>
<td>Serum folate, ng/mL</td>
<td>4.9 (4.0-6.2)</td>
<td>4.9 (4.0-6.6)</td>
</tr>
<tr>
<td>Erythrocyte folate, mg/mL</td>
<td>273.6 (214.5-367.2)</td>
<td>298.6 (238.3-367.6)</td>
</tr>
<tr>
<td>Folate intake, µg/d</td>
<td>54.0 (42.6-59.0)</td>
<td>52.2 (41.6-60.8)</td>
</tr>
<tr>
<td>MTHFR 677C→T genotype, % CC/CT/TT</td>
<td>35.0 (47.5/17.5)</td>
<td>45.1/41.3/13.6</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>1.6 (0.7-3.1)</td>
<td>1.1 (0.6-2.4)</td>
</tr>
<tr>
<td>sICAM-1, µg/L</td>
<td>139 (118-160)</td>
<td>139 (114-165)</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>139 (118-160)</td>
<td>139 (114-165)</td>
</tr>
<tr>
<td>Oxidized LDL, mg/L</td>
<td>13 (10-18)</td>
<td>13 (10-18)</td>
</tr>
<tr>
<td>Oxidized LDL/LDL ratio, U/mmol</td>
<td>13 (10-18)</td>
<td>13 (10-18)</td>
</tr>
<tr>
<td>IgG against oxidized LDL, OD450</td>
<td>0.15 (0.09-0.22)</td>
<td>0.14 (0.08-0.24)</td>
</tr>
<tr>
<td>IgM against oxidized LDL, OD450</td>
<td>0.13 (0.07-0.23)</td>
<td>0.14 (0.08-0.24)</td>
</tr>
<tr>
<td>Total cholesterol, mean ± SD, mg/dL</td>
<td>228 ± 42</td>
<td>228 ± 46</td>
</tr>
<tr>
<td>HDL cholesterol, mean ± SD, mg/dL</td>
<td>46 ± 12</td>
<td>50 ± 15</td>
</tr>
<tr>
<td>LDL cholesterol, mean ± SD, mg/dL</td>
<td>158 ± 39</td>
<td>158 ± 42</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>42.4</td>
<td>36.8</td>
</tr>
<tr>
<td>Systolic blood pressure, mean ± SD, mm Hg</td>
<td>133 ± 16</td>
<td>133 ± 17</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Creatinine, mean ± SD, mg/dL</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Alcohol intake, g/d</td>
<td>13 (4-24)</td>
<td>13 (5-23)</td>
</tr>
<tr>
<td>Body mass index, mean ± SD</td>
<td>27 ± 3</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>22.0</td>
<td>18.4</td>
</tr>
<tr>
<td>Prevalent vascular disease, %</td>
<td>15.5</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*Calculated as weight in kilograms divided by the square of height in meters.

### Table 2. Concentrations of Serum Folate, Plasma Homocysteine, and Inflammatory Markers After 1-Year Treatment With Folic Acid or Placebo

<table>
<thead>
<tr>
<th>Variable</th>
<th>Folic Acid</th>
<th>Placebo</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate, mg/dL</td>
<td>26.5 (19.9-37.5)</td>
<td>5.3 (4.4-6.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Homocysteine, mg/L</td>
<td>1.2 (1.1-1.4)</td>
<td>1.7 (1.5-2.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>1.4 (0.9-3.1)</td>
<td>1.2 (0.6-3.1)</td>
<td>.88</td>
</tr>
<tr>
<td>sICAM-1, µg/L</td>
<td>139 (118-161)</td>
<td>139 (118-170)</td>
<td>.69</td>
</tr>
<tr>
<td>Oxidized LDL, U/L</td>
<td>53 (39-71)</td>
<td>55 (41-70)</td>
<td>.92</td>
</tr>
<tr>
<td>Oxidized LDL/LDL ratio, U/mmol</td>
<td>14 (10-19)</td>
<td>14 (10-19)</td>
<td>.90</td>
</tr>
</tbody>
</table>

*Calculated as weight in kilograms divided by the square of height in meters.

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; MTHFR, 5,10-methylenetetrahydrofolate reductase; OD450, optical density at 450 nm; sICAM-1, soluble intercellular adhesion molecule-1.
flamatory markers at baseline. The lipid parameters also did not change owing to treatment (data not shown). At baseline, homocysteine concentrations were 8% higher and serum folate concentrations were 18% lower in MTHFR 677TT homozygotes compared with subjects with the CC genotype. Concentrations of inflammatory markers did not differ between the genotypes (data not shown). Furthermore, the MTHFR 677C→T genotype did not appear to modify the effect of folic acid supplementation on markers of inflammation after 1 year (data not shown).

COMMENT

In this 1-year randomized placebo-controlled trial, marked reduction of slightly elevated homocysteine concentrations through daily folic acid supplementation did not affect plasma concentrations of oxidized LDL, autoantibodies against oxidized LDL, soluble intercellular adhesion molecule-1, or C-reactive protein in an elderly population with a median dietary folate intake below that of the Dutch recommended daily allowance (300 μg/d). Although homocysteine is associated with vascular disease risk in the general population, it is unlikely that homocysteine or folic acid influences these markers of inflammation.

To our knowledge, this randomized controlled trial is the largest one on lowering of homocysteine concentrations to examine the effect on inflammatory markers. In line with 2 previous studies, our results did not detect a decrease in concentrations of C-reactive protein after the lowering of homocysteine concentrations.13,14 Furthermore, to our knowledge, our study is the first to report an absence of an effect of folic acid supplementation on soluble intercellular adhesion molecule-1 levels. Other adhesion molecules like vascular cell adhesion molecule-1 and E-selectin did not respond to half of the homocysteine distribution (data not shown). In the former study, whether this increase was significant relative to the control group is unclear.

There is considerable evidence that C-reactive protein, soluble intercellular adhesion molecule-1, and oxidized LDL are involved in atherogenesis15,16 and predict risk of vascular disease.17,18 Although longitudinal studies on the association of oxidized LDL with vascular disease risk are more scarce.19 Formation of oxidized LDL in vivo leads to the generation of autoantibodies against various forms of oxidized LDL. Findings from experimental and epidemiological research on the relation of autoantibodies against oxidized LDL with atherosclerosis or vascular disease are inconsistent. Plasma levels of autoantibodies against oxidized LDL correlate with the amount of oxidized LDL in lesions, have been associated with the risk of vascular disease,20 although not consistently,21 and localize to atherosclerotic lesions in vivo.16 The mechanism via which autoantibodies against oxidized LDL influence atherosclerosis is not clear but may involve clearance of atherogenic oxidatively modified LDL. The IgG autoantibodies against oxidized LDL were shown to induce macrophage Fcγ receptor-mediated phagocytosis of oxidized LDL.22 On the other hand, autoantibodies against oxidized LDL may block the uptake of oxidized LDL by macrophages.23 Moreover, oxidatively modified LDL has been found to bind to innate pattern recognition receptors such as CD14 and C-reactive protein and activate toll-like receptor 4, which could enhance macrophage function and atherogenesis.24

In our population, erythrocyte folate but not homocysteine concentrations were weakly inversely related to oxidized LDL concentrations; however, folic acid supplementation did not affect concentrations of oxidized LDL or of autoantibodies against oxidized LDL. These findings concur with those of trials in the general population or in patients with coronary artery disease, which found no effect from B vitamin supplementation on in vitro LDL oxidizability or plasma malondialdehyde concentrations, a final product of lipid peroxidation.25-27 Likewise, folic acid supplementation in combination with vitamin B12 supplementation did not decrease urinary levels of 8-epi-prostaglandin F2α, an indicator of oxidative stress, in subjects with cognitive impairment.28 In patients with renal disease, however, oxidative stress appears responsive to high doses of folic acid or folic acid therapy. In these patients, a 40% to 50% decrease in homocysteine concentrations was associated with a 30% to 40% reduction in serum and erythrocyte malondialdehyde concentrations and a 13% reduction in autoantibodies against oxidized LDL.29-31 These trials did not use a placebo-controlled arm and hence should be interpreted with caution. In our study, a comparable decrease in homocysteine concentrations was found (25% decrease), and the decrease in subjects with the MTHFR 677TT genotype was even greater (40% decrease). Nevertheless, concentrations of the inflammatory markers did not respond to folic acid in subjects with the MTHFR 677TT genotype, similar to earlier findings,27 or in subjects in the upper half of the homocysteine distribution (data not shown). Although the relative reduction was similar, the postintervention concentrations of homocysteine in patients with renal disease were much higher compared with those in our own study (approximately 2.7 vs 1.2 mg/L [20 vs 9 μmol/L]). One study in the general population has shown a decrease in C-reactive protein concentrations after multivitamin supplementation, which included 0.8 mg/d of folic acid; this effect was greater in subjects with initial C-reactive protein concentrations of at least 1 mg/L.32 However, even when we confined our analyses to those subjects in the upper half of the distribution of the inflammatory markers or in the lower half of the folate distribution, our results did not change (data not shown). Were we too late in intervening, was the duration not long enough, or is folic acid an inappropriate intervention? We have shown that 1-year folic acid supplementation in the elderly (mean age, 65 years) did not lead to an improvement in markers of inflammation despite a marked decrease in homocysteine concentrations. The trials in patients with renal disease that observed reductions in concentrations of autoantibodies against oxidized LDL and malondialdehyde were shorter than our
Furthermore, a 2-year trial in subjects younger than our population did not detect differences in C-reactive protein and adhesion molecule concentrations (mean age, 47 years). Folic acid is the most effective B vitamin to reduce elevated concentrations of homocysteine, being responsible for a reduction of approximately 25%; the addition of vitamin B6 and vitamin B12 can lower homocysteine concentrations an additional 7%.33 If we extrapolate from the results from other studies that examine the effect of a combination of these forms of vitamin B on markers of inflammation,14,25 we do not expect that the addition of vitamin B6 and vitamin B12 would have altered our findings. Whether a dietary folate approach or supplemental folic acid may determine the success of lowering homocysteine concentrations on inflammatory markers is unclear. Adhering to a folate-rich diet for 16 weeks, compared with folic acid supplementation or a control diet, did not affect malondialdehyde concentrations.27 However, in patients with coronary artery disease, a whole-grain diet compared with the white-rice control diet was associated with a decrease in homocysteine and malondialdehyde concentrations after 16 weeks.34 Similarly, in patients with the metabolic syndrome, 2-year adherence to a Mediterranean diet, a diet typified by folate-rich foods, decreased concentrations of C-reactive protein and other cytokines.35

### CONCLUSIONS

The possible pathogenic mechanism of elevated concentrations of homocysteine in vascular disease remains unclear. Elevated concentrations of homocysteine and decreased concentrations of folate may have other proinflammatory properties in the elderly not captured by the markers measured in this study6; however, their amelioration through folic acid supplementation is unlikely to influence concentrations of C-reactive protein, soluble intercellular adhesion molecule-1, and oxidized LDL directly. The possible pathogenic mechanism remains unsolved, and convincing evidence to support a causal re-
relationship between elevated concentrations of homocysteine and risk of heart disease is absent. Table 3 gives an overview of the randomized controlled trials that examined whether reduction of homocysteine concentrations decreased the risk of vascular disease. The larger studies with longer duration have not been able to detect beneficial effects associated with vitamin B supplementation on clinical end points.40-48 This finding contrasts with those of some of the smaller studies showing a beneficial effect of vitamin B supplementation; these studies have used relatively short intervention periods and have reported a beneficial effect on a variety of surrogate markers for vascular disease risk.49-53 Finally, 1 study has found adverse effects of vitamin B supplementation on major adverse events after percutaneous coronary transluminal angioplasty.45 The risk for vascular disease associated with elevated concentrations of homocysteine is small,45 and many of the intervention studies in Table 3 may lack power to confirm or refute the homocysteine hypothesis, so we will have to wait for trial data to accrue before a meta-analysis can be conducted. A possible protective effect of extra folate acid, direct or via lowering of homocysteine concentrations, will not be due to a decrease in inflammation as measured by the inflammatory markers in our study. Lowering of homocysteine concentrations may affect other inflammatory markers, may affect other pathways leading to vascular disease in the presence of complicated advanced atherosclerosis, or may do nothing beneficial at all.

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REFERENCES


