Lowering Plasma Homocysteine Concentrations of Older Men and Women with Folate, Vitamin B-12, and Vitamin B-6 Does Not Affect the Proportion of (n-3) Long Chain Polyunsaturated Fatty Acids in Plasma Phosphatidylcholine1,2

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Abstract

There is evidence to suggest that folate, homocysteine, or both affect the (n-3) long chain PUFA composition of tissues; however, this evidence is derived largely from experiments with animals and small observational studies in humans. Results from randomized controlled trials are needed. The objective of this study was to determine whether homocysteine lowering with a B vitamin supplement affects the proportion of (n-3) long-chain PUFA in plasma phosphatidylcholine. We conducted a double-blind, placebo-controlled, randomized clinical trial involving 253 participants, 65 y or older, with plasma homocysteine concentrations of at least 13 μmol/L. Participants in the vitamin group (n = 127) took a daily supplement containing 1000 μg folate, 500 μg vitamin B-12, and 10 mg vitamin B-6 for 2 y. The fatty acid composition of plasma phosphatidylcholine was measured at baseline and at 2 y. Plasma homocysteine concentrations during the course of the study were 4.4 μmol/L lower in the vitamin group than in the placebo group. The proportions of eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids in plasma phosphatidylcholine did not differ between the vitamin and placebo groups at 2 y; the mean differences after adjusting for baseline values and sex were −0.03 (99% CI: −0.22, 0.16), 0.03 (99% CI: −0.03, 0.09), and −0.02 (99% CI: −0.27, 0.24) mol%, respectively. Lowering plasma homocysteine concentrations of older men and women with folate, vitamin B-12, and vitamin B-6 had no effect on the proportion of (n-3) long-chain PUFA in plasma phosphatidylcholine. J. Nutr. 138: 551–555, 2008.

Introduction

Results from case control and cohort studies suggest that elevated circulating concentrations of homocysteine are associated with increased risk of cardiovascular disease, independent of traditional risk factors such as smoking, blood pressure, serum lipids, and obesity (1,2). Particularly persuasive evidence, in this respect, is the observation of higher cardiovascular risk in homozygous carriers of the 677C-T polymorphism on the methylenetetrahydrofolate reductase gene, a genotype that confers naturally higher homocysteine concentrations (3,4).

Recently, the causal nature of the association between homocysteine and cardiovascular disease has been called into question, because the results of several large, randomized, controlled trials have not shown reduced incidence of cardiovascular disease after homocysteine-lowering with B-vitamins (folate, B-12, and B-6) (5–7). This apparent contradiction between the observational evidence and results of the clinical trials raises the possibility that the positive association between serum homocysteine and cardiovascular risk in the case control and cohort studies may be confounded by 1 or more unmeasured cardiovascular risk factors.

There has been speculation that one of these confounding risk factors may be plasma (n-3) long chain PUFA. The 2 major (n-3) long chain PUFA, eicosapentaenoic acid and docosahexaenoic acid, exert a range of biochemical and physiological effects consistent with cardioprotection (8). Accumulated evidence from observation studies and randomized controlled trials indicates that (n-3) long chain PUFA lower the risk of cardiovascular disease (9).

Results of experimental animal model studies suggest that low folate status (10) or increased exposure to homocysteine (11) decreases the proportion of (n-3) long chain PUFA in tissues, whereas intramuscular injection of folate (12) increases the...
proportion of these fatty acids. However, the evidence from studies of humans for an association between folate, homocysteine, and plasma or blood cell (n-3) long chain PUFA is limited.

In a study of 22 men with aggressive and hostile behavior, folate concentrations and docosahexaenoic acid in erythrocytes were correlated \( (r = 0.57; P = 0.005) \) (13); however, the results from another study involving 44 patients with depressive disorders showed plasma homocysteine concentrations were not significantly correlated with (n-3) long chain PUFA composition of plasma or erythrocyte lipids (14). Recently, Dullmeijer et al. (15) reported no correlation between plasma or erythrocyte folate concentrations and the proportion of eicosapentaenoic acid or docosahexaenoic acid in plasma cholesterol ester \( (r = 0.04; P = 0.21) \). In a randomized controlled trial, pregnant women supplemented with folic acid from gestation wk 22 until delivery had a very small but significant increase in the proportion of docosahexaenoic acid in total plasma fatty acids (16).

In this study, we used a randomized placebo-controlled trial to test the hypothesis that homocysteine-lowering with the B-vitamins, folate, vitamin B-12, and vitamin B-6 affects the proportion of (n-3) long chain PUFA in plasma phosphatidylcholine.

**Subjects and Methods**

This randomized controlled study was part of a 2-y trial to determine whether B-vitamins improved cognitive function in older men and women (17). The fatty acid composition of plasma phosphatidylcholine is a secondary endpoint of the trial.

**Participants.** Participants 65 y or older were recruited from local service clubs (e.g. Rotary International) and by advertising in newspapers as well as by direct mailings. Participants were excluded if they were taking medications known to interfere with folate metabolism; their physician told them they had probable dementia; they were taking vitamin supplements containing folic acid, vitamin B-12, or vitamin B-6; or they were being treated for or had a history of depression, stroke, transient ischemic attacks, or diabetes. Volunteers were screened for plasma homocysteine and creatinine; those with a fasting homocysteine concentration <13 \( \mu \text{mol/L} \) or a plasma creatinine >133 \( \mu \text{mol/L} \) for men and 115 \( \mu \text{mol/L} \) for women were excluded. Written informed consent was obtained from each participant prior to screening and the University of Otago Human Ethics Committee approved the study.

**Study design.** Participants were stratified using the median values of age and homocysteine concentration from the screening population and randomized to receive a B-vitamin supplement or placebo. Participants were asked to consume 1 capsule daily for 2 y. Participants were provided with a 6-mo supply of capsules and asked to return every 6 mo to obtain a new supply. Participants returned unused capsules every 6 mo and these were counted to assess compliance.

**Supplements.** The treatment capsules contained the microcrystalline cellulose as a filler plus 1000 \( \mu \text{g} \) folate (1-5-methyltetrahydrofolate, calcium salt), 500 \( \mu \text{g} \) vitamin B-12 (cyanocobalamin), and 10 mg vitamin B-6 (pyridoxine). The placebo capsules contained a blend of magnesium stearate and the filler (Merck Eprova). The investigators and the participants were blinded to the contents of the supplements.

**Laboratory methods.** An overnight fasting blood sample was collected at baseline and every 6 mo thereafter. Plasma and serum were obtained by centrifuging the whole blood at 1650 \( \times \) g; 15 min at 4°C within 2 h of collection. Blood samples were stored at -80°C until analyzed. Total homocysteine was measured using the IMx (Abbott Laboratories) fluorescence polarization immunoassay, measuring total i-homocysteine in plasma. Plasma folate concentrations were determined using a microbiological method on 96-well microplates, exactly as described by O’Brien and Kelleher (18) with chloramphenicol resistant *Lactobacillus casei* as the test microorganism. Plasma vitamin B-12 was measured using the ADVIA Centaur vitamin B-12 assay, a competitive immunoassay using direct chemiluminescent technology. Plasma total cholesterol was measured enzymatically using Roche diagnostic kits on a Cobas Mira Analyzer (Roche Diagnostics). CV for these assays were 6.7% for plasma homocysteine, 7.7% for plasma folate, 5.6% for plasma vitamin B-12, and 1.0% for plasma total cholesterol.

Methylene tetrahydrofolate reductase genotype was determined by amplifying DNA using PCR and subject to restriction by *Hinfl* according to the method of Frosst et al. (19).

Lipids were extracted from 400 \( \mu \text{L} \) of plasma according to the method of Bligh and Dyer (20). Plasma phosphatidylcholine was separated by TLC using a solvent system of chloroform:methanol:acetic acid:water \( (50:37.5:5.25:0.25 \text{ by volume}) \) according to Holub and Skeaff (21). The fatty acid composition was analyzed using a DB-225 narrowbore column \( (30-m \times 0.25-mm \text{id.}; \text{film thickness, } 0.25 \mu \text{m}; \text{J&W Scientific}) \) on a HP-6890 gas chromatograph with flame ionization detection (Agilent). The fatty acid results are reported as percent of total fatty acids on a molar basis (i.e. mol%). Precision of the fatty acid measurement was determined by repeat analysis of a pooled plasma sample; 1 pooled sample was analyzed for every 20 samples. The CV for eicosapentaenoic \( [20:5(n-3)] \), docosapentaenoic \( [22:5(n-3)] \), and docosahexaenoic \( [22:6(n-3)] \) acids in plasma phosphatidylcholine were 1.6, 2.7, and 3.7%, respectively.

**Statistical methods.** Differences in characteristics of participants in the vitamin and placebo groups at baseline were determined using a Fisher’s exact test for categorical variables and Student’s \( t \) test for continuous variables. Multiple regression analyses were used to estimate the differences in plasma homocysteine and folate at 2 y after adjustment for baseline values between the placebo group and the treatment group. Likewise, multiple regression analyses, adjusting for baseline fatty acid composition and sex, were used to estimate the differences in the fatty acid composition of plasma phosphatidylcholine at 2 y. Due to the large number of comparisons, differences in fatty acid composition were considered significant at \( P < 0.01 \). All analyses were undertaken using Stata Statistical Software: Release 9 for Macintosh (StataCorp).

The sample size of the study was designed on the primary endpoint—scores on tests of cognition. We estimated that 100 participants in each of the vitamins and placebo group would be required to detect a minimum treatment effect size of 0.4 with a power of 80% and 2-sided \( \alpha \) at 0.05 (17). Values in the text are means (99% CI).

**Results**

Results in Figure 1 show the study participation and follow-up. The fatty acid composition of plasma phosphatidylcholine was...
measured in 253 participants at baseline and 243 participants at y 2. Participants who completed 2 y of the trial (n = 243) reported taking 98% of their tablets. The baseline characteristics of participants are presented in Table 1.

At 2 y, plasma homocysteine was lower in the vitamin group than the placebo group (–4.4 μmol/L; 99% CI: −5.5, −3.4 μmol/L) (Fig. 2A). Plasma folate concentrations were significantly higher in the vitamin than the placebo group (50; 99% CI: 44, 56 nmol/L) (Fig. 2B).

None of the proportions of fatty acids in phosphatidylycerline measured differed between the placebo and vitamin groups after 2 y of treatment (Table 2). The adjusted mean differences in the proportion of eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids between the vitamin and the placebo group were −0.03 (99% CI: −0.22, 0.16), 0.03 (99% CI: −0.03, 0.09), and −0.02 (99% CI: −0.27, 0.24) mol%, respectively.

Discussion

The results of our study showed that lowering plasma homocysteine concentrations by 4.4 μmol/L for 2 y with high intakes of folate, vitamin B-12, and vitamin B-6 did not alter the (n-3) long chain fatty acid composition of plasma phosphatidylycerline. This finding does not support the hypothesis that homocysteine or high intakes of folate, vitamin B-12, or vitamin B-6 influence the metabolism of (n-3) fatty acids in the body.

Krauss-Etschmann et al. (16) recently reported the outcome of a 2 × 2 factorial, placebo-controlled trial of fish oil and folate supplementation on plasma docosahexaenoic acid in which pregnant women consumed 400 μg of 5-methyltetrahydrofolate daily from wk 22 of gestation to delivery. There was a folate treatment × time interaction (P = 0.047) on the proportion of docosahexaenoic acid but not eicosapentaenoic acid (P = 0.081) in maternal plasma; however, the magnitude of the effect of folate on docosahexaenoic acid was very small. In contrast to the finding in maternal plasma, there was no effect of folate supplementation on docosahexaenoic acid proportions in cord blood plasma (P = 0.095). Krauss-Etschmann et al. (16) suggested that folate supplementation may increase maternal docosahexaenoic acid, a possibility that warrants elucidation in larger trials. It is possible that the difference between these findings and those we report simply reflects the different physiological state of the participants in the 2 studies, pregnant compared with nonpregnant. We also cannot exclude the possibility that our study reflects the absence of long-term effects, whereas the results reported by Kraus-Etschmann reflect transient changes.

TABLE 1  Baseline characteristics of older men and women in the placebo and B vitamin groups

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Placebo</th>
<th>Vitamins</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>125</td>
<td>124</td>
<td>0.79</td>
</tr>
<tr>
<td>Age at screening, y</td>
<td>73.4 ± 5.7</td>
<td>73.6 ± 5.8</td>
<td>0.79</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>65 (52)</td>
<td>47 (37)</td>
<td>0.02</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>1 (1)</td>
<td>6 (5)</td>
<td>0.12</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.8 ± 3.7</td>
<td>26.9 ± 4.3</td>
<td>0.49</td>
</tr>
<tr>
<td>MTHFR C677T TT³, n (%)</td>
<td>11 (14)</td>
<td>20 (16)</td>
<td>0.23</td>
</tr>
<tr>
<td>Plasma homocysteine, μmol/L</td>
<td>16.3 ± 4.4</td>
<td>16.8 ± 5.4</td>
<td>0.43</td>
</tr>
<tr>
<td>Plasma folate, μmol/L</td>
<td>23 ± 11</td>
<td>22 ± 11</td>
<td>0.78</td>
</tr>
<tr>
<td>Plasma vitamin B-12, μmol/L</td>
<td>294 ± 102</td>
<td>280 ± 100</td>
<td>0.74</td>
</tr>
<tr>
<td>Plasma total cholesterol, μmol/L</td>
<td>6.01 ± 1.35</td>
<td>6.36 ± 1.25</td>
<td>0.03</td>
</tr>
</tbody>
</table>

1 Values are means ± SD or n (%).
2 Differences were tested by using chi-square tests for categorical variables and t tests for continuous variables.
3 MTHFR, methylene tetrahydrofolate reductase C677T polymorphism, homozygous TT genotype.

FIGURE 2  Plasma homocysteine (A), folate (B), and vitamin B-12 (C) concentrations in older men and women who took a B vitamin supplement (n = 117–127) or a placebo (n = 119–126) for 2 y. Values are means ±99% CI. *Different from the placebo group after adjusting for baseline values, P < 0.001.
Our trial had several design features that made it particularly suited for testing the hypothesis that folate, homocysteine, or both affect docosahexaenoic acid status. The 2-y duration of our trial ensured that plasma vitamin concentrations were maintained at high and steady-state concentrations in plasma (22) and presumably in other tissues, such as liver, for long enough to detect an effect on plasma docosahexaenoic acid if one existed. Participants had high plasma homocysteine concentrations at baseline, 13 μmol/L or higher, and the vitamin supplement lowered homocysteine concentration by 4.4 μmol/L compared with placebo. Despite the long duration of supplementation and large difference in homocysteine concentrations between the treatment and placebo groups, there was no difference in docosahexaenoic acid in plasma phosphatidylcholine at 2 y; even the outer CI of the mean difference (−0.27 mol% and 0.24 mol%) exclude meaningful effects when compared with those caused by small changes in intake of docosahexaenoic acid (23).

The hypothesis that folate deficiency or increased homocysteine concentrations affect (n-3) long chain PUFA composition of tissues originated from results of experiments with animals exposed to folate-deficient diets or extremely high concentrations of homocysteine, physiological conditions and exposures that are generalizable to these cells.

In conclusion, existing evidence for the effect of folate, homocysteine, or both on docosahexaenoic acid composition of tissue is equivocal. The results of our study provide convincing clinical trial evidence that a folate, vitamin B-12, and vitamin B-6 supplement lowers plasma homocysteine concentrations but does not alter docosahexaenoic acid composition of plasma phosphatidylcholine.

### Literature Cited


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**TABLE 2** Fatty acid composition of plasma phosphatidylcholine in older men and women who took a B vitamin supplement or a placebo for 2 y

<table>
<thead>
<tr>
<th>Fatty acid Placebo, n = 126</th>
<th>Vitamin, n = 127</th>
<th>Placebo, n = 120</th>
<th>Vitamin, n = 123</th>
<th>Adjusted difference</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.43 ± 0.16</td>
<td>0.43 ± 0.16</td>
<td>0.46 ± 0.12</td>
<td>0.45 ± 0.13</td>
<td>−0.01 (−0.05 to 0.02)</td>
</tr>
<tr>
<td>16:0</td>
<td>30.83 ± 1.46</td>
<td>30.25 ± 1.31</td>
<td>31.10 ± 1.41</td>
<td>30.82 ± 1.52</td>
<td>−0.12 (−0.53 to 0.29)</td>
</tr>
<tr>
<td>18:0</td>
<td>12.85 ± 0.98</td>
<td>12.84 ± 1.09</td>
<td>12.62 ± 1.00</td>
<td>12.75 ± 1.14</td>
<td>0.08 (−0.18 to 0.34)</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>11.71 ± 1.56</td>
<td>11.64 ± 1.51</td>
<td>12.03 ± 1.72</td>
<td>11.68 ± 1.72</td>
<td>−0.25 (−0.71 to 0.21)</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>21.75 ± 3.14</td>
<td>22.50 ± 2.99</td>
<td>21.72 ± 3.41</td>
<td>22.18 ± 3.20</td>
<td>−0.15 (−0.90 to 0.60)</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.33 ± 0.13</td>
<td>0.34 ± 0.11</td>
<td>0.33 ± 0.13</td>
<td>0.35 ± 0.19</td>
<td>0.01 (−0.04 to 0.06)</td>
</tr>
<tr>
<td>20:3(n-6)</td>
<td>3.43 ± 0.83</td>
<td>3.44 ± 0.68</td>
<td>3.38 ± 0.76</td>
<td>3.42 ± 0.74</td>
<td>0.02 (−0.14 to 0.17)</td>
</tr>
<tr>
<td>20:4(n-6)</td>
<td>8.50 ± 1.96</td>
<td>8.32 ± 1.70</td>
<td>8.23 ± 1.93</td>
<td>8.45 ± 1.99</td>
<td>0.32 (−0.12 to 0.75)</td>
</tr>
<tr>
<td>20:5(n-3)</td>
<td>1.82 ± 0.66</td>
<td>1.54 ± 0.50</td>
<td>1.54 ± 0.69</td>
<td>1.49 ± 0.55</td>
<td>−0.03 (−0.22 to 0.16)</td>
</tr>
<tr>
<td>22:4(n-6)</td>
<td>0.25 ± 0.06</td>
<td>0.25 ± 0.06</td>
<td>0.25 ± 0.06</td>
<td>0.25 ± 0.06</td>
<td>0.01 (−0.01 to 0.02)</td>
</tr>
<tr>
<td>22:5(n-6)</td>
<td>0.17 ± 0.05</td>
<td>0.16 ± 0.04</td>
<td>0.19 ± 0.10</td>
<td>0.18 ± 0.04</td>
<td>0.00 (−0.03 to 0.02)</td>
</tr>
<tr>
<td>22:5(n-3)</td>
<td>1.14 ± 0.22</td>
<td>1.08 ± 0.21</td>
<td>1.05 ± 0.26</td>
<td>1.04 ± 0.21</td>
<td>0.03 (−0.03 to 0.09)</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>3.64 ± 0.85</td>
<td>3.45 ± 0.83</td>
<td>3.35 ± 0.95</td>
<td>3.23 ± 0.82</td>
<td>−0.02 (−0.27 to 0.24)</td>
</tr>
</tbody>
</table>

1 Values are means ± SD or mean (99% CI).

2 Adjusted for baseline values and sex using multiple linear regression.
support causal role for homocysteine and preventive potential of folate? BMJ. 2003;335:1053.


