Differences in Erythrocyte Folate Concentrations in Older Adults Reached Steady-State within One Year in a Two-Year, Controlled, 1 mg/d Folate Supplementation Trial1–3

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Abstract
Daily supplementation with folate increases erythrocyte folate concentrations; however, the time to reach steady-state concentrations has not been empirically demonstrated. Previous predictions of time to steady state or time to 90% steady-state concentration, based on modeling changes in erythrocyte folate during short-term trials, range widely from 40 to 86 wk. We sought to determine the time to steady-state erythrocyte folate concentrations following the initiation of daily folate supplementation using data collected from a 2-y, double-blind, placebo-controlled, randomized trial involving 276 participants aged 65 y or older. The daily supplement contained 1 mg of folate. Erythrocyte folate concentrations were measured, using a microbiological assay, at baseline and at 6, 12, 18, and 24 mo. The mean plasma and erythrocyte folate concentrations in the folate-supplemented group were higher than in the placebo group at 6, 12, 18, and 24 mo (P < 0.001). Adjusted for baseline differences, the difference in erythrocyte folate concentrations between the folate and placebo group at 6 mo was 1.78 μmol/L (95% CI: 1.62–1.95 μmol/L). The difference increased significantly to 2.02 μmol/L (95% CI: 1.85–2.18 μmol/L) at 12 mo. This difference (between the folate and placebo groups) did not significantly change after a further year of folate supplementation; at 18 mo, it was 2.09 μmol/L (95% CI: 1.92–2.27 μmol/L) and at 24 mo it was 1.98 μmol/L (95% CI: 1.18–2.15 μmol/L). Twelve months of daily folate supplementation with 1 mg is sufficient time to cause erythrocyte folate concentrations to reach a new steady state. J. Nutr. 142: 1633–1637, 2012.

Introduction
Folate is an essential nutrient involved in a range of one-carbon metabolic reactions; its role in cardiovascular disease, cancer, neurocognitive diseases, and congenital defects of the neural tube is the focus of considerable research. Concentrations of this vitamin in plasma and erythrocytes are commonly measured to assess the folate status of individuals and populations, with erythrocyte concentrations considered a better measure of usual or long-term status (1).

Folate supplementation, either as folic acid or as 5-methyltetrahydrofolate, increases plasma and erythrocyte folate concentrations. Steady-state concentrations in plasma are reached after 12 wk of supplementation (2,3); however, the duration of supplementation required for erythrocyte folate concentrations to reach a new steady state is uncertain, because the time-course studies have been too short to reveal a plateau (2–10). Thus, the current evidence underestimates the true quantitative relationship between folic acid intake and erythrocyte folate concentration.

In the absence of empirical results, mathematical modeling of the change in erythrocyte concentrations over time has been used to estimate the time to steady state (9,11). Pietrzik et al. (11) suggest, on the basis of the combined results from 2 folate supplementation trials of 16 and 24 wk duration, that 40 wk is required for erythrocyte folate concentrations to reach a plateau, whereas Houghton et al. (9) suggest, on the basis of a 40-wk trial, that as long as 86 wk is needed to reach 90% of steady-state concentrations. The validity and reliability of the predictions have been debated (12,13). The widely different...
predictions of time to steady state suggest the models have inherent limitations and may be unreliable. In this regard, empirical evidence from a folate supplementation trial of sufficient duration to document the attainment of a new steady state in erythrocyte folate concentrations would help advance our understanding about the issue.

For this purpose, we returned to data collected from an earlier 2-y, randomized, double-blind, placebo-controlled trial of folate supplementation and cognitive function during which blood samples were collected at baseline and every 6 mo. We previously reported the plasma folate results from this trial (14) and now extend this to an examination of the changes in erythrocyte folate concentrations over the 2-y period of supplementation.

Participants and Methods

Participants. The 2-y, double-blind, placebo-controlled, randomized trial was previously described (14). The trial was carried out in Dunedin, New Zealand between August 2002 and December 2004 and was approved by the University of Otago Human Ethics committee. All participants gave informed written consent.

Two hundred and seventy-six participants aged 65 y or older were recruited through advertisements, direct mail, and service clubs. Potential participants were screened for high plasma homocysteine concentrations; only participants with fasting plasma homocysteine concentrations $\geq 13 \mu$mol/L were eligible to participate. Exclusion criteria included suspected dementia; diabetes; use of medications known to affect folate metabolism; use of dietary supplements containing folic acid, vitamin B-12, or vitamin B-6; a history of stroke or transient ischemic attacks; and treated depression. Eligible participants were stratified according to the median values for age and homocysteine concentration in the screening sample and participants in each stratum were randomized to receive either the folate capsule containing 1 mg of (6S)-5-methyltetrahydrofolate, as well as 500 $\mu$g of vitamin B-12 and 10 mg of vitamin B-6, or a placebo. Participants were asked to take 1 capsule/ d for 2 y. Capsules were packaged in blister packs and compliance was assessed by counting returned capsules. To monitor the stability of the folate supplement, capsules that had been stored at 25°C throughout the trial were analyzed every 3 mo for the first year of the trial and every 6 mo in the final year.

Laboratory analysis. At baseline and every 6 mo thereafter, a fasting blood sample was collected from participants via venipuncture into a tube containing EDTA. Plasma was obtained by centrifuging whole blood within 2 h of collection at 1650 $\times$ g for 15 min at 4°C. Whole blood and plasma aliquots were stored at −80°C until analyzed.

Whole blood and plasma folate concentrations were measured by microbiological assay with the use of the test organism Lactobacillus Rhamnosus as described by O’Broin and Kelleher (15). The National Institute for Biological Standards and Control international standard for whole blood folate (NIBSC 95/528) was used to generate the standard curve. The CV for the assay was 7.7%. Follow-up folate analysis was conducted over 2 distinct time periods: the first batch-run included samples collected at 0, 6, and 12 mo and the second batch-run included samples collected at 18 and 24 mo. A systematic error occurred in the analysis of the erythrocyte folate concentration in the second batch-run of samples. The mean whole blood folate concentration in the placebo group was higher in the second (18 and 24 mo) compared with the first batch-run (0, 6, and 12 mo). This difference was a systematic error, probably in the dilution of the whole blood samples, because plasma folate concentrations in the placebo group in the first and second batch-runs were not different; furthermore, the plasma quality control samples did not differ between batch-runs. Given that our trial was placebo controlled, the systematic error in the measurement of erythrocyte folate concentrations in the second batch of samples applies equally to placebo and folate groups. Thus, the difference between folate and placebo groups was unaffected by the systematic error.

Statistical analysis. The purpose of the analysis was to test if the folate concentration (plasma or erythrocyte) in the folate group differed from the placebo group at 6, 12, 18, and 24 mo and then to test if the differences in folate concentrations between the folate and placebo groups changed from one time point to the other. All analyses were carried out in Stata, release 10 (StataCorp). A mixed model with participant as a random effect was used to analyze the data. The model included effects for treatment and time and baseline concentration. An interaction between treatment and time was included in the model. If this interaction term was significant, the difference in folate concentration between the folate and placebo groups at 6 mo was compared with the difference at 12, 18, and 24 mo; values were obtained from the model. Similarly, the difference at 12 mo between the folate and placebo groups was compared with those at 18 and 24 mo, and the difference at 18 mo was compared with that at 24 mo. The values reported in the text are mean difference (95%CI). Bonferroni correction was used to adjust the 95%CI for multiple comparisons.

Results

The retention of participants during the original trial of cognitive performance was previously reported (14). Two hundred and fifty-three participants were included in that analysis on the basis of completing the cognitive performance tests at 1 y. The present analysis includes 5 more participants who had plasma and erythrocyte folate concentrations measured at 6 mo but dropped out before 1 y.

The baseline characteristics of the participants in this trial were previously reported (14). The folate and placebo groups were balanced for most characteristics, but there was a larger percentage of women in the placebo group compared with the folate group (Table 1).

Compliance with supplement use was high throughout the trial; between baseline and 24 mo, the mean number of capsules missed per participant in each 6-mo time period was <6, giving a compliance rate of $\geq$97%. Compliance was not different between the folate and placebo groups. The concentration of 5-methyltetrahydrofolate in the supplement was stable throughout the duration of the trial. The amount of folate in the capsule was between 1.02 and 1.00 mg at each monitored point in time from 3 to 24 mo.

The plasma results were also previously reported (14). The mean plasma and erythrocyte folate concentrations in the folate-supplemented group were significantly higher than those in the placebo group at 6, 12, 18, and 24 mo (Table 2). After adjustment for baseline values, the mean difference in the plasma folate concentration between the folate and placebo groups did not differ across 6, 12, 18, and 24 mo ($P$-interaction $= 0.58$ between treatment and time). During the 6–24 mo of the final year of the folate-supplementation period, the mean difference in folate concentrations between the folate and placebo groups was between 1.02 and 1.00 mg at each monitored point in time from 3 to 24 mo.

| TABLE 1 Baseline characteristics of older adults enrolled in a 2-y folate supplementation trial $^1$ |
|-------------------------------------------------|---------------------------------|---------------------------------|
| Placebo group | Folate group |
| Age, y     | 73 ± 6 | 74 ± 6 |
| Women, n (%) | 67 (52) | 48 (37) |
| BMI, kg/m$^2$ | 26 ± 4 | 27 ± 4 |
| Plasma folate, nmol/L | 23 ± 12 | 22 ± 11 |
| Erythrocyte folate, $\mu$mol/L | 0.99 ± 0.42 | 0.98 ± 0.47 |
| Plasma homocysteine, $\mu$mol/L | 16.4 ± 4.5 | 16.7 ± 5.4 |
| Plasma vitamin B-12, $\mu$mol/L | 0.28 ± 0.10 | 0.28 ± 0.10 |

$^1$ Values are mean ± SD unless otherwise stated.
Discussion

The results of our 2-y folate supplementation trial, during which erythrocyte folate concentrations were measured at baseline and every 6 mo, showed that the difference in mean erythrocyte folate concentration between the folate and placebo groups increased from 6 to 12 mo but was unchanged thereafter. The absence of a change in the mean erythrocyte folate concentration during the second year of folate supplementation indicates that steady-state erythrocyte folate concentrations were achieved by 12 mo. The large number of participants in our study, low participant dropout, documented stability of the folate supplements, and high supplement compliance rates add confidence to the veracity of the results.

Results from 9 previous trials (2–8,10,16) ranging from 12 to 40 wk of duration and incorporating doses of folic acid or 5-methyltetrahydrofolate between 100 and 5000 μg/d did not reveal a plateau in erythrocyte folate concentrations. The results of one study stand in contrast. Nguyen et al. (17) showed in a small (n = 38), uncontrolled, open-labeled, 30-wk supplementation trial that erythrocyte folate concentrations appeared to reach a new steady state by 12 wk of supplementation with 1.1 or 5 mg/d of folic acid. The reason for the difference in this result from the others is unclear. The higher doses used by Nguyen et al. (17) do not appear to account for the discrepancy, because, in addition to our trial of 1 mg/d folate, Hao et al. (2) also administered a high dose of folic acid (4 mg/d) for 6 mo yet did not find erythrocyte folate concentrations had reached a maximum. Participants in our trial consumed 5-methyltetrahydrofolate in contrast to the Nguyen et al. (17) trial in which participants received folic acid. However, the results of supplementation trials suggest that changes in erythrocyte folate do not differ between the 2 forms of folate (3,4,18–20). The mean erythrocyte folate concentration at baseline in our study participants was similar to that reported by Nguyen et al. (17): 1.0 μmol/L compared with ~1.1 μmol/L, respectively. Yet in our trial, the erythrocyte folate concentrations increased by 1.78 μmol/L after 6 mo of supplementation with 1 mg/d folate but by only 1.2 μmol/L after a similar period (30 wk) of supplementation with 5 mg/d folate in the trial by Nguyen et al. (17). It is possible that the lower increase found by Nguyen et al. (17) with a higher dose of folate might reflect a decrease in compliance with supplement use during the later weeks of the trial.

Because we did not measure erythrocyte folate concentrations at multiple time points between 6 and 12 mo, we can conclude only that steady state was reached by 12 mo; our...
results do not show the precise time between 6 and 12 mo when it was achieved. Other investigators (9,11) have attempted to predict the time to steady state by modeling change in erythrocyte folate concentrations during folate supplementation trials lasting <1 y. Pietrzik et al. (11) predicted that 40 wk of folate supplementation was required for erythrocyte folate to reach steady-state concentrations. This prediction was based on results from 2 folate supplementation trials of 16 wk (800 \( \mu \)g/d folic acid) and 24 wk (400 \( \mu \)g/d folic acid or 416 \( \mu \)g/d 5-methyltetrahydrofolate). Houghton et al. (9) predicted, based on results from a 40-wk folic acid supplementation trial, that erythrocyte folate concentrations would reach 90% of steady-state concentrations after 74 wk with 140 \( \mu \)g/d and 86 wk with 400 \( \mu \)g/d. The 2 groups’ predictions of time to steady state vary by 2-fold. Other than recognizing the inherent limitations and imprecision of modeling change in erythrocyte concentrations based on trials of insufficient duration, it is difficult to account for the extreme differences in predictions of time to steady state. Minor differences in participant characteristics and dose or form of folate across the trials are unlikely to account for the widely varying predictions. Study participants in the trials by Pietrzik et al. (11) and Houghton et al. (9) were nonpregnant women 18–35 y and 18–40 y, respectively. The doses and forms of folate also overlapped, though 25% of the participants in the trials by Pietrzik et al. (11) consumed the higher dose of 800 \( \mu \)g/d.

To progress our understanding about time to steady-state erythrocyte concentrations, it may be informative to start with empirical evidence, as reported herein, and evaluate the validity of the modeling predictions accordingly. In this regard, our results are consistent with the 40 wk prediction. The reason they differ from the prediction of 74 and 86 wk is not apparent. The older age, 65 y and above, of our participants and higher dose, 1 mg/d, of folate seem unlikely explanations, because these factors also differed from the trials by Pietrzik et al. (11).

Our trial, which used a 1 mg/d folate supplement, was not designed to investigate whether the time to steady-state erythrocyte concentration differs by dose of folate. A kinetic model derived from a small \((n = 18)\), 10-wk, controlled study of folate metabolism in nonpregnant women indicated that the turnover of folate pools was faster when folate intakes were higher (21). These results suggest that chronic daily supplementation with higher doses of folate should lead to more rapid achievement of steady state. However, the predictions modeled on the results by Houghton et al. (9) indicated that the higher dose of folate, 400 \( \mu \)g/d, required an additional 12 wk to reach 90% of erythrocyte folate steady state compared with 140 \( \mu \)g/d. In light of these discrepancies, understanding how the dose of folate affects time to steady state may evolve only from the results of large, well-designed trials of sufficient duration, probably 1.5 to 2 y, with varying doses of folate.

The mean difference that we observed in plasma folate between the folate and placebo groups, 52 nmol/L throughout the study, with daily supplementation of 1 mg 5-methyltetrahydrofolate is comparable with that in other trials that used similar doses of folic acid. After 3 mo of supplementation with 1 mg/d folic acid, Wald et al. (22) observed a change in 55 nmol/L for serum folate. In 2 longer term trials of 36- and 38-mo median duration, supplementation with 800 \( \mu \)g/d folic acid produced changes of 53 and 50 nmol/L, respectively, for serum folate (23,24). Jung et al. (25) reported that erythrocyte folate concentrations were 1.42 \( \mu \)mol/L higher in the folate group compared with the placebo-supplemented group after 3 y of daily supplementation with 800 \( \mu \)g/d of folic acid. With a dose of 1 mg/d folate, we found the mean difference in erythrocyte folate between the folate and placebo groups from 1 to 2 y was 2.03 \( \mu \)mol/L.

In conclusion, our results showed that 12 mo of daily folate supplementation with 1 mg folate produced steady-state erythrocyte folate concentration. These results may be relevant for the design of surveys to monitor changing folic acid intakes in populations. For example, the lag between the introduction of folic acid fortification of a food supply and the measurement of erythrocyte folate in the population should be at least 12 mo; otherwise, the impact of folate fortification on folate status will be underestimated. Likewise, at least 12 mo of supplementation are needed to establish a valid and quantitative dose-response relationship between folate intake and erythrocyte folate concentrations. Results from further trials are needed to determine the effect of dose of folate on the time to steady-state erythrocyte folate concentrations.

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Literature Cited

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