Vitamin B<sub>12</sub> and Folate and the Risk of Anemia in Old Age

The Leiden 85-Plus Study

Wendy P. J. den Elzen, MSc; Rudi G. J. Westendorp, MD, PhD; Marijke Frölich, PhD; Wouter de Ruijter, MD; Willem J. J. Assendelft, MD, PhD; Jacobijn Gussekloo, MD, PhD

**Background:** Screening for deficiencies in vitamin B<sub>12</sub> and folate is advocated to prevent anemia in very elderly individuals. However, the effects of vitamin B<sub>12</sub> and folate deficiency on the development of anemia in old age have not yet been established.

**Methods:** The current study is embedded in the Leiden 85-Plus Study, a population-based prospective study of subjects aged 85 years. Levels of vitamin B<sub>12</sub>, folate, and homocysteine were determined at baseline. Hemoglobin levels and mean corpuscular volume (MCV) were determined annually during 5 years of follow-up.

**Results:** We analyzed data from 423 subjects who did not use any form of cyanocobalamin, hydroxocobalamin, or folic acid supplementation, neither at baseline nor during follow-up. Folate deficiency (<7 nmol/L; n=34) and elevated homocysteine levels (>13.5 µmol/L; n=194) were associated with anemia at baseline (adjusted odds ratio [OR], 2.44; 95% confidence interval [CI], 1.06-5.61; and adjusted OR, 1.82; 95% CI, 1.08-3.06, respectively), but vitamin B<sub>12</sub> deficiency (<150 pmol/L; n=68) was not (adjusted OR, 1.51; 95% CI, 0.79-2.87). Furthermore, vitamin B<sub>12</sub> deficiency was not associated with the development of anemia during follow-up (adjusted HR, 0.92; 95% CI, 0.46-1.82) or with changes in MCV (adjusted linear mixed model; P=.77). Both folate deficiency and elevated homocysteine levels were associated with the development of anemia from age 85 years onward (adjusted HR, 3.33; 95% CI, 1.55-7.14; and adjusted HR, 1.70; 95% CI, 1.01-2.88, respectively), but not with an increase in MCV over time (P>.30).

**Conclusion:** In the general population of very elderly individuals, anemia in 85-year-old subjects is associated with folate deficiency and elevated homocysteine levels but not with vitamin B<sub>12</sub> deficiency.

Arch Intern Med. 2008;168(20):2238-2244
Leiden, the Netherlands. From September 1997 through September 1999, 705 inhabitants of Leiden reached the age of 85 years and were eligible for participation in the study. No other selection criteria were applied. Fourteen subjects died prior to enrollment in the study, and 92 subjects refused to participate. Seven subjects died before blood sample collection, and 30 subjects refused blood sampling. Vitamin B12 levels could not be measured for 3 subjects owing to analytical difficulties. As a result, data for 559 subjects were available at baseline.

Subjects were visited annually (at ages 85-90 years) at their place of residence to participate in face-to-face interviews, to perform several (cognitive) tests (eg, Mini-Mental State Examination17,18), and to obtain a venous blood sample. General practitioners and nursing home physicians were interviewed annually to obtain information on patients' medical history. Mortality data were obtained from the municipal registry. All subjects gave their informed consent, and the medical ethics committee of Leiden University Medical Center approved the study.

Pharmacy records were studied annually, and subjects were interviewed to evaluate the use of any form of (over-the-counter) supplements containing cyanocobalamin, hydroxocobalamin, or folic acid (including multivitamins) at baseline and during the follow-up period. A total of 136 subjects who used any form of cyanocobalamin, hydroxocobalamin, or folic acid supplements at baseline (n = 92) or during follow-up (n = 44) were excluded from the present study. Treating physicians were not informed about the individual levels of vitamin B12, folate, and homocysteine during the study because the analysis of serum samples was performed 2 to 3 years after blood samples were drawn.

Figure 1 shows the annual number of subjects from ages 85 through 90 years for whom hemoglobin (Hb) levels and mean corpuscular volume (MCV) were determined, starting with 423 subjects at age 85 years (baseline). The annual mortality rate was approximately 10%, and the annual refusal rate was below 5%.

MAIN LABORATORY MEASUREMENTS

The Hb levels and MCV were determined annually (at ages 85-90 years) with an automated analysis system (Coulter Counter; Coulter Electronics, Hialeah, Florida). Anemia was defined according to World Health Organization criteria19 (Hb <12 g/dL for women and Hb <13 g/dL for men). An MCV higher than 100 µm² was considered to be elevated. (To convert Hb to millimoles per liter, multiply by 0.625; to convert Hb to grams per liter, multiply by 10.0; to convert MCV to femtoliters, multiply by 1.0.)

Serum samples were stored at −80°C. Serum levels of vitamin B12 and folate at age 85 years were determined in 1 batch using the Dual Count Solid Phase No-Boil Assay (Diagnostic Products Corp, Los Angeles, California). Homocysteine levels for all subjects at age 85 years were measured in 1 batch with a fluorescence polarization immunoassay on an IMx analyzer (Abbott, Abbott Park, Illinois) after reduction to the free form. Vitamin B12 deficiency was defined as serum vitamin B12 levels lower than 150 pmol/L. Folate deficiency was defined as serum folate levels lower than 7 mmol/L. Serum homocysteine levels higher than 13.5 µmol/L were considered to be elevated. (To convert serum vitamin B12 to picograms per milliliter, divide by 0.7378; to convert folate to nanograms per milliliter, divide by 2.466.)

POSSIBLE CONFOUNDERS

Sex, level of education, income, institutionalization, the presence of (chronic) disease, and renal function were considered possible confounding variables in the present study. Information on the presence of (chronic) disease at age 85 years (stroke, myocardial infarction, severe cognitive impairment, diabetes mellitus, Parkinson disease, hip fracture, arthritis, obstructive lung disease, and malignant lesion) was obtained from general practitioners, nursing home physicians, and pharmacy records.20 The presence of severe cognitive impairment was based on diagnosis by the general practitioner or on a Mini-Mental State Examination score of less than 19. C-reactive protein (CRP) levels were measured as an additional marker of health status with a fully automated Hitachi 747 system (Hitachi, Tokyo, Japan). Creatinine clearance was measured as a proxy for renal function and was determined by the Cockcroft-Gault equation.21

STATISTICAL ANALYSIS

Cross-sectional Analyses

Differences in proportions of subjects with anemia and proportions of subjects with elevated MCV between subjects with vitamin B12 deficiency vs those without, with folate deficiency vs those without, and with elevated homocysteine levels vs those without at age 85 years were tested with χ² tests. Differences in Hb levels and MCV between groups were tested with Mann-Whitney tests. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the relation between vitamin B12 deficiency and anemia, folate deficiency and anemia, and elevated homocysteine levels and anemia at the age of 85 years. Logistic regression models were used to adjust for possible confounders (sex, level of education, income, institutionalization, the presence of ≥1 [chronic] diseases, CRP levels, and creatinine clearance).

Prospective Analyses

For the prospective analyses, subjects with anemia at baseline (ie, at age 85 years) were excluded. The effects of vitamin B12 deficiency, folate deficiency, and elevated homocysteine level at baseline (age 85 years) on the development of anemia during follow-up were investigated with Kaplan-Meier curves and Cox proportional hazard models. If a new case of anemia was identi-
At age 85 years, vitamin B₁₂ deficiency and folate deficiency were both associated with the presence of elevated homocysteine levels (ORs, 1.87 [95% CI, 1.10-3.16] and 10.3 [95% CI, 3.56-29.8], respectively). Vitamin B₁₂ deficiency was not associated with the presence of anemia (crude OR, 1.23 [95% CI, 0.69-2.18]; adjusted OR, 1.51 [95% CI, 0.79-2.87]; Table 2). There were no differences in Hb levels between subjects with vitamin B₁₂ deficiency and those with vitamin B₁₂ levels within reference range (P = .59). Subjects with vitamin B₁₂ deficiency had a higher median MCV than those with vitamin B₁₂ levels within reference range (93 µm³ [IQR, 90-96 µm³] vs 91 µm³ [IQR, 88-94 µm³]; P = .01).

Folate deficiency was associated with anemia at age 85 years (crude OR, 2.79 [95% CI, 1.37-5.69]; adjusted OR, 2.44 [95% CI, 1.06-5.61]). Subjects with folate deficiency had lower Hb levels (P < .01) and higher MCV (P = .07) compared with subjects with folate levels within reference range.

Subjects with elevated homocysteine levels had an increased risk of anemia (crude OR, 2.81 [95% CI, 1.79-4.42]; adjusted OR, 1.82 [95% CI, 1.08-3.06]) and lower Hb levels compared with subjects with homocysteine levels within reference range (P < .01). No differences were observed in MCV between subjects with elevated homocysteine levels and subjects with homocysteine levels within reference range (P = .91). In an adjusted logistic regression model including vitamin B₁₂ deficiency, folate deficiency, and elevated homocysteine levels, no independent association with anemia was found (ORs, respectively, 1.40 [95% CI, 0.73-2.70], 2.02 [95% CI, 0.85-4.77], and 1.56 [95% CI, 0.90-2.70]).

**PROSPECTIVE DATA**

Of the 313 subjects without anemia at baseline, 72 subjects developed anemia in 1004 observed person-years (incidence rate: 7.2 per 100 person-years at risk). Figure 2 shows the occurrence of anemia during follow-up, with respect to vitamin B₁₂, folate, and homocysteine status at baseline. Vitamin B₁₂ deficiency was not associated with the incidence of anemia during follow-up (crude HR, 0.85 [95% CI, 0.43-1.65]). Furthermore, adjustment for sex, level of education, income, institutionalization, the presence of 1 or more (chronic) diseases, CRP levels, and creatinine clearance did not change the estimate.
Subjects with folate deficiency had an increased risk of developing anemia over time compared with those with folate levels within reference range (crude HR, 3.16 [95% CI, 1.57-6.37]; adjusted HR, 3.33 [95% CI, 1.55-7.14]). Subjects with elevated homocysteine levels were also at an increased risk of developing anemia (crude HR, 1.96 [95% CI, 1.23-3.12]; adjusted HR, 1.70 [95% CI, 1.01-2.88]).

In an adjusted Cox regression model including vitamin B12 deficiency, folate deficiency, and elevated homocysteine levels, only folate deficiency was independently associated with the incidence of anemia (HRs, respectively, 0.85 [95% CI, 0.43-1.69], 2.77 [95% CI, 1.24-6.21], and 1.48 [95% CI, 0.85-2.55]).

Figure 3 shows Hb levels and MCV during the 5-year follow-up period with respect to the presence or absence of vitamin B12 deficiency, folate deficiency, and elevated homocysteine levels at baseline. The analyses were adjusted for sex, level of education, income, institutionalization, the presence of (chronic) diseases, CRP levels, and creatinine clearance. In subjects with vitamin B12 deficiency, changes in Hb levels were similar to those with vitamin B12 levels within reference range (P = .38). Subjects with folate deficiency had an accelerated decline in Hb levels compared with subjects with folate levels within reference range (additional annual decline, −0.19 g/dL [95% CI, −0.30 to −0.07]; P = .05). Subjects with elevated homocysteine levels also showed an accelerated decline in Hb levels when compared with subjects with homocysteine levels within reference range (additional annual decline, −0.10 g/dL [95% CI, −0.15 to −0.04]; P = .02). After excluding subjects with folate deficiency from the analyses, elevated homocysteine levels were no longer associated with the decline in Hb level (P = .13).

Table 2. Hemoglobin (Hb) Levels and MCV Depending on the Presence of Vitamin B12 Deficiency, Folate Deficiency, and Elevated Homocysteine Levels at Age 85 Years

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vitamin B12</th>
<th>Folate</th>
<th>Homocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Hb level, g/dL (IQR)</td>
<td>13.0 (12.2-13.8)</td>
<td>13.0 (12.2-13.8)</td>
<td>12.4 (11.6-13.1)</td>
</tr>
<tr>
<td>RR (n=355)</td>
<td>13.3 (12.5-13.8)</td>
<td>12.8 (11.8-13.6)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Deficiency (n=68)</td>
<td>25.4</td>
<td>29.4</td>
<td>47.1</td>
</tr>
<tr>
<td>P Value</td>
<td>.59</td>
<td>.48</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Median MCV µm³ (IQR)</td>
<td>91 (88-94)</td>
<td>93 (90-96)</td>
<td>92 (90-96)</td>
</tr>
<tr>
<td>RR (n=389)</td>
<td>91 (89-94)</td>
<td>91 (88-95)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Deficiency (n=34)</td>
<td>2.5</td>
<td>1.5</td>
<td>2.2</td>
</tr>
<tr>
<td>P Value</td>
<td>.60</td>
<td>.21</td>
<td>.26</td>
</tr>
<tr>
<td>MCV &gt;100 µm³, %</td>
<td>1.0</td>
<td>5.9</td>
<td>2.6</td>
</tr>
<tr>
<td>100 µm³, %</td>
<td>2.5</td>
<td>2.1</td>
<td>.79</td>
</tr>
</tbody>
</table>

Abbreviations: IQR, interquartile range; MCV, mean corpuscular volume; RR, reference range.

Conversion factors: To convert folate to nanograms per milliliter, divide by 2.266; to convert Hb to millimoles per liter, multiply by 0.625; to convert serum vitamin B12 to picograms per milliliter, divide by 0.7378; to convert Hb to grams per liter, multiply by 10.0; to convert MCV to femtoliters, multiply by 1.0.

Data are presented as median (IQR) or percentage; P values were obtained by Mann-Whitney (continuous variables) or χ² tests (dichotomous variables).

Vitamin B12 level lower than 150 pmol/L.
Folate level lower than 7 nmol/L.
Homocysteine level greater than 13.5 µmol/L.
Anemia defined according to World Health Organization criteria: Hb level lower than 12 g/dL for women and lower than 13 g/dL for men.

Conversion factors: To convert folate to nanograms per milliliter, divide by 2.266; to convert Hb to millimoles per liter, multiply by 0.625; to convert serum vitamin B12 to picograms per milliliter, divide by 0.7378; to convert Hb to grams per liter, multiply by 10.0; to convert MCV to femtoliters, multiply by 1.0.

A. The effect of vitamin B12 deficiency (<=150 pmol/L); hazard ratio (HR), 0.85; 95% confidence interval (CI), 0.43-1.65. B. Folate deficiency (<7 nmol/L); HR, 3.16; 95% CI, 1.57-6.37. C. Elevated homocysteine levels (>13.5 µmol/L); HR, 1.96; 95% CI, 1.23-3.12. To convert serum vitamin B12 to picograms per milliliter, divide by 0.7378; to convert folate to nanograms per milliliter, divide by 2.266.
There were no differences in changes in MCV between subjects with vitamin B12 deficiency vs those without ($P = .77$), or between subjects with folate deficiency vs those without ($P = .39$), or between subjects with elevated homocysteine levels vs those without ($P = .73$). The present study shows that among very elderly individuals in the general population, both folate deficiency and elevated homocysteine levels were associated with...
the presence of anemia at baseline and the development of anemia during follow-up. No such relationship was present between vitamin B12 deficiency and anemia.

Although (macrocytic) anemia is one of the most generally known consequences of vitamin B12 deficiency, we did not find an association between vitamin B12 deficiency and the presence or development of anemia within the general population of very elderly persons. The present data are, however, in line with clinical trials in mainly elderly subjects with low or subnormally low vitamin B12 levels (detected by screening), without any other clinical symptoms, which showed that cyanocobalamin administration had no effect on Hb levels. In a previous study, we also showed that low vitamin B12 levels at age 85 years do not predict accelerated deterioration in cognitive function. These observational findings were in line with other observational studies and with several randomized controlled trials that showed no effect of cyanocobalamin administration on cognitive function as well. This accumulating evidence suggests that clinicians should reconsider starting cyanocobalamin or hydroxocobalamin supplementation in very elderly patients (≥85 years) with low vitamin B12 levels.

Because the metabolic pathways of vitamin B12, folate, and homocysteine are tightly entwined, it is difficult to unravel which deficiency is the direct cause of anemia. When combining vitamin B12 deficiency, folate deficiency, and elevated homocysteine levels in 1 Cox proportional hazard model, only folate deficiency remained independently associated with the development of anemia in our study population. One possible explanation is that folate is more critical for DNA synthesis than vitamin B12. Within the cell, folate is converted into 5-methyl-tetrahydrofolate; vitamin B12 is converted into methylcobalamin, which is a coenzyme for methionine synthesis. Methionine synthase converts homocysteine into methionine by transferring a methyl group from 5-methyl-tetrahydrofolate to homocysteine, thereby lowering homocysteine levels. This reaction converts 5-methyl-tetrahydrofolate into tetrahydrofolate (THF); THF is essential for the formation of thymidylate and purine synthesis, both of which are required for DNA replication. When THF production is decreased, DNA synthesis is delayed, and anemia develops owing to ineffective erythropoiesis and destruction of abnormal circulating red cells. Thus, folate is a direct precursor of the components of DNA, whereas vitamin B12 is only indirectly involved in DNA synthesis by assisting in the conversion of 5-methyl-tetrahydrofolate into THF. Because vitamin B12 is a precursor of a coenzyme, it can assist many subsequent enzymatic reactions, and even severely low levels of vitamin B12 may be sufficient to produce adequate levels of THF. In this light, folate acid supplementation could increase the level of THF and directly restore erythropoiesis, but the effects of cyanocobalamin or hydroxocobalamin supplementation on erythropoiesis may be smaller. Because the strong association between elevated homocysteine levels and anemia disappears after adjustment for the presence of folate deficiency, elevated homocysteine levels will identify older subjects at increased risk of developing anemia, but homocysteine does not seem to be causally related to anemia in old age.

Strong points of the present study are its prospective design, an almost complete follow-up, and the population-based setting, allowing us to unravel the temporal relationship between vitamin B12 deficiency, folate deficiency, and subsequent changes in Hb levels and MCV in the general population of older persons. In addition, we measured serum homocysteine levels because it is well known that serum levels of vitamin B12 and folate may not accurately reflect the vitamins’ true status within tissues.

It may be considered a limitation of our study that we evaluated only subjects aged 85 years or older. However, it is important to study anemia in very elderly subjects, not only because they are the fastest growing segment of the general population in industrialized societies but also because the prevalence of anemia and deficiencies in vitamin B12 and folate rapidly increase with age. More important, results from studies in elderly subjects who are younger than 85 years cannot be extrapolated to those 85 years or older because the risk of common determinants of disease and mortality in middle age, such as hypothyroidism, hypertension, and hypercholesterolemia, have already been shown to reverse or disappear in those 85 years or older. For the same reasons, the results of the present study should not be extrapolated to the population of those younger than 85 years.

The small number of subjects with folate deficiency, especially in the prospective analyses, may also be considered a potential limitation of our study. Yet, the fact that even with this low number of subjects we still find a statistically significant association between folate deficiency and the development of anemia (adjusted HR, 3.33; 95% CI, 1.55-7.14) and changes in Hb levels over time (P = 0.05) suggests that folate deficiency is a strong risk factor for developing anemia in old age.

Another limitation of our study could be that vitamin B12, folate, and homocysteine levels were not measured annually. Nevertheless, we believe that correction of low vitamin B12 and folate levels is not underlying the lack of association between low vitamin B12 levels and the development of anemia during follow-up. First, subjects known to have used cyanocobalamin, hydroxocobalamin, or folic acid supplements at baseline or during follow-up were excluded from the analyses. Second, treating physicians were not informed about the results of the vitamin B12, folate, and homocysteine measurements during the study because the laboratory analyses were performed 2 to 3 years after blood samples were drawn.

In conclusion, anemia is an important health issue in elderly individuals because it is highly prevalent in this sector of the population and is associated with poor functional status and increased mortality. Based on our findings, however, screening and subsequent treatment of low vitamin B12 levels may not have any beneficial effect on the incidence of anemia in old age. Although the biochemical pathways suggest that folic acid supplementation is beneficial, it remains to be seen if folate acid fortification of grain and cereal products (a practice in the United States has) has a positive effect on the incidence of anemia in the very elderly population.
Accepted for Publication: April 20, 2008.
Correspondence: Wendie P. J. den Elzen, MSc, Department of Public Health and Primary Care, Leiden University Medical Center, Post Zone V-0-P, PO Box 9600, 2300 RC Leiden, the Netherlands (w.p.j.den_elzen@lumc.nl).
Author Contributions: Dr Gussekloo had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Westendorp and Gussekloo. Acquisition of data: Westendorp, Frolich, and Gussekloo. Analysis and interpretation of data: den Elzen, Westendorp, Frolich, de Ruijter, Assendelft, and Gussekloo. Drafting of the manuscript: den Elzen and Gussekloo. Critical revision of the manuscript for important intellectual content: den Elzen, Westendorp, Frolich, de Ruijter, Assendelft, and Gussekloo. Statistical analysis: den Elzen, Westendorp, and Gussekloo. Obtained funding: Westendorp and Gussekloo. Administrative, technical, and material support: den Elzen and Frolich. Study supervision: Westendorp, Assendelft, and Gussekloo.
Financial Disclosure: None reported.
Funding/Support: The Leiden 85-Plus Study was funded in part by the Dutch Ministry of Health, Welfare, and Sports. Homocysteine measurements were funded in part by an unrestricted grant from Abbott Laboratories.

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