Vitamin B12 and folate and the risk of anemia in old age. The Leiden 85-plus Study

Wendy PJ den Elzen, Rudi GJ Westendorp, Marijke Frölich, Wouter de Ruijter, Willem JJ Assendelft, Jacobijn Gussekloo

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ABSTRACT

Background Screening for deficiencies in vitamin B12 and folate is advocated to prevent anemia in very elderly individuals. However, the effects of vitamin B12 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 B12, 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 B12 deficiency (<150 pmol/L; n=68) was not (adjusted OR 1.51 [95% CI 0.79-2.87]). Furthermore, vitamin B12 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=0.77). 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>0.30).

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 B12 deficiency.
INTRODUCTION

The prevalence of anemia in elderly individuals aged 65 years or older is around 10% and increases with age to above 20% in subjects aged 85 years or older.1;2 Evidence of coincidental nutritional deficiencies (iron, vitamin B12, and folate deficiency) can be found in clinical examinations of about one-third of older persons with anemia.2

Because deficiencies in vitamin B12 and folate are associated not only with macrocytic anemia but also with dementia, peripheral neuropathy, subacute combined degeneration, and cardiovascular disease (owing to elevated homocysteine levels),3-6 low levels of these vitamins are usually supplemented.4;5;7 And, because of the high prevalence of both deficiencies in elderly individuals (>10% among people aged 75 years),8-12 screening for deficiencies in vitamin B12 and folate as part of a geriatric workup has also often been recommended.13;14

Although the prevalence of vitamin B12 and folate deficiency and the prevalence of anemia are highest in the oldest age groups (≥85 years), to our knowledge, the association between vitamin B12 and folate deficiency and anemia in old age has not been prospectively studied previously. Therefore, we investigated whether deficiencies in vitamin B12 and folate are associated with the occurrence of anemia in the general population of very elderly persons (≥85 years). Because serum levels of vitamin B12 and folate alone may not accurately reflect vitamin status at tissue level, we also studied the effect of elevated serum homocysteine concentration on anemia, which is considered to be a more sensitive marker of these deficiencies at tissue level.6;15;16

METHODS

Study population and procedures

The present study is embedded in the Leiden 85-plus Study, a population-based prospective follow-up study of 85-year-old inhabitants of 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 (e.g. 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 criteria (Hb <12 g/dL for women and Hb <13 g/dL for men). An MCV higher than 100 µm³ 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 nmol/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 2.266.)

**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,
Vitamin B12, folate and risk of anemia

Parkinson disease, hip fracture, arthritis, obstructive lung disease, and malignant lesion) was obtained from general practitioners, nursing home physicians, and pharmacy records. 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.

Table 1. Annual number of subjects for whom hemoglobin levels and mean corpuscular volume were determined from age 85 through 90 years.

<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>n</th>
<th>Refused</th>
<th>Died</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td>423</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>86</td>
<td>368</td>
<td>10</td>
<td>42</td>
</tr>
<tr>
<td>87</td>
<td>316</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>88</td>
<td>275</td>
<td>13</td>
<td>36</td>
</tr>
<tr>
<td>89</td>
<td>226</td>
<td>13</td>
<td>36</td>
</tr>
<tr>
<td>90</td>
<td>190</td>
<td>13</td>
<td>23</td>
</tr>
</tbody>
</table>

Figure 1. Annual number of subjects for whom hemoglobin levels and mean corpuscular volume were determined from age 85 through 90 years.

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 $\chi^2$-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 $\geq 1$ [chronic] diseases, CRP levels, and creatinine clearance).

Prospective analyses

For the prospective analyses, subjects with anemia at baseline (i.e. 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 identified, it was assumed that the anemia had developed halfway through the annual follow-up period. The Cox proportional hazard models were also adjusted for possible confounders.

Between-group differences in changes in Hb levels and MCV during follow-up were estimated with linear mixed models, with adjustment for possible confounding variables. Results were presented as predicted means and their standard errors. For the models examining changes in MCV, subjects at age 85 years with MCV >100 µm$^3$ were excluded.

Data analyses were performed using SPSS statistical software (version 12.0.1 for Windows; SPSS Inc, Chicago, Illinois).

RESULTS

Study population

Table 1 describes the baseline characteristics of the study population. At age 85 years, 34.0% of the population were male, and 20.3% were institutionalized into care homes for the elderly. The prevalence of anemia at baseline was 26.0% (n=110). Most subjects with anemia had normocytic anemia (MCV 80-100 µm$^3$ [92.7%]). Macrocytic anemia (MCV >100 µm$^3$) was observed in only 2 subjects (1.8%). Six subjects (5.5%) had microcytic anemia (MCV <80 µm$^3$).

The median serum vitamin B12 level was 264.6 pmol/L (interquartile range [IQR] 183.4-369.6 pmol/L); 68 subjects (16.1%) had vitamin B12 deficiency (<150 pmol/L). The median folate level was 11.9 nmol/L (IQR 9.3-16.1 nmol/L); 34 subjects (8.0%) had folate deficiency (<7 nmol/L). Elevated homocysteine levels (>13.5 µmol/L) were observed in 194 subjects (45.9% of the study population).
**Table 1.** Baseline characteristics of 423 subjects aged 85 years*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sociodemographic characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>144 (34.0)</td>
</tr>
<tr>
<td>Low level of education (elementary school only)†</td>
<td>276 (65.7)</td>
</tr>
<tr>
<td>Low income (less than $1100 per month)‡</td>
<td>220 (52.9)</td>
</tr>
<tr>
<td>Institutionalized</td>
<td>86 (20.3)</td>
</tr>
<tr>
<td><strong>Hematological characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Anemia§</td>
<td>110 (26.0)</td>
</tr>
<tr>
<td>Macrocytic anemia (MCV &gt;100 µm³)</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>Normocytic anemia (MCV 80-100 µm³)</td>
<td>102 (92.7)</td>
</tr>
<tr>
<td>Microcytic anemia (MCV &lt;80 µm³)</td>
<td>6 (5.5)</td>
</tr>
<tr>
<td>Hb, g/dL</td>
<td>13.0 (12.2-13.8)</td>
</tr>
<tr>
<td>MCV, µm³</td>
<td>91 (88-94)</td>
</tr>
<tr>
<td>MCV &gt;100 µm³</td>
<td>10 (2.4)</td>
</tr>
<tr>
<td><strong>Vitamin characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Serum vitamin B12, pmol/L</td>
<td>264.6 (183.4-369.6)</td>
</tr>
<tr>
<td>Vitamin B12 deficiency (&lt;150 pmol/L)</td>
<td>68 (16.1)</td>
</tr>
<tr>
<td>Serum folate, mmol/L</td>
<td>11.9 (9.3-16.1)</td>
</tr>
<tr>
<td>Folate deficiency (&lt;7 nmol/L)</td>
<td>34 (8.0)</td>
</tr>
<tr>
<td>Serum homocysteine, µmol/L</td>
<td>13.1 (10.8-16.5)</td>
</tr>
<tr>
<td>Elevated homocysteine (&gt;13.5 µmol/L)</td>
<td>194 (45.9)</td>
</tr>
<tr>
<td><strong>Other clinical characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>(Chronic) diseases ‖</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>101 (24.0)</td>
</tr>
<tr>
<td>One</td>
<td>176 (41.9)</td>
</tr>
<tr>
<td>Two or more</td>
<td>143 (34.0)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>4 (1-8)</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min¶</td>
<td>44.3 (37.4-52.0)</td>
</tr>
</tbody>
</table>

Abbreviations: CRP, C-reactive protein; Hb, hemoglobin; MCV, mean corpuscular volume
Conversion factors: to convert serum vitamin B12 to picograms per milliliter, divide by 0.7378; to convert C-reactive protein to nanomoles per liter, multiply by 9.524; to convert folate to nanograms per milliliter, divide by 2.266; 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.
* Continuous data are presented as median (interquartile range); categorical data are presented as number (percentage).
† There are 3 missing values
‡ There are 7 missing values
§ 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
‖ Stroke, myocardial infarction, severe cognitive impairment, diabetes mellitus, Parkinson disease, hip fracture, arthritis, obstructive lung disease and malignant lesion; 3 missing values
¶ The Cockcroft-Gault Formula was used; there are 11 missing values
Cross-sectional data

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=0.59). 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=0.01).

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<0.01) and higher MCV (p=0.07) 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=0.01). No differences were observed in MCV between subjects with elevated homocysteine levels and subjects with homocysteine levels within reference range (p=0.91).

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 (adjusted HR 0.92 [95% CI 0.46-1.82]).
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></th>
<th>Vitamin B12</th>
<th>Folate</th>
<th>Homocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR (n=355)</td>
<td>Deficiency† (n=68)</td>
<td>p</td>
</tr>
<tr>
<td>Median Hb level, g/dL (IQR)</td>
<td>13.0 (12.2-13.8)</td>
<td>13.0 (12.2-13.6)</td>
<td>0.99</td>
</tr>
<tr>
<td>Anemia, %</td>
<td>25.4</td>
<td>29.4</td>
<td>0.48</td>
</tr>
<tr>
<td>Median MCV, µm³ (IQR)</td>
<td>91 (88-94)</td>
<td>93 (90-96)</td>
<td>0.01</td>
</tr>
<tr>
<td>MCV &gt;100 µm³, %</td>
<td>2.5</td>
<td>1.5</td>
<td>0.60</td>
</tr>
</tbody>
</table>

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

*Data are presented as median (interquartile range) or %; P-values were obtained by Mann-Whitney U-tests (continuous variables) or by 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
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=0.38). 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=0.05). 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=0.02). After excluding subjects with folate deficiency from the analyses, elevated homocysteine levels were no longer associated with the decline in Hb level (p=0.13).

There were no differences in changes in MCV between subjects with vitamin B12 deficiency vs those without (p=0.77), or between subjects with folate deficiency vs those without (p=0.39), or between subjects with elevated homocysteine levels vs those without (p=0.73).

**COMMENT**

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.\textsuperscript{22-24} In a previous study,\textsuperscript{25} 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.\textsuperscript{26-28} 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 a 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 synthase. 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.\textsuperscript{5,29} When THF production is decreased, DNA synthesis is delayed, and anemia develops owing to ineffective erythropoiesis and destruction of abnormal circulating red cells.\textsuperscript{30,31} 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, folic 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 disappeared 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.
Figure 2. The effect of vitamin B12 deficiency, folate deficiency, and elevated homocysteine levels on anemia during follow-up in subjects without anemia at age 85 years (n=313).
A. The effect of vitamin B₁₂ deficiency (<150 pmol/L); B. Folate deficiency (<7 nmol/L); C. Elevated homocysteine levels (>13.5 µmol/L).
Figure 3. Changes in hemoglobin (Hb) levels and mean corpuscular volume (MCV) during follow-up. Left panels: Changes in hemoglobin levels during follow-up depending on the presence of vitamin B12 deficiency (<150 pmol/L) (A), folate deficiency (<7 nmol/L) (C), and elevated homocysteine levels (>13.5 µmol/L) (E) at age 85 years. Subjects with anemia at age 85 years (n = 110) were excluded from the analyses. Right panels: Changes in MCV during follow-up depending on the presence of vitamin B12 deficiency (<150 pmol/L) (B), folate deficiency (<7 nmol/L) (D), and elevated homocysteine levels (>13.5 µmol/L) (F) at age 85 years. Subjects with MCV greater than 100 μm$^3$ at age 85 years (n = 10) were excluded from the statistical analyses. All changes were adjusted for sex, level of education, income, institutionalization, the presence of 1 or more (chronic) diseases (ie, stroke, myocardial infarction, severe cognitive impairment, diabetes mellitus, Parkinson disease, hip fracture, arthritis, obstructive lung disease, and malignant lesion), C-reactive protein levels, and creatinine clearance at baseline.
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 occurrence of anemia in old age, but early detection of folate deficiency by screening may identify elderly individuals at risk of developing anemia. Although the biochemical pathways suggest that folic acid
supplementation is beneficial, it remains to be seen if folic acid fortification of grain and cereal products (a practice in the United States\textsuperscript{44,45}) has a positive effect on the incidence of anemia in the very elderly population.

**FUNDING**

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