Free and Protein-Bound Cobalamin Absorption in Healthy Middle-aged and Older Subjects

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OBJECTIVE: To study free- and protein-bound cobalamin absorption and the correlation with atrophic gastritis in healthy middle-aged and older subjects.

DESIGN: A cross-sectional study.

PARTICIPANTS: Fifty-two healthy subjects, aged 26 to 87 years, apparently free from conditions known to influence the cobalamin status. Middle-aged subjects were defined as those younger than 65 years of age (median age 57 years) and older subjects as those 65 years and older (median age 75 years).

MEASUREMENTS: Protein-bound cobalamin absorption was assessed by 48-hour urinary excretion method following oral administration of scrambled egg yolk, labeled in vivo with ^{57}Co-cobalamin by injecting a hen with ^{57}Co-cyanocobalamin. The percentage of ^{57}Co-cobalamin bound to protein was 65%. Free cobalamin absorption was assessed by 48-hour urinary excretion method following oral administration of crystalline ^{57}Co-cyanocobalamin. Plasma cobalamin, folate and fasting plasma gastrin, and pepsinogen A and C concentrations were determined.

RESULTS: The median urinary excretion of egg yolk ^{57}Co-cobalamin in middle-aged subjects was 12.3% (25th and 75th percentiles 10.5%-14.5%) compared with 11.7% (25th and 75th percentiles 9.8%-13.6%) in older subjects (P = .283). The median urinary excretion after administration of free ^{57}Co-cobalamin in middle-aged subjects was 25.7% (25th and 75th percentiles 20.6%-30.7%) compared with 27.9% (25th and 75th percentiles 21.4%-34.5%) in older subjects (P = .694). Neither egg yolk nor free ^{57}Co-cobalamin excretion correlated with age.

A ratio of pepsinogen A to pepsinogen C less than 1.6, indicating atrophic gastritis, was found in 13 subjects. Within the atrophic gastritis group, 11 subjects had a pepsinogen A concentration greater than or equal to 17 μg/L, indicating mild to moderate atrophic gastritis, and two subjects had a pepsinogen A concentration less than 17 μg/L, indicating severe atrophic gastritis or gastric atrophy. All subjects had normal fasting plasma gastrin concentrations. Free and egg yolk ^{57}Co-cobalamin excretions were not reduced in the atrophic gastritis group when compared with the non-atrophic gastritis group.

Median plasma cobalamin concentration was not significantly lower in older subjects (P = .205). Nonetheless, plasma cobalamin concentration correlated negatively with age (r = -.36; P = .008).

CONCLUSIONS: We demonstrated no significant difference in either free or protein-bound cobalamin absorption between healthy middle-aged and older adults. In addition, no alteration in cobalamin absorption was found in subjects identified as having mild to moderate atrophic gastritis. Therefore, based on our results, the high prevalence of low cobalamin levels in older people cannot be explained by either the aging process or mild to moderate atrophic gastritis. J Am Geriatr Soc 44:949–953, 1996.

Many investigators have reported on the strikingly increased prevalence of low serum cobalamin concentrations in older people.[1-6] Recent studies have presented evidence that these low serum cobalamin levels, in most cases, reflect tissue cobalamin deficiency.[6,7] Moreover, it has been recognized that cobalamin treatment of patients with low serum cobalamin levels, but without the classical features of deficiency, may correct a number of subtle metabolic, neurological, and psychiatric abnormalities.[8] Therefore, it is of special interest to investigate the underlying mechanism of the low serum cobalamin levels found frequently in older people. Several pathophysiological possibilities can come into consideration. Dietary causes generally do not play an important role.[9] In addition, the intestinal absorption of free cobalamin, as measured by the classical Schilling test, is usually not decreased in older patients with low cobalamin levels.[10-15] Recently, however, low cobalamin levels have been related to protein-bound or food cobalamin malabsorption.[12,16-18] Protein-bound cobalamin malabsorption is thought to result from an impaired release of cobalamin from its binders in food, because of reduced gastric acid secretion, in the presence of adequate intrinsic factor secretion.[19-22] Indeed, subjects with a- or hypochlorhydria, most commonly caused by atrophic gastritis, have lower serum cobalamin levels.[23] Since the prevalence of atrophic gastritis increases with age, it has been hypothesized that low serum cobalamin levels in older people are the result of decreased protein-bound malabsorption caused by atrophic gastritis. However, as yet, only a few studies (and with conflicting results), have

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specifically addressed this question.[16-18,24] Therefore, we carried out a study on cobalamin absorption, both free and protein-bound, and its relation to atrophic gastritis in healthy middle-aged and older subjects. We used scrambled egg yolk labeled in vivo with ^{57}Co-cobalamin to investigate the protein-bound cobalamin absorption.[25] We selected this test meal because in vivo labeling, as opposed to in vitro labeling, ensures that ^{57}Co-cobalamin is bound naturally to its specific binders. Furthermore, scrambled egg yolk, when compared with raw chicken serum, is a commonly used food. We demonstrate that free and protein-bound cobalamin absorption in healthy volunteers are not affected by either age or mild to moderate atrophic gastritis.

METHODS

Preparation of Protein-Bound ^{57}Co-Cobalamin

On three consecutive days, 250 kBq ^{57}Co-cyanocobalamin (Kodak Clinical Diagnostics LTD, Amersham) was injected into the breast of an egg-laying hen (in total 750 kBq = 20 μCi). Radioactivity in eggs was detected 1 or 2 days after the first injection, reaching peak activity at days 6 to 8. Eggs numbered 2 to 16 had a total activity of 300 kBq ^{57}Co-cobalamin. Yolks were separated from the egg whites. Because no radioactivity was recovered in egg whites, only yolks were used to prepare the test meal. The yolks were homogenized and fried in a pan under constant stirring (scrambled) until a medium dry texture was obtained. Twelve to fourteen portions of about 12 g each, containing approximately 18 kBq (0.5 μCi) ^{57}Co-cobalamin, were packaged in plastic containers and stored in a freezer at −20°C until served. The amount of radioactivity was measured for each egg portion.

Assessment of Protein-Binding in Raw and Fried Egg Yolk

Thin layer chromatography was performed with raw in vivo labeled egg yolk and free ^{57}Co-cyanocobalamin on silica gel impregnated glass fiber sheets (Gelman Sciences, Michigan) using 0.15 *M* acetate buffer, pH 6.0. The sheet was cut into marked 1-cm segments, which were counted for radioactivity in a sodium-iodide well crystal.

To determine the effect of heat on protein binding, 1 g of raw and 1 g of scrambled in vivo labeled egg yolk were homogenized in a blender with 20 mL of 0.15 *M* acetate buffer and subsequently centrifuged at 15.000 *g* for 30 minutes at room temperature. The same procedure was performed after defrosting and heating frozen scrambled egg yolk for 30 seconds in a microwave oven (power 1300 W). Radioactivity was counted in the residue and supernatant and expressed as percentages of the radioactivity in the whole sample. All the above procedures were performed for two different samples of egg yolk from different batches.

Serological Measurements

Assays of plasma cobalamin, plasma folate, and folate in erythrocyte were performed by competitive radioisotope binding techniques using purified hog intrinsic factor as the cobalamin binder and purified β-lactoglobulin as the folate binder (Solid Phase Boil DualCount, Diagnostic Products Corporation, Los Angeles, CA).

Plasma samples were collected from fasted subjects and stored at −70°C. Fasting plasma gastrin was measured by radioimmunoassay,[26] reference range 10-70 pmol/L. Plasma pepsinogen A and pepsinogen C concentration were measured by sensitive and specific radioimmunoassays.[27] The reference range for pepsinogen A is 17 to 120 μg/L, for pepsinogen C, 1 to 40 μg/L, and for the ratio of pepsinogen A to pepsinogen C, greater than 1.6.[28]

Protocol

On the test day a portion of frozen scrambled egg yolk was defrosted and heated in a microwave oven for 30 seconds and served to the overnight-fasting subject for oral consumption. No food was allowed for the next 2 hours. Nonradioactive cyanocobalamin (Pharmachemie B.V. Haarlem Holland OPG Groep) was administered in a dose of 1 mg by intramuscular injection at t = 0 h and t = 24 h. Subjects collected urine from t = 0 h to t = 48 h.

The volumes of the urine collections were measured. In an 800-mL specimen from each 24-hour collection, radioactivity was counted for 1 minute on a well shielded sodium-iodide crystal. This was compared, under identical geometric conditions, with radioactivity of a freshly made, known ^{57}Co-standard. The urinary excretion of ^{57}Co-cobalamin was expressed as a percentage of the administered dose.

Measurement of free cobalamin absorption was performed after oral administration of a capsule containing 0.5 μCi (0.5-1 μg) free or crystalline ^{57}Co-cyanocobalamin (Mallinckrodt Medical, Inc. St. Louis, MO) to the overnight-fasting subject. Subsequently, the same procedure as described for the protein-bound cobalamin absorption test was followed. We were unable to test free cobalamin absorption in three younger subjects.

In all subjects the free cobalamin absorption test was performed at least 1 week after the egg yolk cobalamin absorption test. Pre-test urine specimens were all checked for radioactivity. Urinary creatinine excretions (mmol/24 hours) were measured to check for complete urine collection. Based on low urinary creatinine concentrations, incomplete collection was suspected in three absorption tests. However, the results of these tests were similar to the rest.

Subjects

The study protocol was approved by the Committee for Experimental Research with Humans of the University Hospital Nijmegen. All subjects gave written informed consent.

Healthy volunteers were recruited from the general population by means of advertisements in local newspapers. All participants were asked about their medical history, current medication, and alcohol use and underwent physical and laboratory examinations. The following exclusion criteria were applied: inability to comply with the protocol for urine collection (incontinence, cognitive impairment, impaired renal function (serum creatinine > 120 μmol/L)); pregnancy; vegetarianism; liver disease; alcoholism; previous stomach or intestinal surgery; intestinal diseases; chronic diarrhea; unexplained loss of weight; anemia; cobalamin or folate deficiency in the past, and ongoing treatment with cobalamin, folic acid, oral contraceptives, antacids, antibiotics, chloral hydrate, vitamin C, anticonvulsants, metformin, potassium salt, or colestyramine. The subjects lived at home, were self-sufficient and fully ambulant, and had no current illness. All subjects had normal hemoglobin, serum creatinine, ALAT, serum albumin, and serum iron concentrations. One older male subject had a mean cell volume of 105 fl; he had normal cobalamin and folate concentrations.

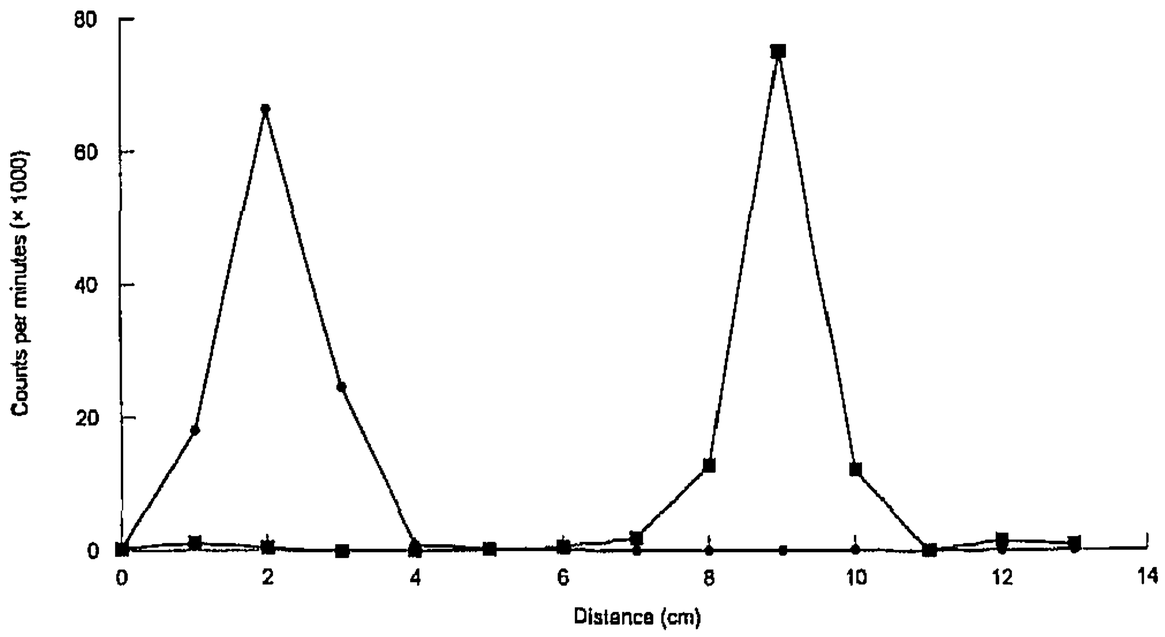

Figure 1. Thin layer chromatography of *in vivo* ^{57}Co-labeled raw egg yolk (•—•) and free ^{57}Co-cyanocobalamin (■—■). Measured radioactivity is plotted against the distance on the sheet.

Statistical Analysis

The results are presented as medians with the 25th and 75th percentiles. The Wilcoxon Rank Sum Test was used for comparing continuous variables of unpaired samples. Confidence intervals for differences in the mean were calculated. The Wilcoxon Signed Rank Test was used for comparing continuous variables of paired samples. The chi-square test was used to assess dependence between categorical variables. The relation between two continuous variables was studied with the Spearman correlation coefficient. A *P* value of .05 or less was considered significant.

RESULTS

Protein-Binding and the Effect of Heating

The results of thin layer chromatography of raw in vivo labeled egg yolk and free ^{57}Co-cyanocobalamin are shown in Figure 1. Most radioactivity of in vivo labeled raw egg yolk was recovered on the first segments of the sheet. In contrast, all radioactivity of free ^{57}Co-cyanocobalamin was recovered at the end of the sheet. Radioactivity in the residue of raw egg yolk was 92%. Radioactivity in the residue of scrambled egg yolk was 65%. Defrosting and heating of the frozen scrambled egg yolk meal in the microwave oven for 30 seconds did not alter this percentage.

Subjects

Fifty-two subjects were included in the study (Table 1). The subjects were divided into two groups: those younger than 65 years (middle-aged subjects, median age 57 years, range 26 to 65 years, n = 24) and those 65 years and older (older subjects, median age 75 years, range 66 to 87 years, n = 28).

Median plasma cobalamin concentration was lower in older subjects, although statistical significance was not reached ($P = .205$). Nonetheless, plasma cobalamin concentration correlated negatively with age (Spearman $r = -.36$; $P = .008$; n = 52). The relationship between plasma cobalamin concentrations (y) and age (x) is given by the equation y = 339-1.5x. Accordingly, the estimated decline in plasma cobalamin concentrations per year is 1.5 pmol/L.

The ratio of pepsinogen A to pepsinogen C was less than 1.6, indicating atrophic gastritis, in 13 subjects, 8 of whom were 65 years or older. In the atrophic gastritis group, 11 subjects had a pepsinogen A concentration equal to or greater than 17 μg/L, indicating mild to moderate atrophic gastritis. Two subjects had a pepsinogen A concentration less than 17 μg/L, indicating severe atrophic gastritis or gastric atrophy. The atrophic gastritis group had significantly higher plasma gastrin levels ($P = .026$). However, all subjects had normal (less than 70 pmol/L) fasting plasma gastrin levels. The ratio of pepsinogen A to pepsinogen C did not correlate with age or plasma cobalamin levels.

Egg Yolk and Free Cobalamin Absorption Test Results

The results of the egg yolk and free cobalamin absorption tests are presented in Table 2. The median urinary excretion of ^{57}Co-cobalamin after administration of egg yolk cobalamin was 12.3% in middle-aged subjects and 11.7% in older subjects ($P = .283$). The 95% confidence interval for the difference in mean egg yolk ^{57}Co-cobalamin excretion between middle-aged (12.9 ± 3.2, mean ± SD) and older subjects (12.1 ± 3.1, mean ± SD) was −0.958 to 2.558. The median urinary excretion of ^{57}Co-cobalamin following the oral administration of free cobalamin was 25.7% in middle-aged subjects and 27.9% in older subjects ($P = .694$). The 95% confidence interval for the difference in mean free ^{57}Co-cobalamin excretion between the middle-aged (26.1 ± 7.2, mean ± SD) and older subjects (27.1 ± 7.5, mean ± SD) was −5.282 to 3.282. The scatterplot showed no relation between the urinary excretion of ^{57}Co-cobalamin in both absorption tests and age. Furthermore, neither egg yolk nor free ^{57}Co-cobalamin excretion correlated with age. No correlations could be demonstrated between the ^{57}Co-excretion on

Table 1. Clinical and Laboratory Characteristics of Healthy Middle-Aged and Older Subjects*

	Middle-Aged Subjects	Older Subjects	*P* Value
Sex (female/male)	12/12	13/15	.797
Age (y)	57 (39–62)	75 (69–78)	
Plasma cobalamin (pmol/L)	250 (190–300)	220 (170–280)	.205
P-folate (nmol/L)	14 (11–24)	15 (12–17)	.883
E-folate (nmol/L)	565 (500–650)	565 (420–780)	.783
Plasma gastrin (pmol/L)	20 (14–37)	19 (16–25)	.785
Plasma PG A (μg/L)	30 (26–51)	47 (38–60)	.067
Plasma PG C (μg/L)	14 (10–25)	18 (14–25)	.228
PG A/C ratio	2.9 (1.6–3.1)	2.9 (1.3–3.3)	.824

*Results in number of subjects or median and 25th and 75th percentiles (in brackets).
Abbreviations: P-folate, plasma folate; E-folate, folate in erytrocytes; PG, pepsinogen.

Table 2. Results of the Egg Yolk and Free Cobalamin Absorption Tests by 24-Hour Periods*

	Middle-Aged Subjects	Older Subjects	*P* Value
Egg yolk cobalamin absorption (%)	n = 24	n = 28	
First 24-hour period	8.4 (7.2–9.8)	7.8 (6.4–9.4)	.409
Second 24-hour period	4.2 (3.3–5.1)	3.8 (3.0–4.5)	.308
Total 48-hour period	12.3 (10.5–14.5)	11.7 (9.8–13.6)	.283
Free cobalamin absorption (%)	n = 21	n = 28	
First 24-hour period	17.2 (12.9–21.0)	18.5 (14.9–23.6)	.342
Second 24-hour period	8.0 (6.7–10.7)	8.8 (5.7–10.0)	.832
Total 48-hour period	25.7 (20.6–30.7)	27.9 (21.4–34.5)	.694

*Results in median and 25th and 75th percentiles (in brackets).

the one hand and plasma cobalamin, gastrin and ratio of pepsinogen A to pepsinogen C on the other. No significant difference between men and women could be demonstrated.

The median egg yolk ^{57}Co-cobalamin excretion in 13 subjects with atrophic gastritis was 12.1% (25th and 75th percentiles 9.8%-14.7%) compared with 11.9% (25th and 75th percentiles 10.2%-14.2%) in the non-atrophic gastritis group ($P = .892$). The median free ^{57}Co-cobalamin excretion in the atrophic gastritis group was 26.7% (25th and 75th percentiles 21.5%-34.4%) compared with 27.0% (25th and 75th percentiles 19.9%-26.7%) in the non-atrophic gastritis group ($P = .695$). The results of the egg yolk cobalamin absorption test in two older subjects with severe atrophic gastritis were 9.9% and 13.7%. Both subjects had normal plasma gastrin concentrations. The ratio of pepsinogen A to pepsinogen C did not correlate with free or egg yolk ^{57}Co-cobalamin excretion.

DISCUSSION

Investigators have hypothesized that the high prevalence of reduced serum cobalamin levels in older people is the result of decreased protein-bound cobalamin absorption attributable to an age-related increase in atrophic gastritis. In this study, we have demonstrated that plasma cobalamin levels decreased with advancing age in healthy middle-aged and older adults whereas neither free nor protein-bound cobalamin absorption was significantly affected by either age or mild to moderate atrophic gastritis. Our finding that free cobalamin absorption does not decline with age is in agreement with previous reports.[29,30] However, in literature there is at present no consensus on protein-bound cobalamin malabsorption as a cause for the increased number of older people with low serum cobalamin levels. To explain the divergent findings, one has to take into account several factors related either to the 'test-meal' conditions or to the prevalence and severity of atrophic gastritis in the study populations.

Many variations of the protein-bound absorption test have been described, from raw in vitro labeled chicken serum to cooked in vivo labeled trout. The test-meals differ in a number of elements that are known to influence the protein-bound cobalamin absorption: cobalamin binders, cobalamin form, degree of protein-binding, preparation (i.e., fried, cooked, boiled etc.), and volume. In the present investigation, scrambled egg yolk labeled in vivo with ^{57}Co-cobalamin was used. We selected this test meal because in vivo labeling, rather than in vitro labeling, ensures that ^{57}Co-cobalamin is bound naturally to its specific binders. Furthermore, scrambled egg yolk, when compared with raw chicken serum, is a commonly used food. However, the degree of protein-binding in our test meal decreased from 92% to 65% after scrambling. Therefore, a higher amount of free ^{57}Co-cobalamin may well have contributed to higher absorption percentages compared with test meals that are not heated. Nonetheless, we deliberately decided to heat our test-meal because most foods containing cobalamin — liver, meat, and eggs — are heated before consumption. Furthermore, administration of raw chicken material to older people was considered ethically unjust in view of a possible contamination of the test meal with *Salmonella*. In sum, the absorption of protein-bound cobalamin varies with the test-meal, and no consensus exist on the specific demands for a test-meal as an appropriate model for the effect of protein-binding on cobalamin absorption. Therefore, standardization of the protein-bound cobalamin absorption test seems warranted to render future studies comparable.

Atrophic gastritis is frequently considered to be the likely mechanism of protein-bound cobalamin malabsorption. We assessed the presence and severity of atrophic gastritis indirectly by serological measurements of plasma pepsinogen A and pepsinogen C. The determination of the ratio of pepsinogen A to pepsinogen C is considered a "serological biopsy" of the gastric fundic mucosa since a good correlation has been reported between the histologic and functional status of the stomach on the one hand and the pepsinogen concentrations on the other.[31] Thus, we found that the cobalamin absorption from scrambled egg yolk in healthy subjects was not affected by mild to moderate atrophic gastritis. Therefore, protein-bound cobalamin malabsorption attributable to mild to moderate atrophic gastritis seems a less likely explanation for the reduced cobalamin levels in older people. It is possible that the release of cobalamin from its protein-binders by gastric acid and/or pepsin is not completely impaired in subjects with milder forms of atrophic gastritis. However, not all subjects with a- or hypochlorhydria have protein-bound cobalamin malabsorption, and not all subjects with this malabsorption have gastric dysfunction.[18,24] These observations suggest that several other conditions are probably involved in determining protein-bound cobalamin malabsorption, such as bacterial overgrowth,[32] *Helicobacter pylori* infection,[33] or secretory pancreatic abnormalities.[34] To summarize, the relative importance of acid-pepsin digestion for the release of cobalamin from its binders remains to be established.

Our finding that protein-bound cobalamin absorption is not related to age is in agreement with Jones et al.[17] and Carmel

et al.[18] In contrast, Dawson et al.[16] reported reduced protein-bound cobalamin absorption in older patients when compared with younger volunteers. However, a small number of subjects were studied, and statistical significance was not reached. A significant decline in protein-bound cobalamin absorption was reported by Scarlett et al.,[24] though their subjects were older than the volunteers in the present investigation (mean age of their middle-aged subjects was 64 years and of the older subjects, 83 years). In addition, many of their subjects older than age 55 years probably had severe atrophic gastritis since almost half had elevated serum gastrin levels. Therefore, the age effect on protein-bound cobalamin absorption observed in the study by Scarlett and co-workers,[24] but not in the present study, may reflect a difference in the prevalence and severity of atrophic gastritis in the study populations. In the five studies, including the present investigation, that have addressed the relation between age and protein-bound cobalamin absorption, four different test-meals were used. Jones et al.[17] and Dawson et al.[16] used in vitro labeled chicken serum. Carmel et al.[18] used scrambled in vitro labeled egg yolk, and Scarlett et al.[24] administered raw in vitro labeled whole egg mixed with 150 mL of milk. It is possible that age has a distinct influence on cobalamin absorption as measured by these different test-meals. Since cobalamin absorption differs with each of these meals it is also possible that the influence of age varies. It would, therefore, be of interest to study the effect of age on protein-bound cobalamin absorption by administering different test meals to each participant.

In conclusion, based on our results, the high prevalence of low cobalamin levels in older people cannot be explained either by the aging process or by mild to moderate atrophic gastritis. On the other hand, we can not rule out the effect of severe atrophic gastritis. Indeed, Krasinski et al.[35] have demonstrated that the minority of older people with atrophic gastritis have low serum cobalamin levels, whereas the majority of older people with low serum cobalamin levels have severe atrophic gastritis. To explain the observed age-related decline in plasma cobalamin levels in the present study, additional influences on the cobalamin status have to be taken into consideration. For example, the intake of cobalamin with different (prepared) foods, the time elapsed from the onset of atrophic gastritis, the functional and structural integrity of the serum cobalamin binding-proteins, and, finally, the amount of cobalamin stored in the liver.

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