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Diagnostic Accuracy of Holotranscobalamin, Methylmalonic Acid, Serum Cobalamin, and Other Indicators of Tissue Vitamin B_{12} Status in the Elderly

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BACKGROUND: Vitamin B_{12} deficiency is common among the elderly, and early detection is clinically important. However, clinical signs and symptoms have limited diagnostic accuracy and there is no accepted reference test method.

METHODS: In elderly subjects (n = 700; age range 63–97 years), we investigated the ability of serum cobalamin, holotranscobalamin (holoTC), total homocysteine (tHcy), methylmalonic acid (MMA), serum and erythrocyte folate, and other hematologic variables to discriminate cobalamin deficiency, defined as red blood cell cobalamin <33 pmol/L.

RESULTS: Serum holoTC was the best predictor, with area under the ROC curve (95% CI) 0.90 (0.86–0.93), and this was significantly better ($P \leq 0.0002$) than the next best predictors; serum cobalamin, 0.80 (0.75–0.85), and MMA, 0.78 (0.72–0.83). For these 3 analytes, we constructed a 3-zone partition of positive and negative zones and a deliberate indeterminate zone between. The boundaries were values of each test that resulted in a posttest probability of deficiency of 60% and a posttest probability of no deficiency of 98%. The proportion of indeterminate observations for holoTC, cobalamin, and MMA was 14%, 45%, and 50%, respectively. Within the holoTC indeterminate zone (defined as 20–30 pmol/L), discriminant analysis selected only erythrocyte folate, which correctly allocated 65% (58/89) of the observations. Renal dysfunction compromised the diagnostic accuracy of MMA but not holoTC or serum cobalamin.

CONCLUSIONS: This study supports the use of holoTC as the first-line diagnostic procedure for vitamin B_{12} status.

Vitamin B_{12} deficiency is considered to be a serious public health issue among elderly populations; however, there is no consensus on diagnosis. The classic signs of deficiency, such as macrocytosis, anemia, and an abnormal low serum vitamin B_{12} (cobalamin) concentration, are quite often absent *(1)*. Furthermore, neurological rather than hematologic symptoms may be the only clinical indications *(2)*, and a relationship between low concentrations of cobalamin and neurological symptoms has been reported *(3, 4)*. In clinical practice, however, many elderly patients present with diffuse nonspecific symptoms, and cobalamin deficiency is only one of several differential diagnoses *(5)*. Thus, it is difficult to increase the pretest probability of disease on the basis of signs and symptoms alone.

Serum cobalamin measures cobalamin bound to the 2 circulating binding proteins, haptocorrin and transcobalamin (TC)[5], and it is only the approximately 20%–30% of cobalamin bound to TC (i.e., holotranscobalamin, the holoTC fraction) for which there is a receptor-mediated cellular uptake. The function of haptocorrin is currently unknown, and low haptocorrin concentrations, found in approximately 15% of persons with low serum cobalamin, could be one of the most common causes of low cobalamin concentrations *(6)*. Thus the cobalamin assay is recognized to be potentially unreliable *(7)*. Methylmalonic acid (MMA) and total homocysteine (tHcy) concentrations are increased in vitamin B_{12} deficiency and are generally considered more sensitive indicators of vitamin B_{12} status than serum cobalamin, but questions of specificity have been raised, particularly among the elderly, in whom renal function may be a confounding factor *(8–11)*. HoloTC has been postulated to be the earliest marker of negative vitamin B_{12} balance *(12)*, and in the last few years, reliable methods for estimating holoTC

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[5] Nonstandard abbreviations: TC, transcobalamin; holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine; eGFR(CG), estimated glomerular filtration rate by Cockcroft–Gault equation; PPV, positive predictive value; NPV, negative predictive value; Hct, hematocrit; Hb, hemoglobin; AUC, area under the curve; MCV, mean corpuscular volume; LR, likelihood ratio.

have become available *(13, 14)*. The true performance of these tests is still debated, however, and it is necessary to establish how much additional value is provided by these newer markers over existing procedures and how they might be used in clinical practice.

Vitamin B_{12} is an essential component in erythropoiesis and is rapidly incorporated into immature erythrocytes *(15)*. The red cell cobalamin concentration provides an estimate of tissue vitamin B_{12} status over the past 120 days, analogous to the red cell folate concentration. Harrison *(16–18)* studied red cell cobalamin concentrations in pernicious anemia, iron deficiency, and folate deficiency and showed responses in cobalamin content that closely mimicked the change in reticulocyte concentration as a result of treatment. A later small study found that there was some correlation between holoTC concentrations and red cell cobalamin concentrations and that holoTC was a better indicator of red cell B_{12} depletion than plasma cobalamin was *(19)*.

In our novel approach, total serum cobalamin, holoTC, MMA, tHcy, folate, and hematologic indices were evaluated against a reference standard of red cell cobalamin concentrations in a population of elderly subjects. Our aim was to establish a diagnostic strategy for this common clinical condition.

Materials and Methods

STUDY POPULATION

Outpatients attending the memory clinic in the Geriatric Unit of St. James Hospital, Dublin, were recruited as part of an ongoing observational cohort study designed to collect environmental, medical, metabolic, and genotype data on 2000 individuals >60 years old with mild to moderate cognitive impairment. The first 700 subjects recruited comprised the study population (December 2008 to October 2009). Nonfasting blood samples were collected along with information on diet, lifestyle, and medical history. The study protocol was approved by the Dublin Federated Hospitals Research Ethics committee, and all individuals gave written informed consent.

A separate reference population of 120 healthy volunteers (64 women, with 27 using oral contraceptives, and 56 men) was recruited among the employees of Axis-Shield and medical students at the local hospital, age range 18–62 years (October 2009 to March 2010). This population was used to determine a reference interval for the red cell cobalamin assay. We also chose to establish reference intervals for holoTC, because it is a relatively novel marker, and for the microbiological total serum cobalamin assay used, because there were no reports in the literature. Institutional ethics approval and written informed consent were obtained for all volunteers.

BIOCHEMICAL MEASUREMENTS

All blood samples were processed within 3 h of sampling. MMA determination was performed at the University of Bergen; all other biochemical measurements were carried out at Trinity College, Dublin. Red cell cobalamin, red cell folate, and MMA/tHcy concentrations were determined on 3 separate EDTA blood samples maintained at 4 °C. For determination of holoTC, cobalamin, and serum folate, bloods were collected into clotting tubes. After processing, serum, plasma, and red cell fractions were stored frozen at −80 °C until assayed. Hematologic and renal function data were available through the cohort study database. Estimated glomerular filtration rate [eGFR(CG)] was calculated by use of the Cockcroft–Gault equation.

We adapted the red cell cobalamin assay from Tisman et al. *(19)* and had interassay CVs of ≤11.7% in measured cobalamin in 4 pooled washed red cell samples (at 92, 156, 227, and 300 pmol/L cobalamin) over 3 experiments, and CVs of ≤5.9% in their extracted supernatants stored at −80 °C for 1 week before assay. Intraassay CVs of 6 repeated estimates in the 4 concentrations noted above were <5.1%. Interassay CVs of 2 pooled washed red cell samples at 66 and 91 pmol/L over 40 assays during the course of the study were 14.9% and 14.6%, respectively (see Supplemental Description 1 in the Data Supplement, which accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue6).

We determined serum and red cell folate concentrations by microbiological assay using a chloramphenicol-resistant strain of *Lactobacillus casei* [*L. rhamnosis* (NCIB 10463; ATCC 27773)] *(20)*. Inter- and intraassay CVs were <11.0%. We measured total cobalamin in serum by microbiological assay using a colistin sulfate–resistant strain of *L. leichmanii* (*delbrueckii*) (NCIB 12519, ATCC 43787) *(21, 22)*. Inter- and intraassay CVs were <10.9%. We measured MMA using a GC-MS method based on methylchloroformate derivatization with a between-day CV of <5% *(23)*. We measured tHcy and holoTC on the Abbott AxSYM *(14, 24)*. The between-assay CVs were <5.2 and 11.1%, respectively.

Cutoff values for the metabolites tHcy and MMA, in relation to possible vitamin B_{12} deficiency, are not well defined. We chose 0.36 μmol/L for MMA *(11)* and 15 μmol/L for tHcy *(25)*. For holoTC, serum cobalamin, and red cell cobalamin, we used the lower limit of the 95% central reference interval as established in our reference population. Serum folate and red cell folate cutoffs used were <6.8 nmol/L *(26)* and <340 nmol/L, respectively *(27)*.

Table 1. Descriptive characteristics of the elderly population.

	n	Mean	2.5th to 97.5th percentile	Abnormal, % (cutoff value)
Age, years	700	81	69–92	
Women, n (%)	490 (70)			
Hematocrit	699	0.386	0.304–0.470	
Hemoglobin, g/dL	699	12.5	9.4–15.4	
MCV, fL	699	91.6	79.0–102.9	
Red cell cobalamin, pmol/L	700	64[a]	21–161	9.6 (<33)
HoloTC, pmol/L	699	47[a]	11–171	8.1 (<20)
Serum total cobalamin, pmol/L	700	254[a]	83–674	8.0 (<123)
Serum folate, nmol/L	697	30[a]	7–360	1.7 (<6.8)
Red cell folate, nmol/L	693	1003[a]	371–3077	1.4 (<340)
MMA, μmol/L	700	0.347[a]	0.145–1.511	41.7 (>0.36)
tHcy, μmol/L	700	16.3[a]	9.1–34.0	52.2 (>15.0)
Creatinine, μmol/L	699	89[a]	51–181	
eGFR(CG), mL/min	698	48[a]	18–108	

[a] Geometric mean.

STATISTICAL ANALYSES

The values for red cell cobalamin, serum cobalamin, holoTC, tHcy, MMA, serum folate, and red cell folate in the elderly population were positively skewed and were natural log–transformed before analysis. We summarized continuous variables as means or geometric means with 2.5–97.5 percentiles. A 2-sample *t*-test was used to test for differences in the means. We used the lower limit of the 95% reference interval for red cell cobalamin to dichotomize red cell cobalamin concentrations for construction of ROC plots for the test variables. The associations between the test variables and red cell cobalamin concentrations were further evaluated by stepwise multiple linear regression analysis. Possible violations of the model assumptions were assessed. Stepwise linear discriminant functions, using the same variables as in the stepwise regression model (except holoTC), were developed for observations between holoTC values of 20–30 pmol/L. The discriminant function used to predict vitamin B_{12} deficiency was assessed by sensitivity, specificity, and proportion of results correctly allocated. Stepwise models had a *P* value to enter of 0.05 and removal of 0.10. For the best-performing markers at specific cutoff points, we assessed sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) in the whole population and in subgroups defined by eGFR(CG) status. Data were analyzed with Analyze-It and SPSS version 18.

Results

POPULATION CHARACTERISTICS

The reference population was between 18 and 62 (median 31) years old, and 53% of the donors were female. Multivitamin or B vitamin supplements were used by 8%. One volunteer on intramuscular vitamin B_{12} injections was excluded, and 1 sample lacked volume for red cell cobalamin determination. The mean (SD) of the red cell cobalamin concentration in the 118 eligible subjects was 97.2 (32.8) pmol/L. The 95% central reference intervals for serum holoTC, serum cobalamin (both natural log–transformed), and red cell cobalamin (not transformed) in the reference population were determined parametrically (see online Supplemental Table 1). Using these data, the cutoffs for deficiency were defined as 20 pmol/L for holoTC, 123 pmol/L for serum cobalamin, and 33 pmol/L for red cell cobalamin.

A description of the elderly population is presented in Table 1. Of the 700 participants in the study, 490 (70%) were female, and the mean age was 81 (range 63–97) years. Vitamin B_{12}– or folate-containing supplements were used by 6%. The prevalences of low red cell cobalamin, holoTC, and total cobalamin were 9.6%, 8.1%, and 8.0%, respectively. The prevalences for abnormal MMA and tHcy were 41.7% and 52.2%, respectively, and for serum and red cell folate, 1.7% and 1.4%. There were strong positive correlations (see online Supplemental Table 2) between red cell cobala-

Table 2. Mean values (95% CI) in participants with and without vitamin B_{12} deficiency.[a]

Variable	n	Deficient	N	Nondeficient	2-tailed *P*
Age, years	67	82 (80 to 83)	633	81 (80 to 81)	0.206
Hematocrit	66	0.38 (0.37 to 0.39)	633	0.39 (0.38 to 0.39)	0.162
Hemoglobin, g/dL[b]	66	12.0 (11.7 to 12.4)	633	12.5 (12.4 to 12.7)	0.018
MCV, fL	66	90 (88 to 92)	633	92 (91 to 92)	0.024
Red cell cobalamin, pmol/L[c]	67	23.4 (21.9 to 25.0)	633	71.7 (69.4 to 74.1)	
holoTC, pmol/L[c]	67	18.2 (15.6 to 21.2)	632	51.4 (49.0 to 53.9)	<0.0001
Serum cobalamin, pmol/L[c]	67	139 (119 to 162)	633	270 (258 to 284)	<0.0001
Serum folate, nmol/L[c]	67	23.5 (18.6 to 29.6)	630	30.8 (28.7 to 32.9)	0.018
Red cell folate, nmol/L[c]	66	712 (621 to 816)	627	1040 (998 to 1084)	<0.0001
MMA, μmol/L[c]	67	0.651 (0.536 to 0.790)	633	0.325 (0.311 to 0.338)	<0.0001
tHcy, μmol/L[c]	67	22.5 (20.1 to 25.1)	633	15.7 (15.4 to 16.1)	<0.0001
Creatinine, μmol/L[c]	66	85 (79 to 91)	633	90 (87 to 92)	0.188
eGFR(CG), mL/min[c]	66	48 (44 to 53)	632	48 (47 to 50)	0.907

[a] Vitamin B_{12} deficiency was defined as red cell cobalamin <33 pmol/L packed red cells.
[b] For SI value (g/L), multiply by 10.
[c] Geometric mean.

min and holoTC and cobalamin ($r = 0.63$ and 0.51, respectively). Red cell folate was also positively correlated with red cell cobalamin ($r = 0.36$). Red cell cobalamin was negatively correlated with plasma MMA ($r = -0.45$) and tHcy ($r = -0.33$), and there were no significant associations with either creatinine ($r = 0.05$) or eGFR(CG) ($r = -0.02$).

Table 2 compares the means of test results and other variables in individuals who had red cell cobalamin concentrations above and below the cutoff of 33 pmol/L packed red cells. There were no significant differences in age, creatinine, eGFR(CG), or hematocrit (Hct) between the 2 groups. Defining anemia as hemoglobin (Hb) <13 g/dL (130 g/L) for men and 12 g/dL (120 g/L) for women, 38 of 66 (58%) and 266 of 633 (42%) were anemic in each group. All serum markers of vitamin B_{12} status as well as red cell folate were highly significantly different ($P < 0.0001$ for all), and serum folate was also different ($P = 0.018$).

ROC ANALYSES

We used the red cell cobalamin lower limit of 33 pmol/L packed red cells to dichotomize the concentrations into deficient and nondeficient vitamin B_{12} status for the construction of ROC plots. The areas under the curves (AUCs) (see online Supplemental Table 3) demonstrate that holoTC is the best-performing indicator of tissue vitamin B_{12} status (AUC 0.90). The differences in AUC between holoTC and serum cobalamin (0.80), MMA (0.78), and tHcy (0.75) were significant ($P \leq 0.0002$) (see Fig. 1).

MULTIPLE LINEAR REGRESSION

Regression analysis was performed for all variables [log transformed except for Hct, Hb, and mean corpuscular volume (MCV)]. The multiple regression model (see

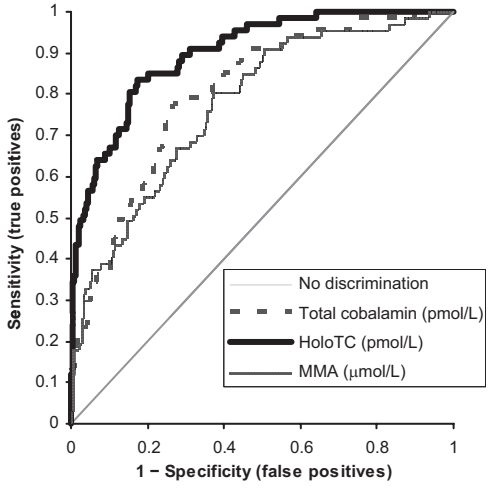

Fig. 1. ROC plots for serum total cobalamin, holoTC, and MMA for vitamin B_{12} deficiency, defined as red cell cobalamin <33 pmol/L.

Total sample size, 699; number of individuals B_{12} deficient, 67. The AUC for holoTC (0.90) was significantly different ($P \leq 0.0002$) from those of both serum total cobalamin (0.80) and MMA (0.78).

Table 3. Diagnostic performance of holoTC, serum cobalamin, and MMA at a single cutoff.

Marker	Cutoff	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
HoloTC	<20 pmol/L	55 (43–67)	96 (94–97)	56 (45–70)	95 (93–97)
Serum cobalamin	<123 pmol/L	33 (22–45)	95 (93–96)	39 (26–53)	93 (91–95)
MMA	>0.36 μmol/L	81 (69–89)	63 (59–66)	19 (14–24)	97 (95–98)

online Supplemental Table 4) containing only log(holoTC) accounted for 43.8% of the variance in red cell cobalamin ($r^2 = 0.44$). The order of addition to the model was log(red cell folate), MCV, log(MMA), log(serum total cobalamin), and log(serum folate), giving r^2 values of 0.47, 0.49, 0.50, 0.51, and 0.52, respectively. Age, weight, sex, creatinine, eGFR(CG), Hct, Hb, and tHcy were not in the model. All except tHcy had a low univariate *r* value, and tHcy was highly correlated with other variables, especially MMA. The residuals for the model with 6 variables were normally distributed (Anderson–Darling $P = 0.71$) and showed no violation of the heterogeneity assumption.

PERFORMANCE OF SELECTED TESTS TO DIAGNOSE COBALAMIN DEFICIENCY

The performance of the 3 best predictor markers (holoTC, MMA, and serum cobalamin) to diagnose vitamin B_{12} deficiency in the elderly population at cutoffs of <20 pmol/L, >0.36 μmol/L, and <123 pmol/L, respectively, is given in Table 3. These data suggest that serum cobalamin has low sensitivity and MMA has low specificity at these cutoff concentrations.

By use of Bayes's theorem, a pretest probability of 9.6%, desirable posttest probabilities of 60% (for ruling-in deficiency) and 2% (for ruling-out deficiency), a desirable positive likelihood ratio (LR+) of 14, and a negative likelihood ratio (LR−) of 0.23 were deduced. The resulting analyte decision thresholds for these likelihood ratios are given in Table 4. The number and proportion of samples that would fall into the respective indeterminate zones were holoTC 13.7% (96/699), serum total cobalamin 44.7% (313/700), and MMA 49.8% (349/700).

DISCRIMINANT ANALYSIS

Eighty-nine samples had holoTC values between 20 and 30 pmol/L (the defined indeterminate zone). Only log(red cell folate) met the criteria to enter the stepwise linear discriminant function (Wilks Λ $P = 0.042$), and all 89 observations had a corresponding log(red cell folate) value. Observations with red cell folate values <778.1 nmol/L were allocated to the B_{12}-deficient category, correctly allocating 65% (sensitivity 65%, specificity 65%).

KIDNEY DISEASE

Using eGFR(CG) levels to categorize kidney function, we then estimated the performance of holoTC, serum total cobalamin, and MMA, at defined cutoffs, for detecting vitamin B_{12} deficiency. We found that the PPV for diagnosing deficiency using the defined cutoffs was higher for holoTC than for serum total cobalamin or MMA regardless of kidney function (Table 5).

Discussion

This is the first study to compare the common circulating markers of vitamin B_{12} status against red cell cobalamin as an index of tissue B_{12} stores. Our results demonstrate that holoTC performs significantly better than all other indicators in this elderly population. Red cell

Table 4. Three-zone decision thresholds for holoTC, serum cobalamin, and MMA based on LR+ of 14 and LR− of 0.23.

Marker	Threshold concentrations	Sensitivity, %	Specificity, %	True positive	True negative	False positive	False negative	PPV, %	NPV, %
HoloTC, pmol/L	19.6	50.7	96.4	34	609	23	33	60	95
	29.9	80.6	84.5	54	534	98	13	36	98
Serum cobalamin, pmol/L	79	13.4	99.1	9	627	6	58	60	92
	238	86.6	57.3	58	363	270	9	18	98
MMA, μmol/L	1.402	17.9	98.7	12	625	8	55	60	92
	0.310	88.1	50.9	59	322	311	8	16	98

Table 5. Performance of holoTC, serum cobalamin, and MMA to detect B12 deficiency in eGFR(CG) ranges.[a]

eGFR(CG) range	n	PPV %	NPV %	Mean[b]
≥60	226 (225 for holoTC)			
HoloTC		66 (41–84)	95 (92–98)	45 (41–49)
Serum cobalamin		31 (12–54)	92 (88–95)	246 (229–264)
MMA		23 (13–34)	95 (91–98)	0.295 (0.275–0.316
30–59	378			
HoloTC		47 (30–65)	94 (91–96)	47 (44–50)
Serum cobalamin		44 (26–62)	94 (91–96)	250 (235–266)
MMA		20 (14–27)	97 (94–99)	0.343 (0.325–0.362)
15–29	94			
HoloTC		78 (40–97)	99 (93–100)	51 (43–60)
Serum cobalamin		50 (7–93)	93 (86–98)	295 (247–351)
MMA		12 (5–22)	97 (94–99)	0.531 (0.457–0.616)

[a] Vitamin B_{12} deficiency was defined as red cell cobalamin <33 pmol/L packed red cells. Cutoffs as in Table 3.
[b] Geometric mean.

cobalamin concentrations have previously been used to indicate tissue vitamin B_{12} levels *(16, 28–30)*. Only the immature red cells (reticulocytes) incorporate the vitamin in a receptor-mediated process *(31)*, and therefore red cell cobalamin is a reflection of status in the youngest blood cells *(29)*. The current study is consistent with the Herbert hypothesis *(29)* of holoTC being a very early marker, since holoTC predicts 44% of the variation in red blood cell cobalamin in the multiple linear regression model. The model suggests that red cell cobalamin has a direct relationship with holoTC, red cell folate, MCV, and serum cobalamin and an inverse relationship with MMA and serum folate. The direction of the MCV and serum folate relationships are perhaps unexpected, but MCV has low diagnostic value in the detection of vitamin B_{12} deficiency *(32)*, and serum folate could be confounded by recent intake or supplementation. Hb concentrations did not enter the regression model.

The prevalence of low red cell cobalamin in this study was 9.6%; similar prevalences were found for holoTC (8.1%) and serum total cobalamin (8.0%). In contrast, and as reported by others *(8)*, the prevalence of abnormal MMA and tHcy was much higher (>40%). In our elderly population, there was a good correlation ($r = 0.63$) between holoTC and red cell cobalamin, similar to that shown by Tisman et al. *(19)* for cancer patients ($r = 0.60$) using an indirect method for estimation of holoTC. We found an even stronger correlation in our healthy reference population ($r = 0.82$).

There is some variation in the literature regarding the reference interval for red cell cobalamin. We found that the mean (SD) of 97.2 (32.8) pmol/L was similar to previously published estimates, but the lower limit of the reference interval we established from these data (33 pmol/L) was somewhat lower than previous published estimates *(16, 30)*. The reference interval estimate for holoTC of 20–125 pmol/L was similar to that published by Brady et al. *(14)* (19–134 pmol/L).

HoloTC was superior ($P \leq 0.0002$) to both serum total cobalamin and MMA for diagnosing tissue B_{12} deficiency in the ROC analysis, whereas MMA and serum total cobalamin were essentially equivalent (AUC 0.90, 0.80, and 0.78, respectively). A previous study in an elderly group concluded that holoTC had modestly better diagnostic accuracy than total serum cobalamin *(33)*. This was based on using MMA as the reference standard, which has known limitations in an elderly population *(11)*. Hematologic indicators of anemia had the lowest AUC, consistent with reports that neurological lesions may be more common among elderly individuals *(1, 2)*. Serum folate (0.61), red cell folate (0.71), and tHcy (0.75) gave intermediate AUCs.

As with most diagnostic tests, there is not perfect discrimination between disease and nondisease. Although the performance of any of these 3 circulating markers at a single cutoff may not be sufficient for screening, we assessed their potential clinical use based on a 3-zone partition with an intentional gray zone *(34)*. We assumed that a posttest probability of around 60% would be sufficient to rule in the disease, on the basis that the treatment is effective and safe, and that a posttest probability of no deficiency of 98% would rule out deficiency. This approach also allows for a direct comparison of the diagnostic performance of each test.

For holoTC, around 14% of samples tested would fall within this gray zone, whereas for serum total cobalamin and MMA the proportions were 45% and 50%, suggesting that these would not be useful tests under this scenario.

It has been long recognized that the serum total cobalamin assay has a wide gray zone. In a study of the Framingham elderly population, a cutoff point of 258 pmol/L was suggested *(35)*. On the other hand, many physicians react only to very low cobalamin concentrations—for example, <74 pmol/L (100 pg/mL) *(36)*. It is tempting to suggest that these are 2 sides of the same coin depending on whether the objective is to maximize positive or negative predictive value. We found that a serum total cobalamin cutoff of 79 pmol/L had a PPV of 60%, whereas a cutoff of 238 pmol/L had an NPV of 98%. Matchar et al. *(36)* reported the PPV of the serum total cobalamin test compared to clinical diagnosis as follows: <133 pmol/L, 22.2%; <118 pmol/L, 30%; <96 pmol/L, 40%; and <74 pmol/L, 50%, results very similar to those found in this study.

Most reports comment that MMA concentrations <0.3 μmol/L rule out disease with high probability *(37)*, and we found that an MMA concentration of 0.31 μmol/L had an NPV of 98%. We suggest, however, that a diagnosis of B_{12} deficiency with high probability would not be reached until very high concentrations of MMA. This result is supported by a report on the use of MMA as a diagnostic tool for vitamin B_{12} deficiency in Denmark, which found that physicians did not tend to react until MMA was >1.0 μmol/L *(38)*. It is a new finding to demonstrate that serum total cobalamin and MMA provide almost equivalent diagnostic performance.

We suggest a diagnostic strategy using holoTC as the front-line test. For values <20 pmol/L, treatment should be initiated. For values ≥30 pmol/L, no treatment is required. Between these limits, a refinement of disease pretest probability (for example using symptoms and history) may allow diagnosis using holoTC gray-zone likelihood ratios. In this study, for holoTC values of 22, 24, and 26 pmol/L, these were 10.2, 7.6, and 5.9 for LR+ and 0.43, 0.39, and 0.35 for LR−, respectively.

Of the biochemical markers, only red cell folate concentrations were useful for further discrimination. Observations with red cell folate values ≤778 nmol/L were allocated to the B_{12}-deficient category, correctly allocating 65% of observations (sensitivity 65%, specificity 65%). A possible explanation for this finding is the "methylfolate trap" hypothesis *(39)*. Whatever the cause, this observation is consistent with reports that 60% of patients with pernicious anemia have low red blood cell folate concentrations *(40)*.

We partitioned all subjects according to kidney function. The mean concentration of holoTC, serum cobalamin, and MMA was increased in the lowest eGFR(CG) range, but there was no evidence of compromised performance for holoTC or serum cobalamin to detect true vitamin B_{12} deficiency as kidney function decreased. HoloTC was the best-performing marker, and even in the most compromised individuals [eGFR(CG) 15–29], holoTC had a PPV of 78%. The PPV of MMA showed a steady decline (23%–20% to 12%) as renal function decreased, suggesting that the diagnostic performance of MMA is, in part, confounded by renal function.

The strengths of this study are that all indicators were tested on all samples, and the sample size was large enough to give adequate power for AUC differences in the ROC analysis.

Although the concentration of red cell cobalamin probably depends on body content of vitamin B_{12}, our knowledge of other determinants is limited. Factors that influence erythrocyte production may affect measured concentrations *(16–18)*. However, we observe that holoTC accounts for a high proportion of the variance in red cell cobalamin concentrations, providing reassurance that the results are valid. It is possible that the relationship between holoTC and red cell cobalamin is greater than we report here in elderly individuals, since the relationship was considerably stronger in our healthy reference population. We did not exclude serum folate–deficient subjects from our multiple regression and discriminant analysis, because it would be uncertain in practice whether these subjects also were cobalamin deficient. There were 12 serum folate–deficient patients, 2 of whom had low red cell cobalamin concentrations, and there were 4 observations in the holoTC indeterminate zone with a serum folate concentration below the cutoff, none of which had low red cell cobalamin concentrations.

In conclusion, the results of the present study of elderly individuals support the use of holoTC as the first-line diagnostic procedure for vitamin B_{12} status, and use of a gray zone may allow screening. Within the holoTC gray zone, the use of red cell folate concentrations provides additional discriminative information in the absence of clinical signs, symptoms, and history. The practicality of such a strategy, however, would depend on standardization of red cell folate determination and using suitably reliable methodology.

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