Measurement of Total Vitamin B_{12} and Holotranscobalamin, Singly and in Combination, in Screening for Metabolic Vitamin B_{12} Deficiency

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Background: The standard screening test for vitamin B_{12} deficiency, measurement of total plasma vitamin B_{12}, has limitations of sensitivity and specificity. Plasma vitamin B_{12} bound to transcobalamin (holoTC) is the fraction of total vitamin B_{12} available for tissue uptake and therefore has been proposed as a potentially useful alternative indicator of vitamin B_{12} status.

Methods: We compared the diagnostic accuracy of total vitamin B_{12}, holoTC, and a combination of both measures to screen for metabolic vitamin B_{12} deficiency in an elderly cohort (age ≥60 years). Plasma methylmalonic acid and homocysteine were used as indicators of vitamin B_{12} deficiency.

Results: Low total vitamin B_{12} (<148 pmol/L) and low holoTC (<35 pmol/L) were observed in 6.5% and 8.0%, and increased methylmalonic acid (>350 nmol/L) and homocysteine (>13 μmol/L) were observed in 12.1% and 17.0% of the study participants. In multiple regression models, holoTC explained 5%–6% more of the observed variance in methylmalonic acid and homocysteine than did total vitamin B_{12} ($P \leq 0.004$). ROC curve analysis indicated that total vitamin B_{12} and holoTC were essentially equivalent in their ability to discriminate persons with and without vitamin B_{12} deficiency. Individuals with low concentrations of both total vitamin B_{12} and holoTC had significantly higher concentrations of methylmalonic acid and homocysteine than did individuals with total vitamin B_{12} and/or holoTC within the reference intervals ($P < 0.001$).

Conclusions: HoloTC and total vitamin B_{12} have equal diagnostic accuracy in screening for metabolic vitamin B_{12} deficiency. Measurement of both holoTC and total vitamin B_{12} provides a better screen for vitamin B_{12} deficiency than either assay alone.

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Vitamin B_{12} deficiency is a major public health problem, particularly among older persons. Conservative estimates indicate that 2%–3% of this population has or will develop pernicious anemia caused by failure of gastric intrinsic factor production and consequent vitamin B_{12} malabsorption *(1, 2)*. Other estimates suggest that the prevalence of vitamin B_{12} deficiency may be as high as 30% among the elderly because of malabsorption of vitamin B_{12} from food caused by chronic gastritis, gastric atrophy, and perhaps other unknown causes *(3, 4)*.

Sensitive and specific assays for determining vitamin B_{12} status are needed because of the high prevalence and the potentially serious complications of vitamin B_{12} deficiency. Typically, vitamin B_{12} deficiency is suspected only when an individual presents with hematologic manifestations of megaloblastic anemia, which occur only in the most severely vitamin B_{12}-depleted individuals *(5)*. Total plasma vitamin B_{12} concentration is the current standard clinical screening test for vitamin B_{12} deficiency. Total vitamin B_{12} concentrations <148 pmol/L (<200 pg/mL) are generally considered deficient. This range is diagnostically useful for the majority of cases of vitamin B_{12} deficiency; however, a proportion of individuals with vitamin B_{12} concentrations that would be considered deficient exhibit no clinical or biochemical evidence of deficiency *(6)*. Conversely, neuropsychiatric *(7)* and met-

abolic abnormalities *(6, 8)* can occur with plasma vitamin B_{12} concentrations within the reference interval.

Methylmalonic acid and homocysteine are increased in vitamin B_{12} deficiency and are generally considered more sensitive indicators of vitamin B_{12} status than is total plasma vitamin B_{12} *(6, 8)*, but issues of specificity limit their utility. There thus is a need for more reliable, sensitive, and specific screening tests to detect vitamin B_{12} deficiency.

There are 2 vitamin B_{12} transport proteins in blood, haptocorrin and transcobalamin (TC).[5] TC is responsible for transport of vitamin B_{12} to the tissues. The vitamin B_{12} associated with TC (holoTC) represents the functionally important fraction of plasma vitamin B_{12}. Herzlich and Herbert *(9)* postulated that the earliest change that occurs when an individual enters into negative vitamin B_{12} balance is a decrease in plasma holoTC concentrations. Definitive proof of the utility of holoTC as an indicator of vitamin B_{12} status has been elusive, however, because a reliable and robust assay has not been available.

Recently, an assay for the direct measurement of holoTC has become commercially available (Axis-Shield ASA) *(10)*. The US Food and Drug Administration has cleared this assay for the diagnosis of vitamin B_{12} deficiency. We examined the utility of plasma holoTC measurement, compared with and in combination with total vitamin B_{12}, in screening for metabolic vitamin B_{12} deficiency in plasma samples collected from an elderly cohort at risk for vitamin B_{12} deficiency.

Materials and Methods

STUDY PARTICIPANTS

The Human Subjects Review Committee at the University of California, Davis, approved participant recruitment and study procedures, and written informed consent was obtained from all study participants. The study population consisted of community-dwelling older adults (age ≥60 years) of Latino ancestry residing in Sacramento, CA, and the surrounding Northern California communities. Participants were recruited over a period of 18 months beginning in February 1998, after mandated folic acid fortification in the United States became effective in January 1998. The details of sampling and recruitment have been described elsewhere *(11)*.

SAMPLE COLLECTION AND ANALYSIS

Fasting blood samples were collected by standard venipuncture into evacuated tubes with and without EDTA and were transported on ice to the University of California, Davis Medical Center Clinical Laboratory for processing within 4 h of collection. Plasma and serum were stored at −80 °C until analysis. Total plasma vitamin B_{12}

concentrations were determined by radioligand binding assay (Quantaphase II; Bio-Rad Diagnostics), plasma holoTC by monoclonal antibody capture assay (HoloTC RIA; Axis-Shield) *(10)*, plasma methylmalonic acid by liquid chromatography–tandem mass spectrometry at ARUP Laboratories (Salt Lake City, UT) *(12)*, total plasma homocysteine by HPLC with postcolumn fluorescence detection *(13)*, erythrocyte folate by automated chemiluminescence assay (ACS 180; Bayer Diagnostics), and serum creatinine by standard spectrophotometric assay. Cutoff values for each metabolite were based on literature reports or standard clinical concentrations. These cutoff values were as follows: total vitamin B_{12} <148 pmol/L (standard clinical value); holoTC <35 pmol/L *(14, 15)*; methylmalonic acid >350 nmol/L *(16)*; homocysteine >13 μmol/L *(17)*; erythrocyte folate <160 μg/L (standard clinical value); and creatinine >97 μmol/L (>1.1 mg/dL) for women and >124 μmol/L (>1.4 mg/dL) for men (standard clinical values).

STATISTICAL ANALYSES

Associations between methylmalonic acid and homocysteine (dependent variables) and total vitamin B_{12} and holoTC (independent variables) were evaluated by multiple linear regression analyses with adjustment for confounding by age, sex, erythrocyte folate (homocysteine models only), and creatinine. R^2 values were determined to indicate the percentage of the variance in the dependent variables explained by each regression model. R^2 values were compared among models by simple linear regression of the sum of the residuals vs the difference of the residuals, with a significant correlation indicating a significant difference in the R^2 values. Standardized coefficients (β values) were determined to compare the strength of the associations between each independent variable and the dependent variables within the regression models. To determine the relative capacities of total vitamin B_{12} and holoTC to discriminate among individuals with likely vitamin B_{12} deficiency (see *Results*) and all other persons, ROC curves were constructed. Areas under the ROC curves (AUCs) were compared with the AUC value indicative of no discrimination (AUC = 0.500). Sensitivities and specificities for detecting individuals with likely vitamin B_{12} deficiency were also determined by use of the established cutoff values for total vitamin B_{12} and holoTC cited above. With these cutoff values for total vitamin B_{12} and holoTC, we also used a general linear model procedure and the Tukey–Kramer multiple comparisons test to calculate and compare geometric means (with 95% confidence intervals) of methylmalonic acid and homocysteine among persons with low or within-reference interval total vitamin B_{12} and low or within-reference interval holoTC. Because the values for methylmalonic acid, homocysteine, total vitamin B_{12}, holoTC, and creatinine did not show a gaussian distribution (i.e., there was tailing toward higher values), these variables were natural log–transformed before the multiple regres-

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[5] Nonstandard abbreviations: TC, transcobalamin; holoTC, holotranscobalamin; AUC, area under the curve; and SALSA, Sacramento Area Latino Study on Aging.

sion and general linear model analyses. Sample sizes for each variable were not equal because sufficient volumes of blood were not available from all participants for each assay (Table 1). Statistical analyses were carried out with data from persons with available measurements for all variables used in any particular analysis, as described in the *Results* section. Statistical significance was defined for all analyses as $P < 0.05$. The statistical analyses were carried out using Statview for Macintosh and Windows (Ver. 5.0.1; Abacus Concept), SAS for Windows (Ver. 7; SAS Institute Inc), and the Analyze-it add-in (Ver. 1.69; Analyze-it Software, Ltd.) to Microsoft Excel (Microsoft Corporation).

Results

A description of the Sacramento Area Latino Study on Aging (SALSA) population is presented in Table 1. Subnormal vitamin B$_{12}$ status, defined as plasma vitamin B$_{12}$ <148 pmol/L (<200 pg/mL), was present in 6.5% of the population. A large proportion (17.0%) had increased plasma homocysteine (>13 μmol/L), although <1% had erythrocyte folate concentrations indicative of deficiency (<160 μg/L). The prevalence of increased serum creatinine [>97 μmol/L (>1.1 mg/dL) in women; >124 μmol/L (>1.4 mg/dL) in men] was 6.5% and 5.4% for women and men, respectively, and was consistent with the age of the population. These data have been reported previously *(18)*. The data in Table 1 not previously reported include plasma holoTC and methylmalonic acid. HoloTC was low (<35 pmol/L) in 8.0%, and plasma methylmalonic acid was increased (>350 nmol/L) in 12.1% of the study participants. The proportion of participants with holoTC above the upper limit of the calibration curve of the assay (>160 pmol/L) was 9.3%. Women had higher median total vitamin B$_{12}$ (324 vs 288 pmol/L; $P < 0.001$) and higher median holoTC (85.6 vs 73.4 pmol/L; $P < 0.001$) than men. Both total vitamin B$_{12}$ ($R^2 = 0.008$; $P < 0.001$) and holoTC ($R^2 = 0.007$; $P = 0.006$) were weakly and inversely associated with age. There was no significant linear association between total vitamin B$_{12}$ or holoTC and creatinine.

To compare total vitamin B$_{12}$ with holoTC as predictors of plasma methylmalonic acid and homocysteine concentrations, we constructed a series of multiple regression models (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue2). Methylmalonic acid and homocysteine serve in these models as metabolic indicators of vitamin B$_{12}$ status. For models 1–3, in which methylmalonic acid serves as the dependent variable, 643 individuals had complete data (i.e., measurements of total vitamin B$_{12}$, holoTC, methylmalonic acid, and creatinine). Persons with holoTC above the upper limit of the calibration curve of the assay (>160 pmol/L) were excluded from the analysis. In model 1, age, sex, and creatinine accounted for 9.8% ($R^2 = 0.098$) of the observed variance in methylmalonic acid within the study sample. The addition of total vitamin B$_{12}$ to the model (model 2) increased the R^2 value from 0.098 to 0.311 ($P < 0.001$). In comparison, substitution of holoTC for total vitamin B$_{12}$ in the model (model 3) increased the R^2 value from 0.098 to 0.372 ($P < 0.001$). Comparing the 2 measurements of vitamin B$_{12}$ status, we found that holoTC significantly improved the predictive capacity of the model by 6.1 percentage points more than did total vitamin B$_{12}$ ($P = 0.004$).

We also calculated standardized coefficients (β values) for each of the independent variables in the methylmalonic acid models. In model 2, total vitamin B$_{12}$ ($\beta = -0.468$) was a stronger predictor of methylmalonic acid than creatinine ($\beta = 0.308$). Similarly, in model 3, holoTC ($\beta = -0.532$) was a stronger predictor of methylmalonic acid than creatinine ($\beta = 0.365$).

For models 4–6 (see Table 2 in the online Data Supplement), in which homocysteine served as the dependent variable, 1016 individuals had complete data (i.e., measurements of total vitamin B$_{12}$, holoTC, homocysteine, erythrocyte folate, and creatinine). Again, persons with

Table 1. SALSA population characteristics.

	n	Median (range)	Percent abnormal (cutoff value)
Sex	1789	58% female	
Age, years	1789	70 (60–101)	
Total vitamin B$_{12}$, pmol/L	1545	307 (16–1453)	6.5 (<148)
HoloTC, pmol/L	1209	79.8 (3.0 to >160)	8.0 (<35)[a]
			9.3 (>160)
Methylmalonic acid, nmol/L	859	170 (30–9090)	12.1 (>350)
Homocysteine, μmol/L	1640	9.8 (4.0–129)	17.0 (>13)
Erythrocyte folate, μg/L	1501	488 (50–900)	0.9 ($\leq$160)
Creatinine,[b] mg/L			
Women	1041	0.7 (0.3–7.7)[a]	6.5 (>11)[b]
Men	748	0.9 (0.4–10.2)	5.4 (>14)[b]

[a] For holoTC, 35 pmol/L was the cutoff for abnormally low and 160 pmol/L was the upper limit of the calibration curve.

[b] Creatinine medians (ranges) in μmol/L: women, 62 (27–681); men, 80 (35–902). Creatinine cutoff values in μmol/L: women >97 μmol/L; men >124 μmol/L.

holoTC above the upper limit of the calibration curve of the assay ($>$160 pmol/L) were excluded from the analysis. In model 4, age, sex, erythrocyte folate, and creatinine explained 31.7% ($R^2 = 0.317$) of the observed variance in homocysteine within the study sample. The addition of total vitamin B_{12} to the model (model 5) increased the R^2 value from 0.317 to 0.433 ($P <0.001$). In comparison, substitution of holoTC for total vitamin B_{12} in the model (model 6) increased the R^2 value from 0.317 to 0.488 ($P <0.001$). Comparing the 2 measurements of vitamin B_{12} status, we found that holoTC significantly improved the predictive capacity of the model by 5.5 percentage points more than did total vitamin B_{12} ($P <0.001$).

We also calculated standardized β values for each of the independent variables in the homocysteine models. In model 5, the strongest predictor of homocysteine was creatinine ($\beta = 0.422$), followed by total vitamin B_{12} ($\beta = -0.352$). Erythrocyte folate was a relatively weak predictor of homocysteine ($\beta = -0.138$). A similar pattern was seen in model 6, in which holoTC replaced total vitamin B_{12}, and the standardized coefficients for creatinine, holoTC, and erythrocyte folate were 0.460, -0.428, and -0.111, respectively.

In separate multiple regression analyses (see Table 3 in the online Data Supplement) of 584 individuals with complete data for total vitamin B_{12}, holoTC, homocysteine, erythrocyte folate, creatinine, and methylmalonic acid, and excluding individuals with holoTC above the upper limit of the calibration curve, we compared methylmalonic acid with total vitamin B_{12} and holoTC as predictors of homocysteine concentrations. The R^2 value for the model that included methylmalonic acid as the independent indicator of vitamin B_{12} status was intermediate ($R^2 = 0.471$) to those of the total vitamin B_{12} ($R^2 = 0.433$) and holoTC ($R^2 = 0.501$) models. The R^2 values for the total vitamin B_{12} and holoTC models were significantly different from each other ($P <0.001$), whereas the R^2 value for the methylmalonic acid model was not significantly different from either the total vitamin B_{12} or the holoTC models.

To further compare total vitamin B_{12} and holoTC as predictors of vitamin B_{12} status, we constructed ROC curves (Fig. 1) that indicated the capacity of each measurement to discriminate between persons with and without vitamin B_{12} deficiency. Because no clinical criteria on which to base a diagnosis of vitamin B_{12} deficiency were available for the SALSA participants, we defined "likely" vitamin B_{12} deficiency by the following characteristics: homocysteine $>$13 μmol/L; methylmalonic acid $>$350 nmol/L; and healthy kidney function as reflected by serum creatinine $\leq$97 μmol/L ($\leq$1.1 mg/dL) in women and $\leq$124 μmol/L ($\leq$1.4 mg/dL) in men. Results from persons with increased creatinine concentrations were excluded from the statistical evaluation because renal dysfunction is known to cause increases, independent of vitamin B_{12} status, in both homocysteine and methylmalonic acid.
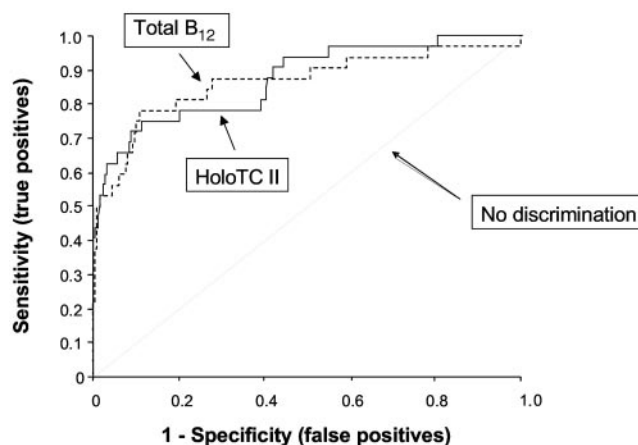

Fig. 1. ROC curves.

ROC curves were produced for total vitamin B_{12} (*dashed line*) and holoTC (*solid line*) to compare the capacity of each measurement to discriminate between persons with and without vitamin B_{12} deficiency. Likely vitamin B_{12} deficiency was defined as methylmalonic acid $>$350 nmol/L, homocysteine $>$13 μmol/L, and normal kidney function as reflected by serum creatinine $\leq$97 μmol/L ($\leq$1.1 mg/dL) in women and $\leq$124 μmol/L ($\leq$1.4 mg/dL) in men. AUCs (SE) were 0.816 (0.047) and 0.828 (0.040) for total B_{12} and holoTC, respectively. Both areas were significantly $>$0.500, the area that signifies no discrimination ($P <0.001$). The total sample size for these analyses was 607. The number of persons with likely vitamin B_{12} deficiency was 37.

Persons with holoTC above the upper limit of the calibration curve ($>$160 pmol/L) also were excluded. Of a total of 609 persons with complete data (i.e., those who had measurements of total vitamin B_{12}, holoTC, methylmalonic acid, and homocysteine, as well as a creatinine concentration within the reference interval), 37 persons (6.1%) had both homocysteine $>$13 μmol/L and methylmalonic acid $>$350 nmol/L. The AUCs (SE) of the ROC curves for total vitamin B_{12} [0.816 (0.047); $P <0.001$] and holoTC [0.828 (0.040); $P <0.001$] indicated that both measurements have similarly good capacities to discriminate between persons with likely vitamin B_{12} deficiency and all others.

We also calculated the relative sensitivities and specificities of total vitamin B_{12}, holoTC, and the combination of total vitamin B_{12} and holoTC to detect likely vitamin B_{12} deficiency, using the established cutoff values for both measurements cited above (total vitamin B_{12} $<$148 pmol/L; holoTC $<$35 pmol/L). For these calculations, holoTC values above the upper limit of the calibration curve were included because they could be classified as $>$35 pmol/L. Total sample size for these calculations was 656 persons. The cutoff value for holoTC had better sensitivity than the cutoff value for total vitamin B_{12} (59.5% vs 48.6%, respectively), whereas the cutoff value for holoTC had slightly lower specificity than the cutoff value for total vitamin B_{12} (93.7% vs 95.3%, respectively). If the cutoff values for total vitamin B_{12} and holoTC were used together such that all persons with low values for one or both of the measurements were combined, the sensitivity to detect likely vitamin B_{12} deficiency was 59.5% (equivalent to that calculated using holoTC alone

and higher than that calculated for total vitamin B$_{12}$ alone) and the specificity was 91.5% (slightly lower than that calculated using total vitamin B$_{12}$ or holoTC alone).

To further evaluate the utility of measuring both total vitamin B$_{12}$ and holoTC in screening for vitamin B$_{12}$ status, we compared geometric means for methylmalonic acid (adjusted for age, sex, and creatinine) and homocysteine (adjusted for age, sex, erythrocyte folate, and creatinine) among persons divided into 4 groups based on the established cutoff values for total vitamin B$_{12}$ and holoTC cited above. For these analyses, persons with holoTC values above the upper limit of the calibration curve were included because these values could be classified as >35 pmol/L. As shown in Figs. 2 and 3, those persons with both low total vitamin B$_{12}$ and low holoTC had significantly higher adjusted geometric mean methylmalonic acid and homocysteine concentrations than those persons with either total vitamin B$_{12}$ or holoTC, or both, in the reference interval ($P < 0.001$). Those individuals with low values for either vitamin B$_{12}$ or holoTC (but not both) had intermediate values for both methylmalonic acid and homocysteine.

Discussion

We used data generated from an elderly cohort at risk for vitamin B$_{12}$ deficiency to compare total vitamin B$_{12}$ and holoTC as screening tests for vitamin B$_{12}$ deficiency. HoloTC accounted for ~5%–6% more of the observed

Fig. 3. Homocysteine concentrations sorted by cutoff values for total vitamin B$_{12}$ and holoTC.

Study participants were divided into 4 groups based on whether their total vitamin B$_{12}$ was less than or more than 148 pmol/L and their holoTC less than or more than 35 pmol/L. The *columns* represent geometric means and the *error bars* the 95% confidence intervals for homocysteine in each group, adjusted for age, sex, erythrocyte folate, and ln(creatinine). The *numbers* above each *column* indicate the actual adjusted geometric means with 95% confidence limits for each group. Sample sizes (n) are indicated for each group. Significant differences in natural log–adjusted mean values among the groups are indicated by a *horizontal line* and corresponding *P* value. *NS*, not significant.
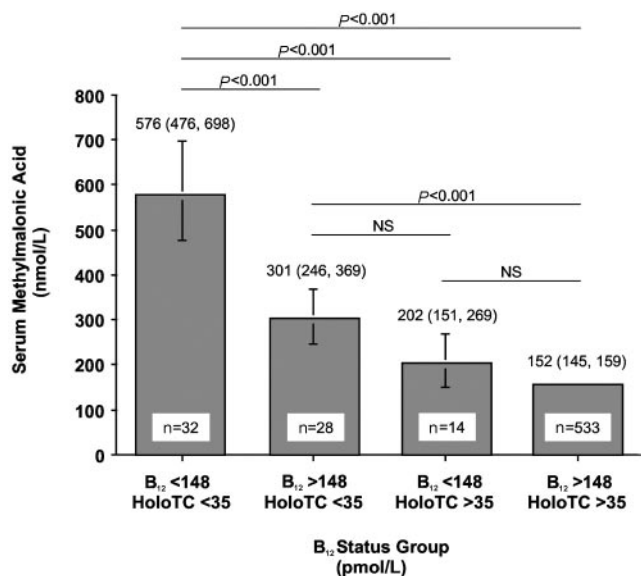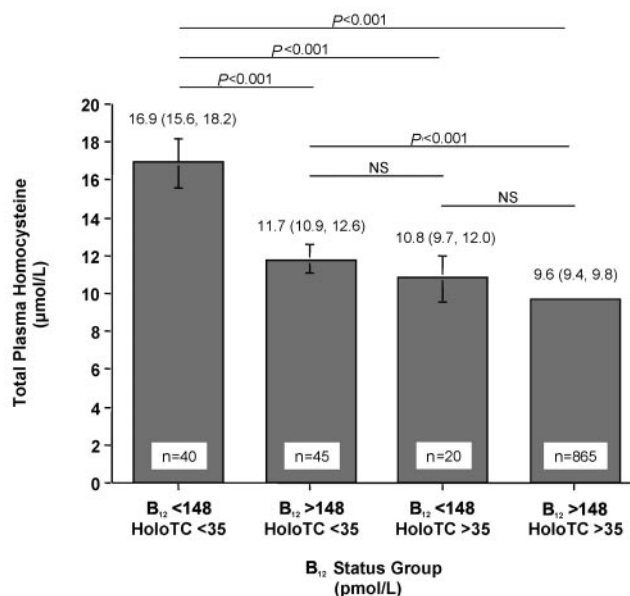

Fig. 2. Methylmalonic acid concentrations sorted by cutoff values for total vitamin B$_{12}$ and holoTC.

Study participants were divided into 4 groups based on whether their total vitamin B$_{12}$ was less than or more than 148 pmol/L and their holoTC less than or more than 35 pmol/L. The *columns* represent geometric means, and the *error bars* represent the 95% confidence intervals for methylmalonic acid in each group adjusted for age, sex, and ln(creatinine). The *numbers* above each *column* indicate the actual adjusted geometric means with 95% confidence limits for each group. Sample sizes (n) are indicated for each group. Significant differences in natural log–adjusted mean values among the groups are indicated by a *horizontal line* and corresponding *P* value. *NS*, not significant.

variance in 2 metabolic indicators of vitamin B$_{12}$ status, methylmalonic acid and homocysteine, than was explained by total vitamin B$_{12}$. Total vitamin B$_{12}$ and holoTC were essentially equivalent for discriminating between persons with and without likely vitamin B$_{12}$ deficiency (defined as increased methylmalonic acid and increased homocysteine in the absence of evidence of renal dysfunction).

When we used total vitamin B$_{12}$ and holoTC together as the initial screen for vitamin B$_{12}$ deficiency, the sensitivity for likely vitamin B$_{12}$ deficiency was greater than that calculated for total vitamin B$_{12}$ alone but was the same as that calculated for holoTC alone. However, the specificity for measurements in which both values were low was slightly less than that calculated for low total vitamin B$_{12}$ alone and for low holoTC alone. Although these findings might suggest that there is no advantage to combining total vitamin B$_{12}$ and holoTC in screening for vitamin B$_{12}$ deficiency, when we performed data analysis that treated the metabolic indicators of vitamin B$_{12}$ status as continuous variables, we found that those persons with low concentrations of both total vitamin B$_{12}$ and holoTC had higher methylmalonic acid and homocysteine concentrations than persons with low concentrations of only one or neither of the measures of vitamin B$_{12}$ status. Persons with either low total vitamin B$_{12}$ or low holoTC, but not both, had intermediate concentrations of methyl-

malonic acid and homocysteine. Under the assumption that higher methylmalonic acid and homocysteine concentrations reflect a greater likelihood (or severity) of vitamin B_{12} deficiency, measuring both total vitamin B_{12} and holoTC allows graded predictive classifications of vitamin B_{12} status to be established. Accordingly, we propose the following designations based on the total vitamin B_{12} and holoTC tests:

(1) Probable vitamin B_{12} deficiency: both total vitamin B_{12} and holoTC low;

(2) Possible vitamin B_{12} deficiency: either total vitamin B_{12} or holoTC low;

(3) Vitamin B_{12} deficiency unlikely: neither total vitamin B_{12} nor holoTC low.

Inclusion of the "possible vitamin B_{12} deficiency" category is important because it takes into consideration the possibility that an individual with a total vitamin B_{12} concentration within the reference interval might nonetheless be at risk for deficiency as indicated by a low holoTC concentration, and vice versa. Measuring both would facilitate identification of more at-risk individuals. Using these designations, a physician could then decide on the necessity for further diagnostic testing (methylmalonic acid, homocysteine, complete blood count, full neurologic evaluation, vitamin B_{12} absorptive capacity) and/or treatment.

Our screening strategy for identifying individuals at high risk of vitamin B_{12} deficiency should be compared with that proposed by Clarke et al. *(16)*. On the basis of methylmalonic acid and homocysteine concentrations, these investigators concluded that individuals with total vitamin B_{12} <150 pmol/L have a high probability and that individuals with total vitamin B_{12} >200 pmol/L have a low probability of being vitamin B_{12} deficient. Individuals in the borderline range of 150–200 pmol/L are identified as high risk for vitamin B_{12} deficiency if they also have increased methylmalonic acid (>350 nmol/L) or increased homocysteine (>15 μmol/L). This strategy has drawbacks. Measurement of methylmalonic acid is performed routinely only in select locations around the world. Measurement of homocysteine, although less expensive and easier to perform, is not as specific for vitamin B_{12} status as are methylmalonic acid measurements. In addition, both methylmalonic acid and homocysteine become increased with renal dysfunction *(6)*; therefore, an individual with renal disease could have a total vitamin B_{12} of 150–200 pmol/L as well as increased methylmalonic acid and homocysteine and not be vitamin B_{12} deficient.

The advantage of our strategy to use both total vitamin B_{12} and holoTC is that it obviates the need to perform the methylmalonic acid and homocysteine assays during screening, considering the inherent limitations of the metabolite assays. In contrast to methylmalonic acid and homocysteine, the holoTC assay is a relatively straightforward, routine radioimmunoassay. Also supporting holoTC measurement over methylmalonic acid is our finding that holoTC is as good as (if not better than) methylmalonic acid as a predictor of plasma homocysteine concentrations. There are some limitations of holoTC at present, however. Unlike total vitamin B_{12}, the holoTC assay has not been automated. In addition, the determinants of holoTC need to be systematically investigated: plasma holoTC increases in renal disease *(15, 19)*, and from this observation, Herrmann et al. *(15)* concluded that holoTC cannot be used as an indicator of vitamin B_{12} status in patients with renal disease. HoloTC may also increase after vitamin B_{12} intake *(20)*, and we *(21)* and others *(22–30)* have observed that the plasma holoTC concentration is affected by a common polymorphism in TC (C776G), which can also influence methylmalonic acid and homocysteine concentrations.

Recently, other research teams have evaluated the utility of holoTC as a screening tool for vitamin B_{12} deficiency *(15, 31–35)*. Herrmann et al. *(15)*, using increased methylmalonic acid (>271 nmol/L) as the indicator of vitamin B_{12} deficiency, compared ROC curves for total vitamin B_{12} and holoTC. In this analysis, the AUC values for holoTC and total vitamin B_{12} were somewhat higher than those obtained in the present study. In addition, using cutoff values for total vitamin B_{12} and holoTC similar to what we used, Herrmann et al. *(15)* calculated respective sensitivities and specificities for increased methylmalonic acid of 0.45 and 0.68 for total vitamin B_{12} and 0.87 and 0.75 for holoTC. These results are consistent with our findings with respect to sensitivity but not specificity. The discrepancies between the studies may be related to different criteria for vitamin B_{12} deficiency used in the 2 studies.

Using the criteria for vitamin B_{12} deficiency of methylmalonic acid >750 nmol/L and homocysteine >15 μmol/L, Lloyd-Wright et al. *(32)* constructed ROC curves for holoTC and total vitamin B_{12} in a population of vegans and omnivores. The AUC values they obtained were also somewhat higher than those in the present study. Again, this difference may be attributable to different definitions of likely vitamin B_{12} deficiency used in each study. Lloyd-Wright et al. *(32)* suggested a screening strategy for vitamin B_{12} deficiency that uses a holoTC measurement alone. Patients with holoTC >50 pmol/L would be classified as "unlikely to suffer from vitamin B_{12} deficiency", and patients with holoTC <25 pmol/L would be classified as "likely to suffer from vitamin B_{12} deficiency". Only patients with results within the indeterminate range of 25–50 pmol/L would require further testing of metabolic markers. This strategy, however, is not appreciably different from that of Clarke et al. *(16)*, who used total vitamin B_{12} instead of holoTC.

Hvas and Nexo *(35)* compared and contrasted total vitamin B_{12} and holoTC as screening assays in persons at risk for vitamin B_{12} deficiency, as indicated by increased methylmalonic acid (>280 nmol/L). Using the criteria for vitamin B_{12} deficiency of methylmalonic acid >750 nmol/L and homocysteine >15 μmol/L, they con-

structed ROC curves for holoTC and total vitamin B$_{12}$. Again, AUC values were somewhat higher than observed in the present study. Two other differences between the studies are notable: First, Hvas and Nexo [35] found that total vitamin B$_{12}$ was more strongly associated with methylmalonic acid and homocysteine than holoTC, whereas we found the opposite. Second, Hvas and Nexo [35] found that creatinine was positively associated with both total vitamin B$_{12}$ and holoTC. In contrast, we found no such correlation. The reasons for these discrepancies between the studies are unclear at this time.

The primary limitation of our study, as well as the studies of Herrmann et al. [15] and Lloyd-Wright et al. [32], is that comprehensive clinical diagnostic criteria were not used to definitively categorize individuals as vitamin B$_{12}$ deficient or vitamin B$_{12}$ adequate. In our study, we did not have access to hematologic or neurologic assessments. Consequently, we could only categorize persons as having likely vitamin B$_{12}$ deficiency based on methylmalonic acid and homocysteine concentrations, while accounting for potential confounding by renal dysfunction. There is the possibility that some percentage of participants were misclassified with respect to their true vitamin B$_{12}$ status. In particular, the sensitivities calculated for both total vitamin B$_{12}$ and holoTC, using the established cutoff values of 148 pmol/L and 35 pmol/L, respectively, might improve with more definitive criteria for identifying true clinical vitamin B$_{12}$ deficiency. It must be noted, however, that Hvas and Nexo [35] did not find significant associations between biochemical markers of vitamin B$_{12}$ deficiency (e.g., total vitamin B$_{12}$, holoTC, methylmalonic acid, and homocysteine) and clinical manifestations of vitamin B$_{12}$ deficiency (e.g., neurologic symptoms, anemia, and gastrointestinal symptoms) and that they observed no improvements in clinical symptoms after 3 months of vitamin B$_{12}$ supplementation despite improvements in the biochemical indices. Solomon [36] has also found poor correlation between biochemical indices of vitamin B$_{12}$ status and clinical response to vitamin B$_{12}$ supplementation. The reasons for such discrepancies between the biochemical indicators and the clinical manifestations are unclear [37].

References

1. Chanarin I. The megaloblastic anemias, 2nd ed. Oxford, UK: Blackwell Scientific Publications, 1979:783pp.
2. Carmel R. Prevalence of undiagnosed pernicious anemia in the elderly. Arch Intern Med 1996;156:1097–100.
3. Baik HW, Russell RM. Vitamin B$_{12}$ deficiency in the elderly. Annu Rev Nutr 1999;19:357–77.
4. Campbell AK, Miller JW, Green R, Haan MN, Allen LH. Plasma vitamin B-12 concentrations in an elderly Latino population are predicted by serum gastrin concentrations and crystalline vitamin B-12 intake. J Nutr 2003;133:2770–6.
5. Marcus DL, Shadick N, Crantz J, Gray M, Hernandez F, Freedman ML. Low serum B$_{12}$ levels in a hematologically normal elderly subpopulation. J Am Geriatr Soc 1987;35:635–8.
6. Green R. Metabolite assays in cobalamin and folate deficiency. Baillieres Clin Haematol 1995;8:533–66.
7. Lindenbaum J, Healton EB, Savage DG, Brust JC, Garrett TJ, Podell ER, et al. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. N Engl J Med 1988;318:1720–8.
8. Allen RH, Stabler SP, Savage DG, Lindenbaum J. Metabolic abnormalities in cobalamin (vitamin B$_{12}$) and folate deficiency. FASEB J 1993;7:1344–53.
9. Herzlich B, Herbert V. Depletion of serum holotranscobalamin II: an early sign of negative vitamin B$_{12}$ balance. Lab Invest 1988;58:332–7.
10. Ulleland M, Eilertsen I, Quadros EV, Rothenberg SP, Fedosov SN, Sundrehagen E, et al. Direct assay for cobalamin bound to transcobalamin (holo-transcobalamin) in serum. Clin Chem 2002;48:526–32.
11. Wu CC, Mungas D, Petkov CI, Eberling JL, Zrelak PA, Buonocore MH, et al. Brain structure and cognition in a community sample of elderly Latinos. Neurology 2002;59:383–91.
12. Kushnir MM, Komaromy-Hiller G, Shushan B, Urry FM, Roberts WL. Analysis of dicarboxylic acids by tandem mass spectrometry: high-throughput quantitative measurement of methylmalonic acid in serum, plasma, and urine. Clin Chem 2001;47:1993–2002.
13. Gilfix BM, Blank DW, Rosenblatt DS. Novel reductant for determination of total plasma homocysteine. Clin Chem 1997;43:687–8.
14. Lindgren A, Kilander A, Bagge E, Nexo E. Holotranscobalamin: a sensitive marker of cobalamin malabsorption. Eur J Clin Invest 1999;29:321–9.
15. Herrmann W, Obeid R, Schorr H, Geisel J. Functional vitamin B$_{12}$ deficiency and determination of holotranscobalamin in populations at risk. Clin Chem Lab Med 2003;41:1478–88.
16. Clarke R, Refsum H, Birks J, Evans JG, Johnston C, Sherliker P, et al. Screening for vitamin B-12 and folate deficiency in older persons. Am J Clin Nutr 2003;77:1241–7.
17. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 1999;340:1449–54.
18. Miller JW, Green R, Ramos MI, Allen LH, Mungas DM, Jagust WJ, et al. Homocysteine and cognitive function in the Sacramento Area Latino Study on Aging (SALSA). Am J Clin Nutr 2003;78:441–7.
19. Carmel R, Vasireddy H, Aurangzeb I, George K. High serum cobalamin levels in the clinical setting: clinical associations and holo-transcobalamin changes. Clin Lab Haematol 2001;23:365–71.
20. Bor MV, Cetin M, Aytac S, Altay C, Nexo E. Nonradioactive vitamin B$_{12}$ absorption test evaluated in controls and in patients with

inherited malabsorption of vitamin B_{12}. Clin Chem 2005;51:
2151–5.

21. Miller JW, Ramos MI, Garrod MG, Flynn MA, Green R. Transcobalamin II 775G>C polymorphism and indices of vitamin B_{12} status in healthy older adults. Blood 2002;100:718–20.

22. Namour F, Guy M, Aimone-Gastin I, de Nonancourt M, Mrabet N, Guéant J-L. Isoelectric phenotype and relative concentration of transcobalamin II isoproteins related to the codon 259 Arg/Pro polymorphism. Biochem Biophys Res Comm 1988;251:769–74.

23. Namour F, Olivier J-L, Abdelmouttaleb I, Adjalla C, Debard R, Salvat C, et al. Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood. Blood 2001; 97:1092–8.

24. Afman LA, van der Put NMJ, Thomas CMG, Trijbels JMF, Blom HJ. Reduced vitamin B_{12} binding by transcobalamin II increases risk of neural tube defects. Q J Med 2001;94:159–66.

25. McCaddon A, Blennow K, Hudson P, Regland B, Hill D. Transcobalamin polymorphism and homocysteine. Blood 2001;98: 3497–9.

26. Namour F, Guéant J-L. Transcobalamin polymorphism, homocysteine, and aging. Blood 2001;98:3499.

27. Afman LA, Lievers KJ, van der Put NMJ, Trijbels JMF, Blom HJ. Single nucleotide polymorphisms in the transcobalamin gene: relationship with transcobalamin concentrations and risk of neural tube defects. Eur J Hum Genet 2002;10:433–8.

28. Lievers KJ, Afman LA, Kluijtmans LA, Boers GH, Verhoef P, den Heijer M, et al. Polymorphisms in the transcobalamin gene: association with plasma homocysteine in healthy individuals and vascular disease patients. Clin Chem 2002;48:1383–9.

29. Wans S, Schuttler K, Jakubiczka S, Muller A, Luley C, Dierkes J. Analysis of the transcobalamin II 776C>G (259P>R) single nucleotide polymorphism by denaturing HPLC in healthy elderly: associations with cobalamin, homocysteine and holo-transcobalamin II. Clin Chem Lab Med 2003;41:1532–6.

30. von Castel-Dunwoody KM, Kauwell GP, Shelnutt KP, Vaughn JD, Griffin ER, Maneval DR, et al. Transcobalamin II 776C>G polymorphism negatively affects vitamin B-12 metabolism. Am J Clin Nutr 2005;81:1436–41.

31. Obeid R, Schorr H, Eckert R, Herrmann W. Vitamin B_{12} status in the elderly as judged by available biochemical markers. Clin Chem 2004;50:238–41.

32. Lloyd-Wright Z, Hvas AM, Moller J, Sanders TAB, Nexo E. Holotranscobalamin as an indicator of dietary vitamin B_{12} deficiency. Clin Chem 2003;49:2076–8.

33. Hvas AM, Nexo E. Holotranscobalamin as a predictor of vitamin B_{12} status. Clin Chem Lab Med 2003;41:1489–92.

34. Nexo E, Hvas A-M, Bleie O, Refsum H, Fedosov SN, Vollset SE, et al. Holotranscobalamin is an early marker of changes in cobalamin homeostasis: a randomized placebo-controlled study. Clin Chem 2002;48:1768–71.

35. Hvas A-M, Nexo E. Holotranscobalamin: a first choice assay for diagnosing early vitamin B_{12} deficiency. J Intern Med 2005;257: 289–98.

36. Solomon LR. Cobalamin-responsive disorders in the ambulatory care setting: unreliability of cobalamin, methylmalonic acid, and homocysteine testing. Blood 2005;105:978–85.

37. Green R. Unreliability of current assays to detect cobalamin deficiency: "nothing gold can stay". Blood 2005;105:910–1.