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

JOSHUA W. MILLER,\textsuperscript{1} MARJORIE G. GARROD,\textsuperscript{1} ALAN L. ROCKWOOD,\textsuperscript{2} MARK M. KUSHNIR,\textsuperscript{2} LINDSAY H. ALLEN,\textsuperscript{3} MARY N. HAAN,\textsuperscript{4} and RALPH GREEN\textsuperscript{1*}

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

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

Results: Low total vitamin B\textsubscript{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 \mu mol/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\textsubscript{12} (\(P \leq 0.004\)). ROC curve analysis indicated that total vitamin B\textsubscript{12} and holoTC were essentially equivalent in their ability to discriminate persons with and without vitamin B\textsubscript{12} deficiency. Individuals with low concentrations of both total vitamin B\textsubscript{12} and holoTC had significantly higher concentrations of methylmalonic acid and homocysteine than did individuals with total vitamin B\textsubscript{12} and/or holoTC within the reference intervals (\(P < 0.001\)).

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

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Vitamin B\textsubscript{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\textsubscript{12} malabsorption (1, 2). Other estimates suggest that the prevalence of vitamin B\textsubscript{12} deficiency may be as high as 30\% among the elderly because of malabsorption of vitamin B\textsubscript{12} from food caused by chronic gastritis, gastric atrophy, and perhaps other unknown causes (3, 4).

Sensitive and specific assays for determining vitamin B\textsubscript{12} status are needed because of the high prevalence and the potentially serious complications of vitamin B\textsubscript{12} deficiency. Typically, vitamin B\textsubscript{12} deficiency is suspected only when an individual presents with hematologic manifestations of megaloblastic anemia, which occur only in the most severely vitamin B\textsubscript{12}-depleted individuals (5). Total plasma vitamin B\textsubscript{12} concentration is the current standard clinical screening test for vitamin B\textsubscript{12} deficiency. Total vitamin B\textsubscript{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\textsubscript{12} deficiency; however, a proportion of individuals with vitamin B\textsubscript{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). 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 \( B_{12} \) less than 148 pmol/L (less than 200 pg/mL), was present in 6.5% of the population. A large proportion (17.0%) had increased plasma homocysteine (greater than 13 \( \mu \)mol/L), although less than 1% had erythrocyte folate concentrations indicative of deficiency (160 \( \mu \)g/L). The prevalence of increased serum creatinine (greater than 97 \( \mu \)mol/L, or greater than 1.4 mg/dL) 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 \( B_{12} \) (\( R^2 = 0.008; \) 0.01) and HoloTC (\( R^2 = 0.007; \) 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 (greater than 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 (\( \beta \) 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

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<th>Table 1. SALSA population characteristics.</th>
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<sup>a</sup> For HoloTC, 35 pmol/L was the cutoff for abnormally low and 160 pmol/L was the upper limit of the calibration curve.

<sup>b</sup> Creatinine medians (ranges) in \( \mu \)mol/L: women, 62 (27–681); men, 80 (35–902). Creatinine cutoff values in \( \mu \)mol/L: women >97 \( \mu \)mol/L; men >124 \( \mu \)mol/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 \(\beta\) 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 \(\mu\)mol/L; methylmalonic acid >350 nmol/L; and healthy kidney function as reflected by serum creatinine ≤97 \(\mu\)mol/L (≤1.1 mg/dL) in women and ≤124 \(\mu\)mol/L (≤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.

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 \(\mu\)mol/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.

**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 \(\mu\)mol/L, and normal kidney function as reflected by serum creatinine ≤97 \(\mu\)mol/L (≤1.1 mg/dL) in women and ≤124 \(\mu\)mol/L (≤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.

Clinical Chemistry 52, No. 2, 2006 281
and higher than that calculated for total vitamin B₁₂ alone) and the specificity was 91.5% (slightly lower than that calculated using total vitamin B₁₂ or holoTC alone).

To further evaluate the utility of measuring both total vitamin B₁₂ and holoTC in screening for vitamin B₁₂ 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 B₁₂ 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 B₁₂ and low holoTC had significantly higher adjusted geometric mean methylmalonic acid and homocysteine concentrations than those persons with either total vitamin B₁₂ or holoTC, or both, in the reference interval (P < 0.001). Those individuals with low values for either vitamin B₁₂ 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 B₁₂ deficiency to compare total vitamin B₁₂ and holoTC as screening tests for vitamin B₁₂ deficiency. HoloTC accounted for ~5%–6% more of the observed variance in 2 metabolic indicators of vitamin B₁₂ status, methylmalonic acid and homocysteine, than was explained by total vitamin B₁₂. Total vitamin B₁₂ and holoTC were essentially equivalent for discriminating between persons with and without likely vitamin B₁₂ deficiency (defined as increased methylmalonic acid and increased homocysteine in the absence of evidence of renal dysfunction).

When we used total vitamin B₁₂ and holoTC together as the initial screen for vitamin B₁₂ deficiency, the sensitivity for likely vitamin B₁₂ deficiency was greater than that calculated for total vitamin B₁₂ 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 B₁₂ alone and for low holoTC alone. Although these findings might suggest that there is no advantage to combining total vitamin B₁₂ and holoTC in screening for vitamin B₁₂ deficiency, when we performed data analysis that treated the metabolic indicators of vitamin B₁₂ status as continuous variables, we found that those persons with low concentrations of both total vitamin B₁₂ and holoTC had higher methylmalonic acid and homocysteine concentrations than persons with low concentrations of only one or neither of the measures of vitamin B₁₂ status. Persons with either low total vitamin B₁₂ or low holoTC, but not both, had intermediate concentrations of methylmalonic acid and homocysteine.
malonic acid and homocysteine. Under the assumption that higher methylmalonic acid and homocysteine concentrations reflect a greater likelihood (or severity) of vitamin B₁₂ deficiency, measuring both total vitamin B₁₂ and holoTC allows graded predictive classifications of vitamin B₁₂ status to be established. Accordingly, we propose the following designations based on the total vitamin B₁₂ and holoTC tests:

1. Probable vitamin B₁₂ deficiency: both total vitamin B₁₂ and holoTC low;
2. Possible vitamin B₁₂ deficiency: either total vitamin B₁₂ or holoTC low;
3. Vitamin B₁₂ deficiency unlikely: neither total vitamin B₁₂ nor holoTC low.

Inclusion of the “possible vitamin B₁₂ deficiency” category is important because it takes into consideration the possibility that an individual with a total vitamin B₁₂ 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 B₁₂ absorptive capacity) and/or treatment.

Our screening strategy for identifying individuals at high risk of vitamin B₁₂ 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 B₁₂ <150 pmol/L have a high probability and that individuals with total vitamin B₁₂ >200 pmol/L have a low probability of being vitamin B₁₂ deficient. Individuals in the borderline range of 150–200 pmol/L are identified as high risk for vitamin B₁₂ 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 B₁₂ 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 B₁₂ of 150–200 pmol/L as well as increased methylmalonic acid and homocysteine and not be vitamin B₁₂ deficient.

The advantage of our strategy to use both total vitamin B₁₂ 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 B₁₂, 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 B₁₂ status in patients with renal disease. HoloTC may also increase after vitamin B₁₂ 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 B₁₂ deficiency (15, 31–35). Herrmann et al. (15), using increased methylmalonic acid (>271 nmol/L) as the indicator of vitamin B₁₂ deficiency, compared ROC curves for total vitamin B₁₂ and holoTC. In this analysis, the AUC values for holoTC and total vitamin B₁₂ were somewhat higher than those obtained in the present study. In addition, using cutoff values for total vitamin B₁₂ 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 B₁₂ 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 B₁₂ deficiency used in the 2 studies.

Using the criteria for vitamin B₁₂ 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 B₁₂ 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 B₁₂ deficiency used in each study. Lloyd-Wright et al. (32) suggested a screening strategy for vitamin B₁₂ deficiency that uses a holoTC measurement alone. Patients with holoTC >50 pmol/L would be classified as “unlikely to suffer from vitamin B₁₂ deficiency”, and patients with holoTC <25 pmol/L would be classified as “likely to suffer from vitamin B₁₂ 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 B₁₂ instead of holoTC.

Hvas and Nexo (35) compared and contrasted total vitamin B₁₂ and holoTC as screening assays in persons at risk for vitamin B₁₂ deficiency, as indicated by increased methylmalonic acid (>280 nmol/L). Using the criteria for vitamin B₁₂ deficiency of methylmalonic acid >750 nmol/L and homocysteine >15 μmol/L, they con-
structured ROC curves for holoTC and total vitamin B₁₂. 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 B₁₂ 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 B₁₂ 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 B₁₂ deficient or vitamin B₁₂ adequate. In our study, we did not have access to hematologic or neurologic assessments. Consequently, we could only categorize persons as having likely vitamin B₁₂ 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 B₁₂ status. In particular, the sensitivities calculated for both total vitamin B₁₂ 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 B₁₂ deficiency. It must be noted, however, that Hvas and Nexo (35) did not find significant associations between biochemical markers of vitamin B₁₂ deficiency (e.g., total vitamin B₁₂, holoTC, methylmalonic acid, and homocysteine) and clinical manifestations of vitamin B₁₂ deficiency (e.g., neurologic symptoms, anemia, and gastrointestinal symptoms) and that they observed no improvements in clinical symptoms after 3 months of vitamin B₁₂ supplementation despite improvements in the biochemical indices. Solomon (36) has also found poor correlation between biochemical indices of vitamin B₁₂ status and clinical response to vitamin B₁₂ supplementation. The reasons for such discrepancies between the biochemical indicators and the clinical manifestations are unclear (37).

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