Clinical Medicine & Research
Volume 11, Number 1: 7-15

clinmedres.org

Original Research

Laboratory Evaluation for Vitamin B_{12} Deficiency: The Case for Cascade Testing

Richard L. Berg, MS and Gene R. Shaw, MD

Objective: Potential vitamin B_{12} deficiency is a common clinical diagnostic problem, and many providers have a low threshold for initiating therapy. The goal of this study was to systematically evaluate current practice patterns regarding the laboratory evaluation of suspected vitamin B_{12} deficiency.

Methods: This retrospective study reviewed the electronic medical records of 192 patients initiated on intramuscular vitamin B_{12} injections.

Results: Only 12 patients had objectively documented hematologic responses: decrease of mean corpuscular volume by ≥5 fL with stable or improved hemoglobin. Another 5 patients had equivocal hematologic responses. There was one plausible neurologic response. Thus, only 18 (9.4%) of 192 patients had data supportive of a clinical response. In these 18 patients, the baseline serum B_{12} level was ≤107 pg/mL; only 3 patients also had a baseline serum methylmalonic acid level, which was ≥1.29 µmol/L in all 3 patients.

Conclusions: Currently, only a small minority of patients initiated on intramuscular vitamin B_{12} supplementation derive any meaningful clinical benefit. Furthermore, current testing recommendations for vitamin B_{12} deficiency are usually not followed. Up-front ordering of a diagnostic testing cascade is recommended to improve compliance; an example is presented with decision points chosen to improve specificity for clinically evident vitamin B_{12} deficiency without loss of sensitivity. Ultimately, a better understanding of vitamin B_{12} physiology is needed to develop and evaluate laboratory tests that more accurately reflect true intracellular vitamin B_{12} status.

Keywords: Holotranscobalamin; Methylmalonic acid/blood; Nutritional status; Vitamin B_{12}

The laboratory evaluation of possible vitamin B_{12} deficiency is often prompted by the presence of macrocytic anemia.[1] In clinically evident vitamin B_{12} deficiency, macrocytosis (mean corpuscular volume [MCV] >99 fL) usually precedes the development of anemia; less commonly neurologic manifestations can occur when both values are normal.[2] However, macrocytosis is a common nonspecific finding in adults undergoing an automated complete blood count (CBC). Even less specific are neurologic abnormalities that may include paresthesias, ataxia, dementia, and depression. Rarely do patients derive any meaningful neurologic response to vitamin B_{12} supplementation (personal communication with author, GRS).

Published estimates of the sensitivity and specificity of serum B_{12} measurement vary widely, largely due to the lack of a gold standard for diagnosis.[3,4] Serum B_{12} levels below 100 pg/mL are reported to have specificity approaching 90% for diagnosing clinically-manifest deficiency.[3] However, in clinical practice, with more chronically ill, elderly patients, the practical specificity may be lower.[5] Using elevated metabolite levels (ie, serum methylmalonic acid [MMA] or

Corresponding Author: Gene R. Shaw, MD; Marshfield Clinic; Department of Lab/Pathology; 1000 North Oak Avenue; Marshfield, WI 54449; Tel: (715) 221-6300; Fax: (715) 387-7121; E-mail: shaw.gene@marshfieldclinic.org

Received: July 24, 2012
Revised: October 1, 2012
Accepted: October 3, 2012

doi:10.3121/cmr.2012.1112

homocysteine [Hcy]) to define deficiency, the "screening" serum B_{12} cutoff used in many diagnostic algorithms has been raised to 250 pg/mL to 300 pg/mL to provide greater sensitivity.[6] With this approach, the prevalence of vitamin B_{12} deficiency may be as high as 15% to 20% in the elderly.[7] Of course, most of these patients will have no hematologic or neurologic manifestations of B_{12} deficiency (ie, subclinical deficiency).[8] Holotranscobalamin (holoTC) has been reported to offer slightly better performance than total serum B_{12} levels in diagnosing deficiency, but these study designs have been criticized, and there is currently no consensus that holoTC should replace conventional serum B_{12} testing.[8,9]

Given the limitations of current testing for vitamin B_{12} deficiency, and because its administration is inexpensive and fairly innocuous, many clinicians have a low threshold for prescribing vitamin B_{12} supplementation (usually by intramuscular injection to bypass issues with absorption). However, this approach may give a false sense of effective medical intervention for both the patient and provider, thereby diverting attention away from further evaluation and appropriate diagnosis.

In recent years, a number of diagnostic algorithms for evaluating vitamin B_{12} deficiency have been promulgated, but adherence to these recommendations in clinical practice is unknown. Also, how well these algorithms actually perform in terms of predicting meaningful response to vitamin B_{12} supplementation is unclear. In this study, we sought to systematically evaluate current practice patterns regarding the laboratory evaluation of suspected vitamin B_{12} deficiency.

Methods

This study was approved with waiver of written informed consent by the Institutional Review Board of Marshfield Clinic, a large, multi-specialty, multi-site group practice with regional centers throughout central and northern Wisconsin. Medical records of 250 potential subjects were electronically identified utilizing the Healthcare Common Procedure Coding System (HCPCS) code for vitamin B_{12} injection (J3420) and International Classification of Diseases (ICD-9-CM) codes related to vitamin B_{12} deficiency (266.2, 281.0-1), with 125 randomly selected for manual review from each of the calendar years 2000 and 2005. Fifty-eight patients were excluded, leaving 192 cases (93 from year 2000 and 99 from year 2005) that formed the basis for this study. Reasons for exclusion were previous vitamin B12 injections within 12 months before the reference date (26 cases), documented alcohol abuse (16 cases), undergoing chemotherapy or hormonal therapy for a malignancy (9 cases), and no documentation that vitamin B_{12} therapy was initiated (7 cases). Very few patients had well-documented neurologic exams, which generally precluded objective evaluation of neurologic improvement.

Vitamin B_{12} was measured on the Bayer Immunol analyzer from June 1999 to August 2004 with a reference range of 185-1000 pg/mL (137-740 pmol/L). Starting in June 2002, the comment "Borderline low B_{12} level (100-250 pg/mL); suggest serum methylmalonic acid if clinically indicated" was addended to all laboratory reports with B_{12} levels in that range. In August 2004, B_{12} testing was moved onto the Beckman Access/DXI instrument which uses a competitive

Table 1. Demographics and baseline laboratory results.

Patients (n)	192
Female (%)	64.1
Age	
median (years)	75.2
range	18–93
B_{12}	
# tested	171
median (pg/mL)	168
range (pg/mL)	26–2000
Hgb	
# tested	165
median (g/dL)	13.1
range (g/dL)	7.5–17.2
MCV	
# tested	165
median (fL)	91.7
range (fL)	63–131
MMA	
# tested	40
median (µmol/L)	0.4
range (µmol/L)	0.1–16
Serum creatinine	
# tested	179
median (mg/dL)	1.0
range (mg/dL)	0.5–5.4

Hgb, hemoglobin; MCV, mean corpuscular volume; MMA, methylmalonic acid

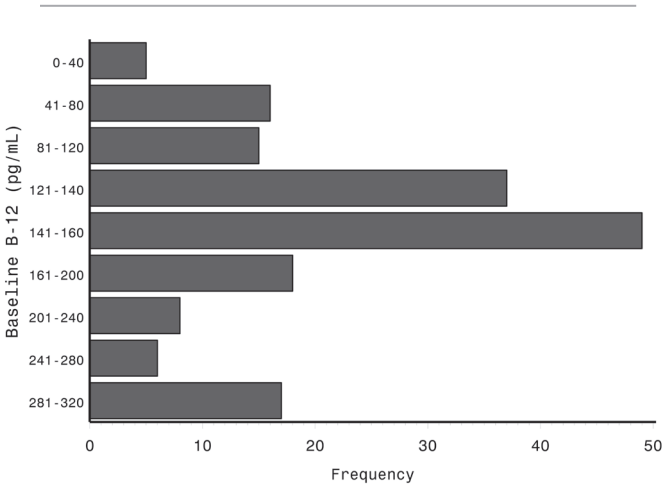

Figure 1. Frequency distribution of baseline values for serum vitamin B_{12}.

Table 2. Diagnostic category by year.

Group*	Year 2000 n (%)	Year 2005 n (%)
1: Minimal/no evidence	33 (35.5)	35 (35.4)
2: Possible	38 (40.9)	51 (51.5)
3: Probable	18 (19.4)	6 (6.1)
4: Confirmed	4 (4.3)	7 (7.1)
Total patients (N)	*93*	*99*

*Groups were defined as follows: Group 1 – (a) no documented testing for vitamin B_{12} status, (b) normal serum vitamin B_{12} (>250 pg/mL) regardless of MMA value, or (c) low serum vitamin B12 (any level ≤ 250 pg/mL) with normal MMA. Group 2 – moderate to borderline low serum vitamin B_{12} (100–250 pg/mL) with no documented testing for MMA. Group 3 *(unconfirmed)* – very low serum vitamin B_{12} (<100 pg/mL), but no documented testing for MMA. Group 4 *(confirmed by laboratory criteria)* – low serum vitamin B_{12} (≤250 pg/mL) with elevated MMA.

immunoassay incorporating intrinsic factor. The correlation between the two methods was: Access = 0.966 x (Immuno1) + 12; (n = 42 lab employees). Based on complaints from Marshfield Clinic neurologists regarding a longstanding history of referrals for low B_{12} results, the lower limit of the reference range was changed at that time to 160 pg/mL.

Methylmalonic acid in serum was determined by liquid chromatography-tandem mass spectrometry using butanol derivatization with deuterated MMA as the internal standard on an Applied Biosystems API 3000 with Analyst 1.4 software. The reference range was 0.0-0.40 µmol/L.

Plasma Hcy was assayed by an enzymatic method measuring S-adenosylmethionine catalyzed by homocysteine-S-methyltransferase. Our laboratory utilized age-adjusted reference ranges (eg, <16.5 for males 61-80 years and <14.8 for females 61-80 years).

Baseline patient data had to be within 6 months before initiation of intramuscular B_{12} therapy. Follow-up data had to be at least 2 months after this date, but not more than 12 months. Hematologic response was defined as a decrease in MCV by ≥5 fL while the hemoglobin (Hgb) remained stable or increased. Based on baseline serum B12 and MMA testing alone, patients were placed into one of four categories derived from current testing recommendations. Minimal or no evidence of B_{12} deficiency (Group 1) was defined as (a) no documented testing for vitamin B_{12} status, (b) normal serum vitamin B_{12} (>250 pg/mL) regardless of MMA value, or (c) low serum vitamin B_{12} (any level ≤250 pg/mL) with normal MMA. Possible vitamin B_{12} deficiency (Group 2) was defined as moderate to borderline low serum vitamin B_{12} (100-250 pg/mL) with no documented testing for MMA. Probable vitamin B_{12} deficiency, unconfirmed (Group 3) was defined as very low serum vitamin B_{12} (<100 pg/mL), but no documented testing for MMA. Lastly, vitamin B_{12} deficiency, confirmed by laboratory criteria (Group 4) was defined as low serum vitamin B_{12} (≤250 pg/mL) with elevated MMA.

Analyses for this study were primarily descriptive, the principle goal being to present the clinical experience at a single institution with respect to the adequacy of laboratory evaluation for patients on vitamin B_{12} supplementation. Associations among laboratory tests were examined graphically with scatter plots and were measured using the Spearman rank correlation coefficient. Results in this report were deemed statistically significant at the 5% level (P<0.05) without adjustment for multiple comparisons.

Results

Patients started on intramuscular vitamin B_{12} were mostly female (64%) and elderly (median age 75 years). Laboratory data were measured before or at the time of diagnosis (ie, at baseline) in the majority of patients (table 1). Figures 1, 2, and 3 show the frequency distribution of baseline values for serum vitamin B_{12}, Hgb, and MCV, respectively. Baseline Hgb was inversely correlated with serum creatinine (Cr): correlation coefficient r = -0.21 (P=0.01). Forty patients had baseline MMA levels; of these, 35 also had baseline Hgb and

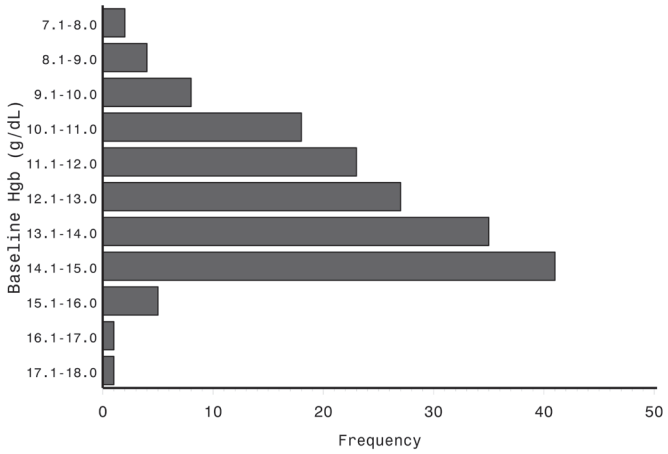

Figure 2. Frequency distribution of baseline values for serum hemoglobin (Hgb).

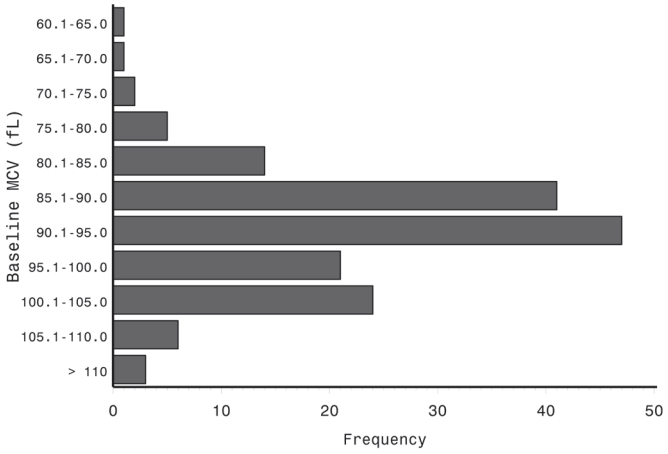

Figure 3. Frequency distribution of baseline values for mean corpuscular volume (MCV).

MCV data. Serum MMA was directly correlated with Cr: r = 0.33 (*P*=0.04). Closer inspection of the relationship between MMA and Cr showed that 11 of 12 patients with a baseline serum Cr >1.6 mg/dL had an elevated serum MMA (>0.40 µmol/L), compared with only 7 of 26 having a Cr <1.5. Although MCV was significantly correlated with MMA (r =0.43, *P*=0.01), no cutoff value for MCV was apparent that could usefully identify patients having an increased MMA. The inverse correlation between MCV and B_{12} did not quite reach statistical significance (r = -0.15, *P*=0.07). No significant relationships were identified between MMA and B_{12} or MMA and Hgb.

Of the 192 patients included in the study, B_{12} therapy was initiated in calendar years 2000 and 2005 for 93 and 99 patients, respectively. Their stratification by diagnostic category (Groups 1-4) is shown in table 2. The lab report addendum suggesting testing of serum MMA for B_{12} levels in the 100-250 pg/mL range did result in 29 of 99 patients (29%) having a baseline serum MMA in 2005, compared with 11 of 93 (12%) in 2000. However, this had little effect on patient classification, and surprisingly, relatively fewer patients with probable or confirmed deficiency (Group 3 or 4) were identified in 2005 (13/99, 13%) than in 2000 (22/93, 24%). No plausible explanation (other than chance) was identified, and therefore, data from 2000 and 2005 were combined in subsequent analyses reported below.

Only 88 patients had both baseline and follow-up B_{12} levels assessed. As illustrated in figure 4, B_{12} levels rose for the vast majority of the patients in Groups 2, 3, and 4, providing laboratory evidence that supplementation was occurring. Follow-up B_{12} levels were ≥100 pg/mL for all but one individual. A significantly greater number of patients had both baseline and follow-up Hgb and MCV assessed: 136 and 131, respectively. There were no significant correlations between the changes in these variables and the B12 increment (data not shown). In contrast, the change in MCV was significantly correlated with the baseline B_{12} level (r = 0.27, *P*=0.003); this relationship is illustrated in figure 5. Vertical lines at 100 pg/mL and 250 pg/mL indicate the cutoffs used for group assignment. Data points in the lower left quadrant represent patients in whom the baseline B_{12} was <100 pg/mL, and MCV decreased as would be expected with a hematologic response to B_{12} supplementation.

Remarkably, 35.4% of patients had no laboratory evidence to justify therapeutic intervention (Group 1), and another 46.3% had only possible B_{12} deficiency (Group 2). Only two of these patients had a hematologic response. Notably, these two patients had the lowest baseline B_{12} values of all the patients in either Group 1 or 2 (103 and 107 pg/mL).

Twenty-four patients had probable B_{12} deficiency defined as a baseline B_{12} <100 pg/mL without measuring MMA (Group 3). Eighteen of these had both baseline and follow-up Hgb and MCV data and are included in figure 5. Eight patients met the criteria for hematologic response, and another four had equivocal hematologic responses (eg, incomplete data or changes not quite fulfilling those criteria.) The one patient with a plausible neurologic response was in this group. She was a woman, age 68 years, in whom paresthesias of both feet had resolved when she was examined 6 weeks after initiation of B_{12} therapy; criteria for hematologic response were not met.

Eleven patients had "confirmed" B_{12} deficiency defined as a serum B_{12} ≤250 and serum MMA >0.40 µmol/L (Group 4). All but one had baseline Hgb and MCV data, but just seven (shown in figure 5) had follow-up hematologic data. Only two of these seven patients had well-documented hematologic responses. Three of the other five patients (all age 75 years or greater) had a serum Cr ≥1.6 that could explain the slightly elevated serum MMA. Four patients had inadequate data to assess for a hematologic response; only one had baseline values suggestive of clinical B_{12} deficiency. In summary, only three of the eleven patients with confirmed B_{12} deficiency had hematologic data to corroborate clinical deficiency.

Of the 40 patients (21%) who had baseline serum MMA levels, five had a serum B_{12} <100 pg/mL, where one could arguably initiate vitamin B_{12} without further testing. One of these patients did not have corroborative evidence of B_{12} deficiency (normal MMA, Hgb, and MCV). Only 11 (5.7%) patients had Hcy testing before initiation of B_{12} therapy. Two of these (with levels of 74.3 µmol/L and 27.2 µmol/L, respectively) appeared to have clinical B_{12} deficiency. Notably, three patients with levels between 19.1 and 28.8 µmol/L had no corresponding laboratory evidence or clinical response to support B_{12} deficiency; one of these three was clearly iron deficient (serum ferritin = 3 ng/mL). Too few patients had

Table 3. Other causes of anemia and their B_{12} levels.

Cause	Number of patients	Number with B_{12} ≤250 pg/ml	Range of B_{12} levels ≤250 pg/ml
Iron deficiency	16	13/15*	57-238
Renal insufficiency	3	3/3	143-238
Autoimmune hemolytic anemia	3	3/3	143-177
Myelodysplastic syndrome	2	1/2	145
Plasma cell myeloma	2	2/2	152, 166
Folate deficiency	1	1/1	86

*One iron deficiency patient without a reliable baseline serum B_{12} level.

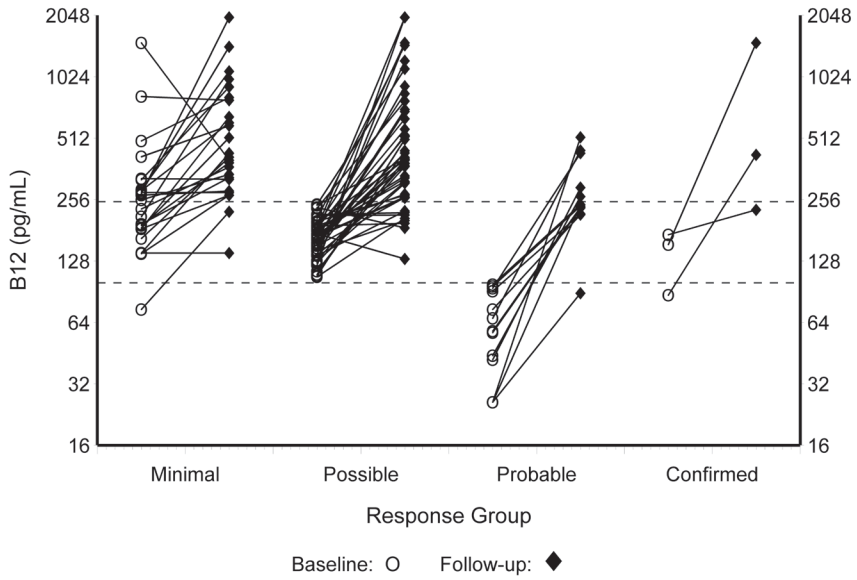

Figure 4. Study of vitamin B_{12} supplementation: B_{12} response group and time.

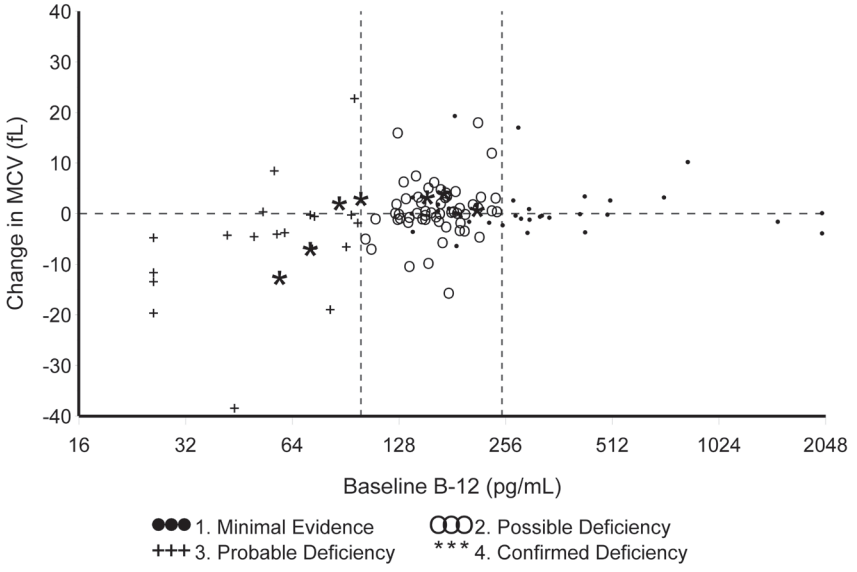

Figure 5. Study of vitamin B_{12} supplementation: mean corpuscular volume (MCV) change versus baseline B_{12}.

these three had evidence of a hematologic response to B_{12} supplementation.

An interesting observation was that 37 patients (27 females and 10 males) had a history of gastrointestinal surgery that may have predisposed them to malabsorption of certain nutrients including vitamin B_{12}. Of these surgical procedures, 24 were gastric bypass or gastrectomy (the vast majority for morbid obesity), and 20 of these patients were women. Of the thirty-five patients that had baseline serum B_{12} levels, five were <100 pg/mL, 27 were between 100 pg/mL and 250 pg/mL, and three were >250 pg/mL. Only three patients had evidence of a hematologic response.

Table 3 summarizes the findings in 27 patients who were started on B_{12} therapy but found to have another cause of anemia. None of the 27 patients in table 3 had clinical data to suggest a meaningful response to B_{12} therapy.

Surprisingly, iron deficiency was nearly as common as clinical B_{12} deficiency in this cohort of 192 patients started on B_{12} injections. In 10 patients this was well-documented, and it was strongly suspected in another six in whom there was incomplete laboratory documentation. In 13 of 15 patients with baseline B_{12} levels, they were 250 pg/mL or below (ranging from 57 pg/mL to 238 pg/mL). The highest MCV in an iron deficient patient was 90.5 fL; whereas, the lowest MCV in a patient with documented or equivocal clinical response to B_{12} supplementation was 91.8 fL.

follow-up MMA or Hcy levels to evaluate for so-called metabolic responses.

Baseline intrinsic factor (IF) antibodies, which would support a more specific diagnosis of pernicious anemia, were evaluated in 15 patients and were positive in 5 patients. In one patient (DAT-positive hemolytic anemia responsive to prednisone), this was a false positive. None of the ten patients with a negative IF antibody test had laboratory evidence or a clinical picture of B_{12} deficiency. The Schilling test, which has become largely unavailable in recent years, was performed on only four patients. It was reportedly positive in three; two of

Three patients had renal insufficiency as the likely cause for their anemia, with serum Cr values between 3.0 mg/dL and 5.4 mg/dL. Three patients had well-documented autoimmune hemolytic anemia with a positive direct antibody test; B_{12} levels ranged between 143 pg/mL and 177 pg/mL, and all three had an elevated MCV due to reticulocytosis. Another patient had a spuriously high MCV of 122.8 fL due to cold agglutinin disease; his MCV data were deleted from the statistical analysis and table 3. Two patients likely had a myelodysplastic syndrome (MDS) explaining their macrocytic anemia. Two patients were diagnosed with plasma cell myeloma within 2 months after starting B_{12} therapy.

Folate testing, either serum folate or red cell folate, was done in a small minority of patients in this study, and only one patient was identified with possible deficiency. This woman, age 91 years, had a slightly low serum folate level of 3.6 ng/mL (reference range 4-20 ng/mL), and baseline values were B_{12} 86 pg/mL, Hgb 11.6 g/dL, and MCV 98.2 fL with no follow-up labs. Her decreased sensation below the knees bilaterally was unchanged with B_{12} and folate supplementation.

In five patients, drugs may have been the cause of borderline to mild macrocytosis (MCV ≥96.1 fL) without anemia. The implicated drugs were chemotherapeutic agents in two, phenytoin in one, both phenytoin and methotrexate in one, and hydroxyurea in one. No patients were identified in which hypersegmented neutrophils were reported in the electronic medical record within 6 months before initiating intramuscular vitamin B_{12} supplementation.

Discussion

The elderly age distribution and female predominance were expected given the demographics of vitamin B_{12} deficiency. Also, some of the female predominance may be attributable to the higher frequency of gastric bypass surgery in women; 20 of 24 such patients were women.

The positive correlation between serum MMA and renal function is well known.[10] Caution is advised in interpreting serum MMA levels in patients with renal insufficiency (eg, Cr >1.6 mg/dL). In one study from the United Kingdom, average MMA levels increased from 0.25 mmol/L in people age 65 years to 74 years, to 0.38 mmol/L in people >85 years.[11] Declining renal function and slightly lower B_{12} levels only partially accounted for this change. Elevated serum Hcy is another metabolite indicator of B_{12} deficiency but is less specific than MMA, and there are not well established diagnostic cutoffs. Like B_{12}, Hcy is largely cleared by the kidneys, and levels are higher in patients with renal insufficiency.[12]

How to manage patients with a history of gastrointestinal surgery is unclear. This study could not evaluate the frequency with which low vitamin B_{12} levels are encountered in this patient cohort, but it seems fairly common. Notably, these patients rarely have any meaningful clinical response to parenteral vitamin B_{12} supplementation. Some might argue that a low threshold for B_{12} therapy is reasonable as a preventative measure, rather than waiting for symptomatic deficiency to develop.[1] This issue deserves further study, particularly with the recent increase in gastric procedures for morbid obesity.

The quagmire surrounding vitamin B_{12} deficiency contrasts sharply with iron deficiency, which is much more common, readily confirmed by laboratory testing, and predictably responsive to supplementation. Notably, iron deficiency was nearly as common as B_{12} deficiency in this cohort of patients started on B_{12} injections. The rarity of folate deficiency is consistent with recent data from the United States and Canada, pointing to the introduction of widespread folic acid fortification of many foods.[13,14]

Hematologists and hematopathologists frequently encounter patients with low B_{12} levels that are found to have another cause for their macrocytosis with or without anemia (eg, hemolysis with reticulocytosis, drug-related macrocytosis, MDS, or other neoplasms). Incorrectly attributing a macrocytic anemia to B_{12} deficiency delays further evaluation that could uncover the true underlying cause, and of course, appropriate treatment is delayed as well.

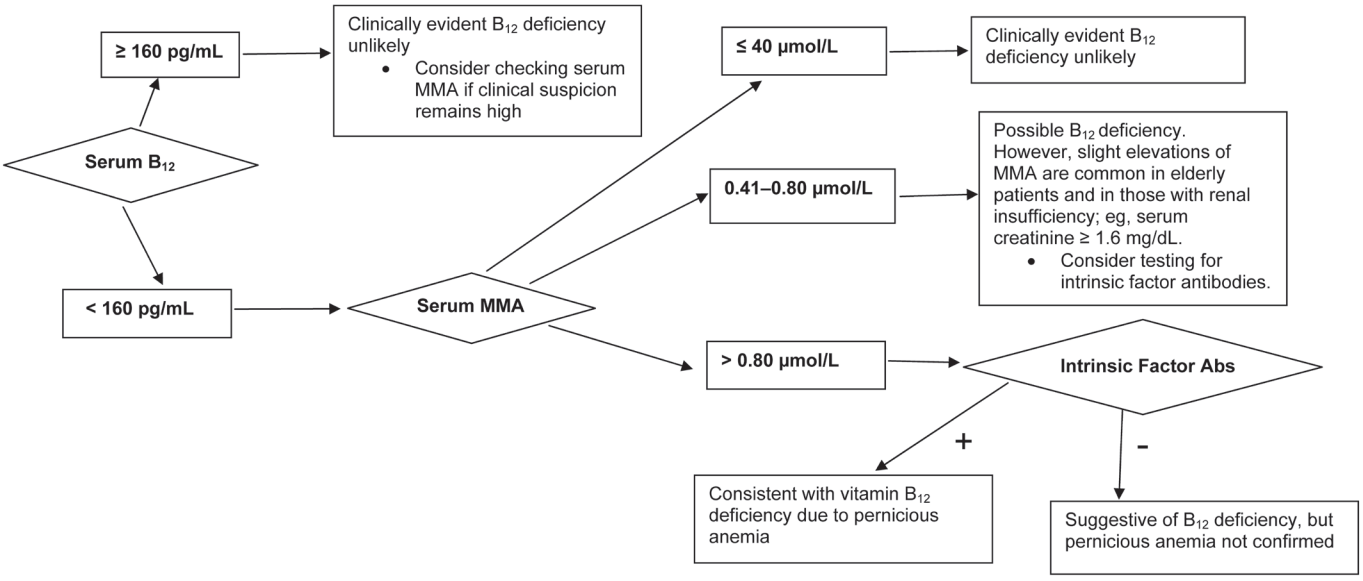

Figure 6. Testing cascade for vitamin B_{12} deficiency.

Myelodysplastic syndromes, particularly 5q syndrome, often present with a macrocytic anemia. The medical records of 13 patients (not included in the 192 patient cohort) with MDS diagnosed at Marshfield Clinic in which cytogenetics revealed a 5q deletion (accompanied by noncomplex cytogenetic abnormalities in two) were reviewed. All 11 of the patients tested at baseline had a normal serum B_{12} level >250 pg/mL. Although these data suggest that low serum B_{12} levels are uncommon in patients with MDS, one author (GRS) has anecdotally encountered several MDS patients with low B_{12} levels that were unresponsive to supplementation. Further testing for MMA could clarify the situation in most cases.

The dramatic presentation and response of patients with full-blown vitamin B_{12} deficiency provides some insight into recognizing diagnostic pitfalls. Two recent cases of pernicious anemia encountered by one author (GRS) during the preparation of this manuscript serve as examples. Both patients had baseline B_{12} levels <50 pg/mL, positive intrinsic factor (IF) antibodies, and severe macrocytic anemia (Hgb <5.8 g/dL and MCV >134.5 fL) with striking poikilocytosis that morphologically raised some concern for an MDS (possibly with myelofibrosis). In the one patient tested, MMA was 32.8 µmol/L and Hcy 94.9 µmol/L. Both patients had tremendous elevations in their lactate dehydrogenase level (>2600 U/L) and slight to moderately increased indirect bilirubin, which raised consideration of a hemolytic anemia. In fact, one patient had a positive direct antiglobulin test (albeit weak reactivity) and an increased reticulocyte count (but not nearly enough to explain the severe macrocytosis). The other patient had a low haptoglobin level. Within 3 weeks of starting parenteral B_{12} therapy, Hgb and MCV of both patients had normalized (>12.2 g/dL and <97.3 fL, respectively).

In the electronic medical record, the reporting of hypersegmented neutrophils was of no help in diagnosing vitamin B_{12} deficiency. This concurs with most reports in the literature stating that the presence of hypersegmented neutrophils on the peripheral blood smear is neither sensitive nor specific for this purpose.[15] However, careful review of a peripheral blood smear by a hematologist or hematopathologist may provide helpful clues regarding the underlying cause of a macrocytic anemia.

Approximately 75% of serum B_{12} is bound to haptocorrin (formerly called transcobalamin I) whose function is unknown.[9] Therefore, total serum B_{12} levels largely reflect B_{12} that is not bioavailable. Total transcobalamin, consisting of holoTC and apo-transcobalamin, is the major carrier protein in the plasma/serum that delivers B_{12} to the tissues. Conceptually, holoTC should more accurately reflect intracellular B_{12} levels. Sequencing of the TCN1 gene for haptocorrin has identified a 999G>T polymorphism with heterozygosity in about 12% of individuals of European ancestry.[16] Based on limited familial studies, heterozygosity for this polymorphism is associated with slightly lower total haptocorrin and, hence, slightly lower total serum B_{12} levels. This polymorphism may help explain why whites, on average, have lower total haptocorrin and B_{12} levels than blacks. A 776C>G polymorphism in the transcobalamin gene (TCN2) that affects holoTC has been described as commonly occurring in the Portuguese population.[17] Extremely rare mutations in the TCN2 gene may result in severe intracellular cobalamin depletion.[18] More work needs to be done to sort out the genetics and pathophysiology related to cobalamin deficiency.[19]

Some, but not all, studies have found marginally better performance (as assessed by receiver operator curves) by holoTC when compared with serum B_{12}.[9,20-22] However, the "gold standards" for classifying patients as B_{12} deficient have been dubious (ie, elevated MMA or Hcy, with minimal or no clinical correlation). One study used red blood cell (RBC) cobalamin as the "gold standard" and found that holoTC slightly outperformed B_{12}. Curiously, techniques to measure RBC cobalamin were reported four decades ago and yet are still not widely available. Rare patients with pernicious anemia have spurious elevations of vitamin B_{12} when using methods based on competitive binding of serum B_{12} with reagent intrinsic factor.[23]

A recent National Health and Nutrition Examination Survey (NHANES) roundtable recommended conventional serum B_{12} over holoTC at this time, since holoTC methods are relatively new and would benefit from additional performance studies.[24] Furthermore, the same roundtable summary expressed caution regarding the common assumption that detecting subclinical deficiency early is important to enable public health interventions that prevent its progression to clinical deficiency. These experts questioned the value of further hypothesis generating epidemiologic studies and stated that prospective clinical trials are urgently needed.[8,25,26]

From a cost-effective public health standpoint, some might advocate widespread oral vitamin B_{12} supplementation without laboratory testing to provide insurance against subclinical B_{12} deficiency.[27] Although the general public and commercial health food suppliers have a seemingly insatiable appetite for "fountain of youth" dietary supplements, it seems premature to recommend mandatory fortification of the food supply without well-conducted, randomized clinical trials. Several patients in the current study were given vitamin B_{12} injections solely at their request, and sometimes for psychological benefit; patient education would seem more appropriate.

In terms of future studies, a relatively small, well designed, prospective clinical trial may be enlightening. Patients could initially be identified by laboratory testing (eg, a cohort of patients with serum B_{12} levels of <200 pg/mL). A comprehensive battery of laboratory tests could be obtained both at baseline and, with the exception of genetic tests, at defined intervals

once treatment had been initiated (eg, MMA, Hcy, holoTC, a full CBC, IF antibodies, ferritin, serum iron/total iron binding capacity, serum folate, RBC folate, and sequencing of the TCN1 and TCN2 genes). For study purposes, perhaps the Schilling test could even be resurrected to better understand the absorption issues. A careful neurologic exam would be documented by a neurologist and repeated at prescribed intervals. Treatment would be standardized.

This retrospective study has several limitations. Patients were selected on the basis of clinical initiation of intramuscular B_{12} treatment. Thus, this was not a random or representative sample of the general population. Laboratory testing before initiation of therapy was inconsistent, and monitoring even more inconsistent. Also, there was no standard treatment protocol, and numerous patients were incompletely treated; either they or their provider discontinued the therapy. In patients deemed to have had a laboratory response to B_{12} supplementation, there was no control group of untreated or placebo-treated patients, so the possibility that high MCV values decreased because of regression toward the mean cannot be ruled out. It was difficult during chart review to capture all confounding variables (eg, not having stable baseline or follow-up CBC data). This could be due to blood loss, medication changes, or other alterations in clinical status. Comprehensive neurologic exams were rarely conducted, so there may have been some patients with small objective improvements that were overlooked. This data may be considered somewhat dated; however, there have been no significant changes in B_{12} testing over the past decade.

In summary, of 192 patients started on intramuscular B_{12} injections, only 35 (18.3%) had laboratory evidence supporting "probable" or "confirmed" deficiency. Furthermore, only 18 patients (9.4%) had data supportive of a clinical response. In these 18 patients, the baseline serum B_{12} level was ≤107 pg/mL; only three had a baseline MMA level, which was ≥1.29 μmol/L for all three. In terms of diagnosing clinically evident vitamin B_{12} deficiency with a reasonable expectation of clinical response, these limited data suggest that current diagnostic algorithms could be modified to increase specificity without sacrificing sensitivity. Additional studies are needed to corroborate these findings and further explore the benefit (if any) in detecting and treating "subclinical" vitamin B_{12} deficiency.

The following are offered as practical suggestions that providers and laboratories may find useful.

1. Do not test patients for B_{12} deficiency without a clear clinical indication; ie, macrocytosis (usually with anemia) or (less commonly) neurologic signs or symptoms potentially referable to vitamin B_{12} deficiency. Patients with microcytosis (or even an MCV <90 fL) should not undergo B_{12} testing solely for anemia.
2. No single test establishes a diagnosis of vitamin B_{12} deficiency. Laboratory cutoffs vary. In this study, all patients with plausible hematologic or neurologic responses had baseline B_{12} levels of ≤107 pg/mL. Yet, even very low serum B_{12} levels (<100 pg/mL) and B_{12} deficiency "confirmed" by an elevated MMA should be viewed cautiously; the complete clinical picture needs to be considered. Keep in mind that patients with serum Cr values ≥1.6 mg/dL frequently have slightly elevated serum MMA and Hcy. IF antibodies have good specificity with a sensitivity of about 50% by most accounts,[3] and this assay is suggested when the initial laboratory data suggests B_{12} deficiency.
3. Laboratories should recommend a B_{12} testing cascade that includes MMA and IF antibodies as indicated to follow-up low B_{12} levels without requiring a separate order by the provider or a second blood draw. An example of such a cascade is shown in figure 6. To increase specificity, the serum B_{12} cutoff has been conservatively adjusted to our laboratory's lower reference range cutoff (160 pg/mL), and the serum MMA cutoff has been increased to 0.80 μmol/L. Sign-out of low serum B_{12} results by a hematopathologist or clinical chemist (much like serum protein electrophoresis) can provide individualized interpretative comments enhancing communication to providers. In our laboratory, which averages between 50 and 60 serum B_{12} results per day, only about 4% of samples have values <160 pg/mL, so this could be accomplished with relatively little additional professional effort.
4. Whatever the exact therapeutic regimen (not addressed in this study), laboratory reevaluation at about 2 to 3 months by repeating the CBC, B_{12} and MMA testing is recommended. For patients with macrocytic anemia, if the CBC has remained the same and the patient has been compliant in receiving B_{12} therapy, then it may be time to reassess. Have the B_{12} and MMA (or Hcy) levels corrected? What is being gained by the patient in continuing parenteral therapy only to "treat" the B_{12} and MMA numbers? Other diagnoses (eg, MDS, hemolytic anemia) should be considered.

Acknowledgements

This study was supported by funds from the Marshfield Clinic Physician Research Funds, Marshfield, Wisconsin. The authors thank Marie Fleisner of the Marshfield Clinic Research Foundation's Office of Scientific Writing and Publication for editorial support in the preparation of this manuscript.

References

1. Carmel R. How I treat cobalamin (vitamin B_{12}) deficiency. Blood 2008;112:2214-2221.
2. Lindenbaum J, Healton EB, Savage DG, Brust JC, Garrett TJ, Podell ER, Marcell PD, Stabler SP, Allen RH. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. N Engl J Med 1988;318:1720-1728.
3. Snow CF. Laboratory diagnosis of vitamin B_{12} and folate deficiency: a guide for the primary care physician. Arch Intern Med 1999;159:1289-1298.

4. Lindenbaum J, Savage DG, Stabler, SP, Allen RH. Diagnosis of cobalamin deficiency: II. Relative sensitivities of serum cobalamin, methylmalonic acid, and total homocysteine concentrations. Am J Hematol 1990;34:99-107.
5. Matchar DB, McCrory DC, Millington DS, Feussner JR. Performance of the serum cobalamin assay for diagnosis of cobalamin deficiency. Am J Med Sci 1994;308:276-283.
6. Klee GG. Cobalamin and folate evaluation: measurement of methylmalonic acid and homocysteine vs vitamin B_{12} and folate. Clin Chem 2000;46:1277-1283.
7. Chanarin I, Metz J. Diagnosis of cobalamin deficiency: the old and the new. Br J Haematol. 1997;97:695-700.
8. Yetley EA, Pfeiffer CM, Phinney KW, Bailey RL, Blackmore S, Bock JL, Brody LC, Carmel R, Curtin LR, Durazo-Arvizu RA, Eckfeldt JH, Green R, Gregory JF 3rd, Hoofnagle AN, Jacobsen DW, Jacques PF, Lacher DA, Molloy AM, Massaro J, Mills JL, Nexo E, Rader JI, Selhub J, Sempos C, Shane B, Stabler S, Stover P, Tamura T, Tedstone A, Thorpe SJ, Coates PM, Johnson CL, Picciano MF. Biomarkers of vitamin B_{12} status in NHANES: a roundtable summary. Am J Clin Nutr 2011;94:313S-321S.
9. Nexo E, Hoffmann-Lucke E. Holotranscobalamin, a marker of vitamin B_{12} status: analytical aspects and clinical utility. Am J Clin Nutr 2011;94:359S-365S.
10. Vogiatzoglou A, Oulhaj A, Smith AD, Nurk E, Drevon CA, Ueland PM, Vollset SE, Tell GS, Refsum H. Determinants of plasma methylmalonic acid in a large population: implications for assessment of vitamin B_{12} status. Clin Chem 2009;55:2198-2206.
11. Bates CJ, Schneede J, Mishra G, Prentice A, Mansoor MA. Relationship between methylmalonic acid, homocysteine, vitamin B_{12} intake and status and socio-economic indices, in a subset of participants in the British National Diet and Nutrition Survey of people aged 65 y and over. Eur J Clin Nutr 2003;57:349-357.
12. Lewerin C, Ljungman S, Nilsson-Ehle H. Glomerular filtration rate as measured by serum cystatin C is an important determinant of plasma homocysteine and serum methylmalonic acid in the elderly. J Intern Med 2007;261:65-73.
13. Shojania AM, von Kuster K. Ordering folate assays is no longer justified for investigation of anemias, in folic acid fortified countries. BMC Res Notes 2010;3:22.
14. 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;340:1449-1454.
15. Carmel R, Green R, Jacobsen DW, Qian GD. Neutrophil nuclear segmentation in mild cobalamin deficiency: relation to metabolic tests of cobalamin status and observations on ethnic differences in neutrophil segmentation. Am J Clin Pathol 1996;106:57-63.
16. Carmel R, Parker J, Kelman Z. Mutations of TCN1 cause transcobalamin in deficiency with low serum cobalamin levels that are indistinguishable from cobalamin deficiency [Abstract]. Blood 2009;114:787.
17. Castro R, Barroso M, Rocha M, Esse R, Ramos R, Ravasco P, Rivera I, de Almeida IT. The TCN2 776CNG polymorphism correlates with vitamin B_{12} cellular delivery in healthy adult populations. Clin Biochem 2010;43:645-649.
18. Ratschmann R, Minkov M, Kis A, Hung C, Rupar T, Mühl A, Fowler B, Nexo E, Bodamer OA. Transcobalamin II deficiency at birth. Mol Genet Metab 2009;98:285-288.
19. Carmel R, Parker J, Kelman Z. Genomic mutations associated with mild and severe deficiencies of transcobalamin I (haptocorrin) that cause mildly and severely low serum cobalamin levels. Br J Haematol. 2009;147:386-391.
20. Obeid R, Herrmann W. Holotranscobalamin in laboratory diagnosis of cobalamin deficiency compared to total cobalamin and methylmalonic acid. Clin Chem Lab Med 2007;45:1746-1750.
21. Schrempf W, Eulitz M, Neumeister V, Siegert G, Koch R, Reichmann H, Storch A. Utility of measuring vitamin B_{12} and its active fraction, holotranscobalamin, in neurological vitamin B_{12} deficiency syndromes. J Neurol 2011;258:393-401.
22. Miller JW, Garrod MG, Rockwood AL, Kushnir MM, Allen LH, Haan MN, Green R. Measurement of total vitamin B_{12} and holotranscobalamin, singly and in combination, in screening for metabolic vitamin B12 deficiency. Clin Chem 2006;52:278-285.
23. Yang DT, Cook RJ. Spurious elevations of vitamin B_{12} with pernicious anemia. N Engl J Med 2012;366:1742-1743.
24. Valente E, Scott JM, Ueland PM, Cunningham C, Casey M, Molloy AM. Diagnostic accuracy of holotranscobalamin, methylmalonic acid, serum cobalamin, and other indicators of tissue vitamin B_{12} status in the elderly. Clin Chem 2011;57:856-863.
25. Bailey RL, Carmel R, Green R, Pfeiffer CM, Cogswell ME, Osterloh JD, Sempos CT, Yetley EA. Monitoring of vitamin B-12 nutritional status in the United States by using plasma methylmalonic acid and serum vitamin B_{12}. Am J Clin Nutr 2011;94:552-561.
26. Carmel R. Biomarkers of cobalamin (vitamin B_{12}) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. Am J Clin Nutr 2011;94:348S-358S.
27. Carmel R. Mandatory fortification of the food supply with cobalamin: an idea whose time has not yet come. J Inherit Metab Dis 2011;34:67-73.

Author Affiliations

Richard L. Berg; Gene R. Shaw†*

**Biomedical Informatics Research Center, Marshfield Clinic Research Foundation, Marshfield, Wisconsin, USA*
†Department of Lab/Pathology, Marshfield Clinic, Marshfield, Wisconsin, USA