Laboratory Evaluation for Vitamin B\textsubscript{12} Deficiency: The Case for Cascade Testing

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Running title: Laboratory testing for B\textsubscript{12} deficiency

Conflict of interest statement: The authors have no financial or personal relationships to disclose related to this manuscript.

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

Objective: Potential vitamin B<sub>12</sub> 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<sub>12</sub> deficiency.

Methods: This retrospective study reviewed the electronic medical records of 192 patients initiated on intramuscular vitamin B<sub>12</sub> 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<sub>12</sub> level was ≤107 pg/mL; only 3 also had a baseline serum methylmalonic acid level, which was ≥1.29 umole/L in all 3.

Conclusions: Currently, only a small minority of patients initiated on intramuscular vitamin B<sub>12</sub> supplementation derive any meaningful clinical benefit. Furthermore, current testing recommendations for vitamin B<sub>12</sub> 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<sub>12</sub> deficiency without loss of sensitivity. Ultimately, a better understanding of vitamin B<sub>12</sub> physiology is needed to develop and evaluate laboratory tests that more accurately reflect true intracellular vitamin B<sub>12</sub> status.

Key Words: Holotranscobalamin; Methylmalonic Acid/blood; Nutritional Status; Vitamin B 12
The laboratory evaluation of possible vitamin B₁₂ deficiency is often prompted by the presence of macrocytic anemia.¹ In clinically evident vitamin B₁₂ 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 B₁₂ supplementation (personal communication with author GRS).

Published estimates of the sensitivity and specificity of serum B₁₂ measurement vary widely, largely due to the lack of a gold standard for diagnosis.³,⁴ Serum B₁₂ 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 homocysteine [Hcy]) to define deficiency, the "screening" serum B₁₂ cutoff used in many diagnostic algorithms has been raised to 250–300 pg/mL to provide greater sensitivity.⁶ With this approach, the prevalence of vitamin B₁₂ deficiency may be as high as 15%–20% in the elderly.⁷ Of course, most of these patients will have no hematologic or neurologic manifestations of B₁₂ deficiency (ie, subclinical deficiency).⁸ Holotranscobalamin (holoTC) has been reported to offer slightly better performance than total serum B₁₂ levels in diagnosing deficiency, but these study designs have been criticized, and there is currently no consensus that holoTC should replace conventional serum B₁₂ testing.⁸,⁹
Given the limitations of current testing for vitamin B<sub>12</sub> deficiency, and because its administration is inexpensive and fairly innocuous, many clinicians have a low threshold for prescribing vitamin B<sub>12</sub> 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<sub>12</sub> 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<sub>12</sub> supplementation is unclear. In this study, we sought to systematically evaluate current practice patterns regarding the laboratory evaluation of suspected vitamin B<sub>12</sub> 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<sub>12</sub> injection (J3420) and International Classification of Diseases (ICD-9-CM) codes related to vitamin B<sub>12</sub> 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 B_{12} 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 Immuno1 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 added 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 immunoassay incorporating intrinsic factor. The correlation between the two methods was: Access = 0.966 \times (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 umol/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-old and <14.8 for females 61-80 years-old.)

Baseline patient data had to be within 6 months before initiation of intramuscular B₁₂ 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 B₁₂ deficiency (Group 1) was defined as (a) no documented testing for vitamin B₁₂ status, (b) normal serum vitamin B₁₂ (>250 pg/mL) regardless of MMA value, or (c) low serum vitamin B₁₂ (any level ≤ 250 pg/mL) with normal MMA. Possible vitamin B₁₂ deficiency (Group 2) was defined as moderate to borderline low serum vitamin B₁₂ (100–250 pg/mL) with no documented testing for MMA. Probable vitamin B₁₂ deficiency, unconfirmed (Group 3) was defined as very low serum vitamin B₁₂ (<100 pg/mL), but no documented testing for MMA. Lastly, vitamin B₁₂ deficiency, confirmed by laboratory criteria (Group 4) was defined as low serum vitamin B₁₂ (≤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 B₁₂ supplementation. Associations among laboratory tests were examined graphically with scatter plots and were measured using the Spearman rank correlation.
Results

Patients started on intramuscular vitamin B\textsubscript{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\textsubscript{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 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 \( \geq 1.6 \) mg/dL had an elevated serum MMA (>0.40 umol/L), compared with only 7 of 26 having a Cr \( \leq 1.5 \). 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\textsubscript{12} did not quite reach statistical significance (\( r = -0.15, P=0.07 \)). No significant relationships were identified between MMA and B\textsubscript{12} or MMA and Hgb.

Of the 192 patients included in the study, B\textsubscript{12} therapy was initiated in calendar years 2000 and 2005 for 93 and 99 patients, respectively. Their stratification by diagnostic category (Groups 1 through 4) is shown in Table 2. The lab report addendum suggesting testing of serum MMA for B\textsubscript{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\textsubscript{12} levels assessed. As illustrated in figure 4,
B\textsubscript{12} levels rose for the vast majority of these patients in Groups 2, 3, and 4, providing laboratory
evidence that supplementation was occurring. Follow-up B\textsubscript{12} levels were $\geq$100 pg/mL for all but
one individual. A significantly greater number of patients had both baseline and follow-up Hgb
and MCV assessed: 136 and 131, respectively. There were no significant correlations between
the changes in these variables and the B\textsubscript{12} increment (data not shown). In contrast, the change in
MCV was significantly correlated with the baseline B\textsubscript{12} level ($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\textsubscript{12} was $<$100 pg/mL, and MCV decreased as would be expected with a
hematologic response to B\textsubscript{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\textsubscript{12} deficiency (Group 2). Only two of these
patients had a hematologic response. Notably, these two patients had the lowest baseline B\textsubscript{12}
values of all the patients in either Group 1 or 2 (103 and 107 pg/mL).
Twenty-four patients had "probable" B₁₂ deficiency defined as a baseline B₁₂ < 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 68-year-old woman in whom paresthesias of both feet had resolved when she was examined 6 weeks after initiation of B₁₂ therapy; criteria for hematologic response were not met.

Eleven patients had "confirmed" B₁₂ deficiency defined as a serum B₁₂ ≤ 250 and serum MMA > 0.40 umol/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 B₁₂ deficiency. In summary, only three of the eleven patients with "confirmed" B₁₂ deficiency had hematologic data to corroborate clinical deficiency.

Of the 40 patients (21%) who had baseline serum MMA levels, 5 had a serum B₁₂ < 100 pg/mL, where one could arguably initiate vitamin B₁₂ without further testing. One of these patients did not have corroborative evidence of B₁₂ deficiency (normal MMA, Hgb, and MCV). Only 11 (5.7%) patients had Hcy testing before initiation of B₁₂ therapy; reference range for plasma homocysteine up to 18.0 umol/L. Two of these (with levels of 74.3 and 27.2 umol/L, respectively) appeared to have clinical B₁₂ deficiency. Notably, three patients with levels
between 19.1 and 28.8 umol/L had no corresponding laboratory evidence or clinical response to support B₁₂ deficiency; one of these three was clearly iron deficient (serum ferritin of 3 ng/mL). Too few patients had 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. 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 B₁₂ 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 these three had evidence of a hematologic response to B₁₂ 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 B₁₂. Of these surgical procedures, 24 were gastric bypass or gastrectomy (the vast majority for morbid obesity), and 20 of these patients were women. Thirty-five patients had baseline serum B₁₂ levels that were <100 pg/mL in 5, between 100 pg/mL and 250 pg/mL in 27, and >250 pg/mL in 3. Only three patients had evidence of a hematologic response.

Table 3 summarizes the findings in 27 patients who were started on B₁₂ 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 B₁₂ therapy.
Surprisingly, iron deficiency was nearly as common as clinical B\textsubscript{12} deficiency in this cohort of 192 patients started on B\textsubscript{12} injections. In ten 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\textsubscript{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\textsubscript{12} supplementation was 91.8 fl.

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\textsubscript{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\textsubscript{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 91-year-old woman had a slightly low serum folate level of 3.6 ng/mL (reference range 4 ng/mL–20 ng/mL), and baseline values were B\textsubscript{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\textsubscript{12} and folate supplementation.

In five patients, drugs may have been the cause of borderline to mild macrocytosis (MCV $\geq 96.1$ 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\textsubscript{12} supplementation.

**Discussion**

The elderly age distribution and female predominance were expected given the demographics of vitamin B\textsubscript{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 $\geq 1.6$ mg/dL). In one study from the United Kingdom, average MMA levels increased from 0.25 mmol/L in people aged 65- to 74-years-of-age to 0.38 mmol/L in people $\geq 85$ years.$^{11}$ Declining renal function and slightly lower B\textsubscript{12} levels only partially accounted for this change. Elevated serum Hcy is another metabolite indicator of B\textsubscript{12} deficiency but is less specific than MMA, and there are not well-established diagnostic cutoffs. Like B\textsubscript{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 B₁₂ levels are encountered in this patient cohort, but it seems fairly common. Notably, these patients rarely have any meaningful clinical response to parenteral vitamin B₁₂ supplementation. Some might argue that a low threshold for B₁₂ 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 B₁₂ 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 B₁₂ deficiency in this cohort of patients started on B₁₂ 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 B₁₂ 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 B₁₂ deficiency delays further evaluation that could uncover the true underlying cause, and of course, appropriate treatment is delayed as well.

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 B₁₂ level above 250 pg/mL. Although these data suggest that low serum B₁₂ levels are uncommon in patients with MDS, one author (GRS) has anecdotally encountered several MDS patients with low B₁₂ 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 B₁₂ 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 B₁₂ 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 umol/L and Hcy 94.9 umol/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 B₁₂ 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 B₁₂ 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 776CNG polymorphism in the transcobalamin gene (TCN2) that affects
holoTC has been described as commonly occurring in the Portuguese population.\textsuperscript{17} Extremely
rare mutations in the TCN2 gene may result in severe intracellular cobalamin depletion.\textsuperscript{18} More
work needs to be done to sort out the genetics and pathophysiology related to cobalamin
deficiency.\textsuperscript{19}

Some, but not all, studies have found marginally better performance (as assessed by receiver
operator curves) by holoTC when compared with serum $B_{12}$.\textsuperscript{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\textsubscript{12} levels of $\leq$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\textsubscript{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 so. 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 B12 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\textsubscript{12} testing over the past decade.
In summary, of 192 patients started on intramuscular B12 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 B12 level was ≤107 pg/mL; only 3 had a baseline MMA level, which was ≥1.29 μmol/L for all 3. In terms of diagnosing clinically evident vitamin B12 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 B12 deficiency.

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

1. Do not test patients for B<sub>12</sub> deficiency without a clear clinical indication; ie, macrocytosis (usually with anemia) or (less commonly) neurologic signs or symptoms potentially referable to vitamin B<sub>12</sub> deficiency. Patients with microcytosis (or even an MCV lower than 90 fL) should not undergo B<sub>12</sub> testing solely for anemia.

2. No single test establishes a diagnosis of vitamin B<sub>12</sub> deficiency. Laboratory cutoffs vary. In this study, all patients with plausible hematologic or neurologic responses had baseline B<sub>12</sub> levels of 107 pg/mL or lower. Yet, even very low serum B<sub>12</sub> levels (<100 pg/mL) and B<sub>12</sub> 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, and this assay is suggested when the initial laboratory data suggests B<sub>12</sub> deficiency.
3. Laboratories should recommend a B₁₂ testing cascade that includes MMA and IF antibodies as indicated to follow-up low B₁₂ 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 B₁₂ 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 umol/L. Sign-out of low serum B₁₂ 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 B₁₂ 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, B₁₂, 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 B₁₂ therapy, then it may be time to reassess.

Have the B₁₂ and MMA (or Hcy) levels corrected? What is being gained by the patient in continuing parenteral therapy only to "treat" the B₁₂ 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 WI. 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.
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Table 1. Demographics and baseline laboratory results.

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<td>Patients (n)</td>
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<td>Female (%)</td>
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<tr>
<td>range (pg/mL)</td>
<td>26–2000</td>
</tr>
<tr>
<td>Hgb</td>
<td></td>
</tr>
<tr>
<td># tested</td>
<td>165</td>
</tr>
<tr>
<td>median (g/dL)</td>
<td>13.1</td>
</tr>
<tr>
<td>range (g/dL)</td>
<td>7.5–17.2</td>
</tr>
<tr>
<td>MCV</td>
<td></td>
</tr>
<tr>
<td># tested</td>
<td>165</td>
</tr>
<tr>
<td>median (fL)</td>
<td>91.7</td>
</tr>
<tr>
<td>range (fL)</td>
<td>63–131</td>
</tr>
<tr>
<td>MMA</td>
<td></td>
</tr>
<tr>
<td># tested</td>
<td>40</td>
</tr>
<tr>
<td>median (umol/L)</td>
<td>0.4</td>
</tr>
<tr>
<td>range (umol/L)</td>
<td>0.1–16</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td></td>
</tr>
<tr>
<td># tested</td>
<td>179</td>
</tr>
<tr>
<td>median (mg/dL)</td>
<td>1.0</td>
</tr>
<tr>
<td>range (mg/dL)</td>
<td>0.5–5.4</td>
</tr>
</tbody>
</table>

Hgb, hemoglobin; MCV, mean corpuscular volume; MMA, methylmalonic acid
Table 2. Diagnostic category by year.

<table>
<thead>
<tr>
<th>Group</th>
<th>2000</th>
<th>2005</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>1: Minimal/no evidence</td>
<td>33 (35.5)</td>
<td>35 (35.4)</td>
</tr>
<tr>
<td>2: Possible</td>
<td>38 (40.9)</td>
<td>51 (51.5)</td>
</tr>
<tr>
<td>3: Probable</td>
<td>18 (19.4)</td>
<td>6 (6.1)</td>
</tr>
<tr>
<td>4: Confirmed</td>
<td>4 (4.3)</td>
<td>7 (7.1)</td>
</tr>
<tr>
<td>Total patients</td>
<td>93</td>
<td>99</td>
</tr>
</tbody>
</table>

Groups were defined as follows: Group 1 – (a) no documented testing for vitamin B<sub>12</sub> status, (b) normal serum vitamin B<sub>12</sub> (>250 pg/mL) regardless of MMA value, or (c) low serum vitamin B<sub>12</sub> (any level < 250 pg/mL) with normal MMA. Group 2 – moderate to borderline low serum vitamin B<sub>12</sub> (100–250 pg/mL) with no documented testing for MMA. Group 3 (unconfirmed) – very low serum vitamin B<sub>12</sub> (<100 pg/mL), but no documented testing for MMA. Group 4 (confirmed by laboratory criteria) – low serum vitamin B<sub>12</sub> (<250 pg/mL) with elevated MMA.
Table 3. Other causes of anemia and their $\text{B}_{12}$ levels.

<table>
<thead>
<tr>
<th>Cause</th>
<th>Number of patients</th>
<th>Number with $\text{B}_{12} \leq 250$ pg/ml</th>
<th>Range of $\text{B}_{12}$ levels $\leq 250$ pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
<td>16</td>
<td>13/15*</td>
<td>57-238</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td>3</td>
<td>3/3</td>
<td>143-238</td>
</tr>
<tr>
<td>Autoimmune hemolytic anemia</td>
<td>3</td>
<td>3/3</td>
<td>143-177</td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>2</td>
<td>1/2</td>
<td>145</td>
</tr>
<tr>
<td>Plasma cell myeloma</td>
<td>2</td>
<td>2/2</td>
<td>152, 166</td>
</tr>
<tr>
<td>Folate deficiency</td>
<td>1</td>
<td>1/1</td>
<td>86</td>
</tr>
</tbody>
</table>

*One iron deficiency patient without a reliable baseline serum $\text{B}_{12}$ level
Figures

Figure 1: Frequency distribution of baseline values for serum vitamin B12.

![Distribution of Baseline B-12](image-url)
**Figure 2:** Frequency distribution of baseline values for serum hemoglobin.
**Figure 3:** Frequency distribution of baseline values for mean corpuscular volume (MCV).
**Figure 4:** Study of vitamin B12 supplementation: mean corpuscular volume (MCV) change vs. baseline B12.
**Figure 5:** Study of vitamin B12 supplementation: B12 response group and time.
Figure 6. Testing cascade for vitamin B\textsubscript{12} deficiency.