COLLEGE of AMERICAN PATHOLOGISTS

Test Ordering Guidelines for Suspected Vitamin B12 and Folate Deficiency

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SYNOPSIS AND RELEVANCE

Clinicians frequently order vitamin B_{12} and folate levels to evaluate anemia and neuropsychiatric symptoms. Vitamin B_{12} deficiency is more prevalent than folate deficiency in the general population as well as in anemic and hospitalized patients.[1] Automated chemistry concentration assays, combined with clinical correlation, may confirm or rule out a deficiency of B_{12} or folate.[1] However, laboratory cut-offs for B_{12} and folate deficiencies lack sensitivity and specificity,[1] and there is overlap of signs and symptoms between these deficiencies and many other illnesses. Pathologists can add value by reviewing peripheral blood smears, recommending and interpreting additional tests, and avoiding unnecessary testing.

OBJECTIVES

As a result of participating in this activity, participants will be able to:

1. Identify the differences between vitamin B_{12} and folate nutrition, absorption, biochemistry, and deficiency.
2. Recognize the hematologic and biochemical consequences of vitamin B_{12} and folate deficiencies.
3. Use additional laboratory tests to resolve clinically discordant or borderline serum/plasma vitamin B12 and folate Results.
4. Understand laboratory approaches to diagnosing pernicious anemia.
5. Employ strategies to reduce unnecessary folate and red blood cell (RBC) folate testing.

BACKGROUND

Vitamins B_{12} and folate are essential cofactors in enzymatic pathways essential for DNA synthesis and other cell functions.[2,3] A deficiency of either vitamin can cause impaired hematopoiesis, or neuropsychiatric disorders, while B_{12} deficiency is more likely to cause progressive, and potentially irreversible, demyelination of dorsolateral spinal column white matter and peripheral nerves.[4]

Clinicians typically order B_{12} and folate levels together in various clinical situations. However, differences in the vitamins' content in foods, gastrointestinal (GI) absorption, and prevalence of deficiencies should direct selective test ordering patterns guided by the patient's history.[1]

Review of Vitamin B_{12}

Microorganisms synthesize vitamin B_{12} in the GI tract of herbivores, and it is concentrated in their tissues and milk.[2] Absorption of B_{12} requires a multi-step process which begins with its release from food by gastric acid and digestion. In the small intestine, where pancreatic secretions create an alkaline environment, free B_{12} then binds to intrinsic factor (IF), that is produced by parietal cells. In the terminal ilium, B_{12}-IF complexes bind to cubam receptors on enterocytes followed by internalization, release into blood, and binding to transcobalamin and haptocorrin.[5] Passive absorption of B_{12} though mucosal surfaces is inefficient and does not compensate for daily losses.

Vitamin B_{12} is a cofactor for two Intracellular enzymes.[2] Methionine synthase requires both B_{12} and folate to remethylate homocysteine (Hcy) forming methionine, which provides methyl groups to multiple synthetic pathways including RNA and DNA. Methylmalonyl-CoA mutase requires B_{12} to convert methylmalonyl-CoA to succinyl-CoA. As a result, blood levels of the enzyme precursors Hcy and methylmalonic acid (MMA) rise during functional B_{12} deficient states.[2]

Causes of B_{12} deficiency are grouped by mechanism and clinical severity. Pernicious anemia, due to autoimmune gastritis and antibodies to IF, is the most common cause of severe B_{12} deficiency, with an estimated prevalence of approximately 4.0% in older European and African adults.[5] Other causes of severe deficiency include bariatric surgery, ileal resection, and inflammatory bowel disease.[6] Causes of mild B_{12} deficiency include malabsorption of protein bound B_{12}, atrophic gastritis, metformin, and vegetarian/vegan diets.[6] Nitrous oxide inactivates B_{12} without affecting blood levels and chronic abuse raises Hcy and MMA levels and can have serious hematologic and neurologic sequelae.[2]

B_{12} deficiency causes ineffective hematopoiesis and shortens red cell survival due to desynchrony between nuclear and cytoplasmic maturation. Maintenance of neuronal myelination is dependent upon vitamin B_{12} as well. Clinical consequences of B_{12} deficiency range from asymptomatic anemia to progressive fatigue and jaundice.[2] Neurologic symptoms are protean. The most common is peripheral paresthesia,[4] which can progress to ataxia and paraplegia.[1] Additional symptoms include cognitive decline and psychiatric disorders.[5] Hematologic and neurologic signs and symptoms of B_{12} deficiency frequently arise and progress independently of each other.[1,2,5,6]

Review of Folate

Dietary sources of folate include plants and fortified grains. Following absorption in the jejunum, folate is taken up by cells and converted to tetrahydrofolate (THF) linked to a polyglutamate chain.[3] THF accepts and donates methyl groups required for multiple cellular processes including DNA and RNA synthesis and conversion of homocysteine to methionine. In B_{12} deficiency, folate is "trapped" as 5-methlyfolate.[6] Folate supplementation to a B_{12} deficient patient can bypass this trap and correct the anemia but not the neurologic damage from B_{12} deficiency.[6]
Insufficient dietary intake is the primary cause of folate deficiency, typically associated with alcohol use disorders and severe malnutrition.[1] Other causes include gastrointestinal malabsorption and increased folate turnover states, eg, pregnancy, chronic hemolytic anemias.[3] The hematologic consequences of folate deficiency are identical to those of B_{12} deficiency. Neuropsychiatric complications are similar to those that occur with B_{12} deficiency, except peripheral neuropathies are rare.[1,4]

Laboratory Investigation of B_{12}/Folate Deficiencies

Macrocytic anemia is a classic presentation of B_{12} or folate deficiency.[7] However, neither anemia nor macrocytosis is a sensitive screening test,[2] and a modest macrocytosis (mean cell volume [MCV] 100-115 fL) is not specific for B_{12}/folate deficiencies.[1] Supportive laboratory findings include elevated red cell distribution width (RDW), low reticulocyte count, low haptoglobin, and elevated lactate dehydrogenase (LDH) and indirect bilirubin, indicative of ineffective erythropoiesis and hemolytic anemia.[1] Peripheral blood smear findings may include hypersegmented neutrophils along with nucleated red cells, oval macrocytes and schistocytes, mimicking a microangiopathic hemolytic process.[7] Bone marrow examination is not required to confirm B_{12}/folate deficiency anemia. However, if performed, findings of marked erythroid hyperplasia, megaloblastic maturation and atypia mimicking dysplasia may be misinterpreted as a hematologic clonal disorder.[7]

Automated serum/plasma B_{12} and folate chemiluminescence assays are widely available. However, due to very low prevalence of folate deficiency from grain fortification, it is not necessary to measure folate in patients who have adequate diets and no evidence for malabsorption.[1] With respect to B_{12} and folate assays, B_{12} competes with labeled B_{12} to bind to purified intrinsic factor[8] and folate competes with labeled folate to bind to folate binding proteins.[3] Many factors, both biologic and analytic, limit the sensitivities and specificities of B_{12} and folate concentrations near the lower limit cut-offs.[1,2,8] Whenever there is clinical uncertainty surrounding a low or "borderline-low normal" B_{12}/folate result, adjuvant tests of enzyme cofactor activities having higher sensitivities may be recommended,[2,3,5,8] as shown in the following table. Immunoassays for Hcy are widely available while MMA is often a send out to a reference laboratory for performance by chromatography/mass spectrometry. Hcy and MMA concentrations may be falsely elevated in patients with end stage renal disease, or concurrent treatment with methotrexate or phenytoin.

Findings / Test	B_{12} Deficiency	Folate Deficiency	No B_{12} or Folate Deficiency
Methylmalonic acid	Elevated	Normal	Normal
Homocysteine	Elevated	Elevated	Normal

"Active B_{12}" assays specifically measure B_{12} bound to holotranscobalamin, which is the biologically active form of serum/plasma B_{12}.[9] However, there is insufficient evidence to recommend active B_{12} measurements in routine practice.[8] Intracellular folate concentration does not change during the life of a circulating red cell. Recent dietary intake affects serum/plasma folate levels while red cell folate levels provide a more stable long-term estimate.[3] However, RBC folate assay complexity and variability, and lack of improved clinical utility compared to serum/plasma

assays have led many laboratories to discontinue the former assay.[10,11] Modifications to the computerized physician order entry system can reduce unnecessary folate and RBC folate orders.[12]

When an alternative etiology for confirmed B_{12} deficiency is not clinically apparent, pernicious anemia (PA) is the presumed cause. PA is a consequence of atrophic gastritis due to autoimmune destruction of gastric body parietal cells. A test to confirm intrinsic factor (IF) deficiency by measuring absorption of radiolabeled B_{12} combined with IF, eg, Schilling test, is no longer performed. A positive result for serum IF autoantibodies confirms autoimmune destruction of parietal cells, ie, pernicious anemia, with 100% specificity and 50-70% sensitivity.[6] It is important to collect blood for IF antibody testing prior to IM B_{12} injections since interference from high B_{12} concentrations can cause false positive IF antibody results. Other markers of atrophic gastritis like parietal cell autoantibodies and elevated gastrin are more sensitive than IF antibodies but are non-specific and are not diagnostic for PA.[1]

INSIGHTS

Whom to test:

- Patients with incidentally discovered or symptomatic anemia without alternative explanations; absence of macrocytic MCV does not exclude B_{12} or folate deficiency
- Patients with new neurologic signs and symptoms of unclear etiologies

What tests to perform:

- CBC (includes MCV and RDW), reticulocyte count, LDH, haptoglobin, total and indirect bilirubin, review of peripheral blood smear
- Plasma/serum total B_{12} concentration
- Plasma/serum total folate concentration only when history supports a deficient diet or malabsorption comorbidities

Subsequent testing:

- When B_{12} or folate concentrations are borderline or discordant with clinical judgement:
  - Metabolites that accumulate due to deficiencies of B_{12} (MMA, HCY) or folate (HCY)
  - To confirm pernicious anemia: IF autoantibody

Tests that are rarely indicated:

- Plasma/serum folate in patients with adequate diet and no GI malabsorption symptoms
- RBC folate
- Active B_{12}

Testing options to monitor response to B_{12} or folate supplementation:

- Within days: decreased LDH, disappearance of hypersegmented neutrophils
- Within approximately 1 week: normalization of MMA (B_{12}) and HCY (B_{12} and folate)
- If anemic at presentation:
  - Within 2-3 weeks: increasing Hgb and reticulocyte count
  - Within 3-6 months: If anemia recovery is incomplete, initiate evaluation for other causes:
    - Iron panel to detect co-existing iron deficiency

INTERVENTIONS

- Provide information to providers regarding the lack of clinical utility of RBC folate levels.
- Provide information to providers about the low incidence of serum/plasma folate deficiency in patients without malabsorption or alcohol use disorders.
- Obtain support from hospital laboratory utilization/stewardship committee to restrict ordering RBC folate testing.
- Remove RBC folate from electronic and paper order systems.
- Implement clinical decision support soft stops to discourage ordering of folate levels in patients with no/low risk of folate deficiency.
- Create clinical decision support algorithms to encourage ordering of supplementary tests to investigate unexplained anemia or neurologic findings and clinically discordant or borderline B_{12}/folate results (see **Appendix A**).

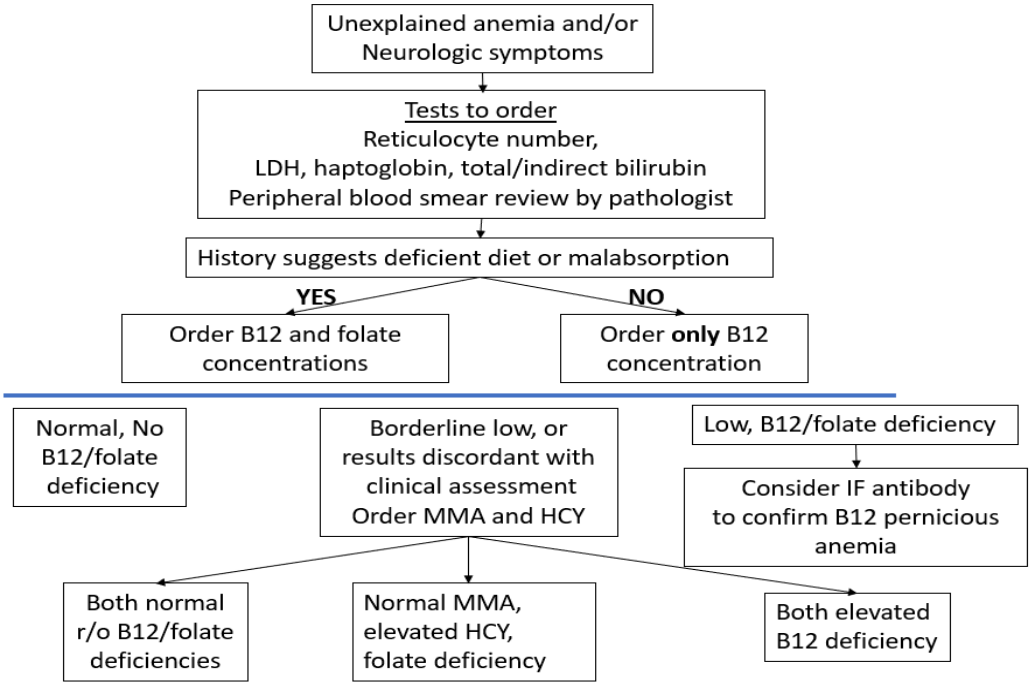
INTERVENTION ANALYSIS

To provide evidence of successful interventions to reduce RBC folate and serum/plasma folate orders:

- Determine the baseline number of RBC folate, serum/plasma folate, and serum/plasma B_{12} orders for a 6-month period before the start of interventions, and compare to respective test ordering volumes for 6 months after interventions, expressed as percent changes (post intervention volume/preintervention test volume x 100).

- Determine the baseline number of times serum/plasma folate and B_{12} are ordered together for a 6-month period before the start of interventions and compare to respective test ordering behavior for 6 months after interventions, expressed as percent change (post intervention volume/preintervention test volume x 100).
- Identify providers or services that continue to frequently order RBC folate or serum/plasma folate for targeted education and peer to peer interventions.
- Determine the baseline number of methylmalonic acid and homocysteine orders for a 6-month period before the start of interventions, and compare to respective test ordering volumes for 6 months after interventions, expressed as percent changes (post intervention volume/preintervention test volume x 100).
- Extract borderline serum/plasma folate and B_{12} results from the laboratory information system database and perform retrospective review of medical records to assess compliance with algorithm guidelines.

APPENDIX A: TESTING ALGORITHM EXAMPLE



QUESTIONS AND ANSWERS

QUESTION 1 OBJECTIVE

Identify the differences between B_{12} and folate nutrition, absorption, biochemistry, and deficiency.

QUESTION 1

Which one of the following statements is true regarding vitamin B_{12} and folate deficiencies?

A. The incidence of folate deficiency is much greater than the incidence of vitamin B_{12} deficiency.
B. Only B_{12} deficiency can cause neurologic signs and symptoms.
C. Anemia and neurologic symptoms begin and progress together in vitamin B_{12} deficient patients.
D. Vegans are at greater risk of developing folate deficiency than vitamin B_{12} deficiency.
E. The antiglycemic medication metformin is associated with decreased absorption of vitamin B_{12}

The correct answer is E. Metformin interferes with calcium dependent absorption of B_{12}-IF complexes in the terminal ileum.

A is incorrect. The incidence of B_{12} deficiency is much higher than the incidence of folate deficiency.

B is incorrect. Both folate and B_{12} deficiencies can present with neurologic complications.

C is incorrect. Neurologic symptoms and anemia can arise and progress independently in patients with B_{12} deficiency.

D is incorrect. A vegan diet would provide ample vegetable sources of folate while excluding sources of B_{12}.

QUESTION 2 OBJECTIVE
Recognize the hematologic and biochemical consequences of B_{12} and folate deficiencies.

QUESTION 2
Which of the following test result patterns is compatible with anemia due to Vitamin B_{12} or folate deficiency?

	Analyte/Index Value Compared to Reference Value			
Option	**LDH**	**Reticulocyte #**	**Haptoglobin**	**RDW**
A	WNL	Elevated	Elevated	Decreased
B	WNL	Decreased	Decreased	WNL
C	Elevated	Decreased	Decreased	Elevated
D	Elevated	Elevated	Decreased	Elevated
E	Decreased	WNL	Decreased	Decreased

Abbreviations:# number. LDH lactate dehydrogenase, RDW red cell distribution width, WNL, within normal limits

The correct answer is C. Ineffective red cell production causes low reticulocyte number, and destruction of defective red cells in the bone marrow hemolysis causes elevated LDH and low haptoglobin. Megaloblastic and damaged red cells cause elevated RDW.
A is incorrect. LDH is elevated due to destruction of defective red cells in the bone marrow and RDW typically increases due to production of macrocytic red cells.
B is incorrect. LDH is elevated due to destruction of defective red cells in the bone marrow. Haptoglobin is low or undetectable due to scavenging free hemoglobin. RDW typically increases due to production of macrocytic red cells.
D is incorrect. Reticulocyte production is low due to ineffective erythropoiesis. RDW typically increases due to production of macrocytic red cells.
E is incorrect. LDH is elevated due to destruction of defective red cells in the bone marrow. Reticulocyte production is low due to ineffective erythropoiesis. RDW typically increases due to production of macrocytic red cells.

QUESTION 3 OBJECTIVE
Use additional laboratory tests to resolve clinically discordant or borderline serum/plasma vitamin B12 and folate results.

QUESTION 3
Which of the following test results would **rule out** a deficiency of Vitamin B_{12} when the serum/plasma level is slightly below the lower limit of the laboratory's reference range?
A. The MCV is < 100 fL.
B. Absence of hypersegmented neutrophils on peripheral smear review.
C. Homocysteine is elevated.
D. MMA is WNL.
E. Normal hemoglobin concentration
The correct answer is D. Methylmalonyl-CoA mutase requires B_{12} to convert methylmalonyl-CoA (MMA) to succinyl-CoA. As a result, blood levels of the enzyme precursor MMA rise during functional B_{12} deficient states.
A is incorrect. Macrocytosis (MCV > 100 fL) is not a sensitive indicator of functional B_{12} deficiency. Therefore, a patient could be functionally B_{12} deficient with a normal MCV.
B is incorrect. Hypersegmented neutrophils are not a sensitive indicator of functional B_{12} deficiency. Therefore, a patient could be functionally B_{12} deficient without the presence of hypersegmented neutrophils.
C is incorrect. Elevated homocysteine could be due to either B_{12} or folate deficiency. Therefore, an elevated Hcy would not rule out B_{12} deficiency.
E is incorrect. Functional B_{12} deficiency can occur without concurrent anemia when testing is done in patients with unexplained neurologic symptoms.

QUESTION 4 OBJECTIVE
Understand laboratory approaches to diagnosing pernicious anemia.

QUESTION 4
Which of the following tests should be used to diagnosis pernicious anemia?
A. Serum parietal cell autoantibodies
B. Schilling test
C. Serum intrinsic factor autoantibodies
D. Stool *Helicobacter pylori* antigen
E. Serum gastrin levels

The correct answer is C. Antibodies to intrinsic factor are highly specific for pernicious anemia being the cause of B_{12} deficiency. While the positive predictive value is very high, sensitivity is not 100% and there can be false negative results.
A is incorrect. Parietal cell autoantibodies are not specific for a diagnosis of pernicious anemia and false positive results can occur.
B is incorrect. The Schilling test was a nuclear medicine method to detect malabsorption of radiolabeled B_{12}. The isotope is no longer available.
D is incorrect. While *H. pylori* can cause atrophic gastritis and decreased B_{12} absorption, detection of *H pylori* antigen in stool is not specific for pernicious anemia.
E is incorrect. Gastrin may be elevated due to many causes of gastritis and achlorhydria and is not specific for pernicious anemia.

QUESTION 5 OBJECTIVE
Employ strategies to reduce unnecessary folate and red blood cell (RBC) folate testing.

QUESTION 5
Which of the following interventions is **least likely** to reduce the use of folate and RBC folate testing?
A. Discontinue in-house RBC folate testing
B. Combine serum/plasma vitamin B12 and folate concentrations into a single test order
C. Clinical decision support intervention to remind clinicians that folate deficiency is rare in the absence of malabsorption or deficient diet
D. Remove RBC folate from the computer physician order entry (CPOE) system
The correct answer is B. A combined folate/ B_{12} order would have the opposite effect.
A is incorrect. While discontinuing in house RBC folate testing would reduce folate testing, there would still be requests to send the test out to a reference laboratory.
C is incorrect. Clinical decision support would likely reduce unnecessary folate testing.
D is incorrect. Removing RBC folate from CPOE would reduce unnecessary RBC folate testing.

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