Peripheral blood evaluation: B_{12} deficiency, anemia, ET, CMML

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April 2019—Diagnoses of essential thrombocythemia and chronic myelomonocytic leukemia were among those covered in four cases in a CAP18 session on practical challenges in peripheral blood evaluation.

Carla S. Wilson, MD, PhD, professor of hematopathology, and Devon S. Chabot-Richards, MD, associate professor of hematopathology and molecular pathology, Department of Pathology, University of New Mexico School of Medicine, walked attendees through seven cases illustrating the benefits of peripheral blood smear evaluation, four of which are reported here. Drs. Wilson and Chabot-Richards are medical directors at TriCore Reference Laboratories, Albuquerque, NM.

The first case was that of a 58-year-old man who presented with a three-month history of weakness, palpitations, and shortness of breath. He complained of bleeding gums, epistaxis, and a tingling sensation throughout his body. His CBC with differential counts revealed marked anemia (Hb 5.6 gm/dL), high RDW (21.2 percent), mild leukopenia (WBC 2.7 × 10^9/L), lymphopenia, adequate neutrophils, and moderate thrombocytopenia (platelet 68 × 10^9/L).

Review of the peripheral blood smear was remarkable for prominent red cell anisopoikilocytosis with schistocytes, oval macrocytes, rare spherocytes, and nonspecific poikilocytes. Polychromasia was not increased and the reticulocyte count was normal, Dr. Wilson said.

Additional studies revealed that the patient had low haptoglobin (<8 mg/dL), elevated LDH (1023 U/L), and elevated indirect bilirubin (3.5; normal range 0.2–1.0) consistent with hemolysis, she said. “We recommended additional testing which showed a serum vitamin B_{12} level of <60 pg/mL [normal 193–986], normal RBC folate level, methylmalonic acid of 3.08 µmol/L [normal 0–0.4], and positive intrinsic factor and parietal cell antibodies. The clinicians were worried about thrombotic thrombocytopenic purpura because of all the schistocytes,” Dr. Wilson said. They sent out for an *ADAMTS13* activity assay; results were normal.

“This is a case of severe vitamin B_{12} deficiency that can mimic microangiopathic hemolytic anemia,” or MAHA, she said. “The key is that you can see a lot of fragmented red cells in vitamin B_{12} deficiency due to increased red cell fragility causing decreased survival in circulation.” (**Fig. 1**)

Severe vitamin B_{12} deficiency affects DNA synthesis in all hematopoietic lineages, and the CBC often shows pancytopenia.

Hypersegmented neutrophils are an important clue to vitamin B_{12} deficiency, as shown in Fig. 1. Hypersegmentation is defined as the presence of six or more lobes in a neutrophil or five lobes in at least five percent of neutrophils. This abnormality is quickly reversed and may not be recognized in smears after initiation of vitamin B_{12} supplementation. Hypersegmented neutrophils are not specific to vitamin B_{12} deficiency. “You can see hypersegmented neutrophils and macrocytic anemia in association with myelodysplastic syndromes or in individuals receiving certain drugs such as hydroxyurea,” Dr. Wilson said.

“How do we distinguish vitamin B_{12} deficiency from microangiopathic hemolytic anemia?” she asked.

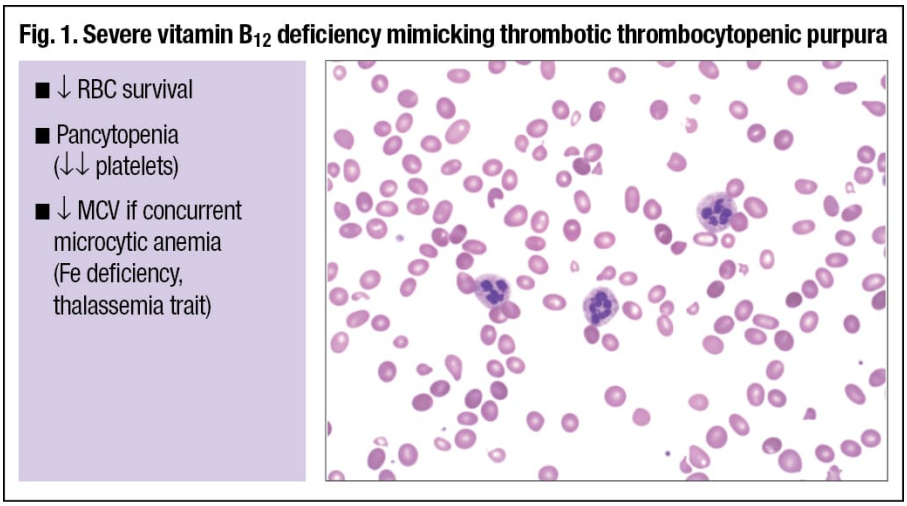
- A high reticulocyte count can be a reliable indicator of MAHA, she said. The reticulocyte percentage may be elevated in B_{12} deficiency but the

absolute reticulocyte number is low due to ineffective hematopoiesis.

- Pancytopenia is rare in MAHA. The degree of anemia is not as severe in MAHA and platelet counts are typically lower than in B_{12} deficiency.
- The MCV in vitamin B_{12} deficiency is usually high and often exceeds 120 fl, although it may be normal in cases of severe hemolysis or with concurrent iron deficiency or thalassemia. Reticulocytosis associated with MAHA only causes a slightly elevated MCV, if at all. The MCV was 114 fl in this case.
- Neutrophil hypersegmentation or neutropenia is not a feature of MAHA.

"The problem with making a diagnosis of vitamin B_{12} deficiency in less severe cases is that no single marker detects deficiency in all patients," she said, and serum vitamin B_{12} testing is neither sensitive nor specific.

Fig. 1. Severe vitamin B_{12} deficiency mimicking thrombotic thrombocytopenic purpura



Reference ranges for serum vitamin B_{12} are based on population studies and may not be adequate for individuals. Another problem is that serum B_{12} assays measure the total B_{12} bound to both transcobalamin (20 percent) and haptocorrin (80 percent). Only the 20 percent of B_{12} bound to transcobalamin is available to tissues through CD320 cell receptors. The 80 percent bound to haptocorrin is metabolically unavailable; serum B_{12} measurements are significantly affected by changes in the haptocorrin protein level. Increased haptocorrin is produced by the liver in some malignancies, and about 15 percent of people normally have decreased levels.

The metabolically active B_{12}-transcobalamin complex is called holotranscobalamin (HTC), and some laboratories, particularly those outside the United States, are testing for HTC rather than total serum B_{12} as a measure of B_{12} deficiency.

"With decreased vitamin B_{12}, you see decreased HTC," Dr. Wilson said. "There is less uptake of B_{12} into tissues, and it starts affecting the B_{12} dependent pathways."

The immunoassay for HTC is being marketed as an active B_{12} assay, and preliminary studies suggest it may have some of the same problems as the existing serum B_{12} assays, most notably when the HTC results fall into an indeterminate range of 25 to 70 pmol/L (depending on the assay). Similar to serum B_{12} assays, results in the intermediate range should be followed up with tests for methylmalonic acid (MMA) preferentially or homocysteine.

MMA is an imperfect test for some individuals, Dr. Wilson said, and particularly for patients with renal disease who do not excrete serum metabolites of MMA. The elevated metabolites can make the patient appear to be B_{12} deficient. "MMA is better than homocysteine because it's usually not elevated in folate deficiency and homocysteine is also affected by renal disease," she said. Automated tests for MMA can detect subtle disturbances

in vitamin B_{12} metabolic pathways and should be combined with serum B_{12} or HTC testing for complete interpretation, Dr. Wilson said.

If faced with discordant vitamin B_{12} laboratory results in a patient with strong clinical features of deficiency, "just tell the clinicians to treat," she advises, to avoid neurological impairment. "Vitamin B_{12} deficiency is easily corrected."

Our next case is a case of anemia," said Dr. Chabot-Richards as she described test results from a 12-year-old African-American girl who presented to the emergency department with acute hip pain. A CT scan and CT angiogram revealed a bony infarction in the femoral neck, and the girl's CBC and peripheral blood smear showed evidence of anemia. Her blood smear was clearly abnormal, with numerous classic sickle cells with pointed ends, as well as other elongated red blood cells with blunt ends. Howell-Jolly bodies and target cells were present. The patient also had an elevated reticulocyte count at 3.7 percent.

"This was sent out for hemoglobin electrophoresis. Alkaline electrophoresis is the classic screening test we do to evaluate for abnormal hemoglobins," Dr. Chabot-Richards said. "It often reflexes to acid depending on our results."

The patient had 42 percent abnormal hemoglobin running in the S/D/G lane and 46 percent running in the C/E/O/A2 lane. Hemoglobin F was two percent. A Sickledex test was positive, and the patient was diagnosed with sickle cell/hemoglobin C disease.

"This is a patient who has co-inheritance of two different β-chain variants: hemoglobin S and hemoglobin C," Dr. Chabot-Richards said. Both are common variants in the African population, so it's possible to see them combined.

The girl had no normal β-chain and was unable to make normal hemoglobin A. The combination of sickle cell and hemoglobin C causes a sickling disorder similar to sickle cell anemia. These patients typically have a slightly milder clinical picture and longer red blood cell survival, which leads to having a higher hemoglobin. Patients also have chronic hemolysis and are at risk for femoral neck necrosis and bone marrow infarction. Compared with patients with sickle cell anemia, patients with sickle cell hemoglobin C disease usually have delayed hyposplenism.

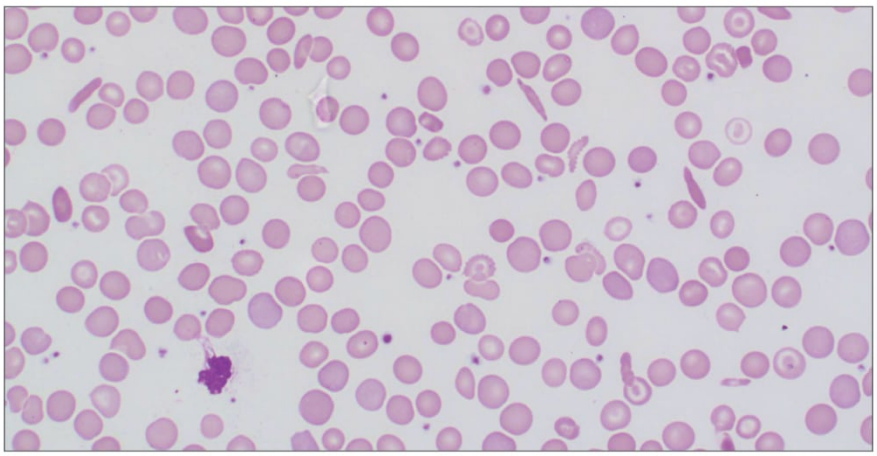
"These patients don't typically have true sickle cells or true hemoglobin C crystals," Dr. Chabot-Richards said of the blood smear findings. "Instead, you see more unusual, elongated cells with rounded ends." Clam- or boat-shaped cells are increased, as are target cells.

In comparison, patients with sickle cell trait have one normal β-chain and one sickle β-chain. Electrophoresis would show 35 to 45 percent hemoglobin S, with the remainder normal hemoglobin A. The majority of sickle cell trait patients are asymptomatic with cells that rarely sickle, and have normal CBC parameters and normal peripheral blood cell appearance. "They do have an increased incidence of renal medullary carcinoma, a risk they share with people who have sickle cell anemia," she said.

Sickle cell anemia patients are homozygous for the S β-chain, and electrophoresis results show greater than 80 percent hemoglobin S. These patients have chronic hemolytic anemia, and their cells sickle in low-oxygen environments. "These patients are typically hyposplenic, so their spleens infarct at a fairly young age," Dr. Chabot-Richards said. Sickle cell anemia patients are also at risk for death from infection, stroke, or respiratory failure due to lung infarction.

Peripheral blood smear findings of a sickle cell anemia patient show numerous elongated cells, much more pointed on the ends than what was seen in the 12-year-old girl (**Fig. 2**). "There is a lot of anisopoikilocytosis, so the cells have a higher red cell distribution width," Dr. Chabot-Richards said.

Fig. 2. Sickle cell anemia blood smear findings



In a comparison of findings of sickle cell anemia patients and sickle C disease patients, she pointed out that while both patients have anemia, it is typically more severe in those with sickle cell anemia. Sickle cell anemia patients also typically have higher reticulocyte counts, higher WBC counts owing to the inflammatory process, higher bilirubin, and higher LDH because of increased hemolysis.

Sickle cell trait is fairly common and can be seen in combination with other hemoglobinopathies, which have different clinical manifestations, such as sickle cell/hemoglobin D. These patients have milder disease and usually a stable hemoglobin level. Sickle cell/hemoglobin O-Arab is a severe sickle disease; patients have one O-Arab β-chain and one sickle β-chain. “Usually, the hemoglobin S is at a slightly higher percentage than the O because the O is such a deleterious mutation,” Dr. Chabot-Richards said.

Another variation is sickle cell C-Harlem, which usually is present at a slightly higher percentage than the S on electrophoresis.

“If you have sickle cell trait together with an α-chain variant, it’s usually clinically silent,” Dr. Chabot-Richards said.

It is not uncommon for patients to have sickle hemoglobin and α-thalassemia co-inherited. These patients have decreased hemolysis and soft tissue damage, so the combination is slightly protective for them, though they have increased bone infarction and osteonecrosis. Overall survival rates are not very different.

If the sickle cell anemia is heterozygous and diagnosed along with an α-chain variant, typically the electrophoresis will show a decreased percentage of hemoglobin S.

Sickle hemoglobin with β-thal-assemia is also not uncommon. Patients who have a sickle cell β-chain with severe β-thalassemia leading to complete loss of protein function are unable to make normal hemoglobin A. They have less hemolysis than sickle cell anemia patients, and their hemoglobin parameters are typically higher. A peripheral blood smear from a patient with β-thalassemia shows a scattering of nucleated red blood cells at low power; sickle cells with pointed ends and numerous target cells are apparent at a higher power.

“Similar to B_{12} deficiency, sometimes there can be confusion with microangiopathic hemolytic anemia,” especially if the patient has very high anisopoikilocytosis, Dr. Chabot-Richards said. With insufficient experience, “it can be hard to distinguish between a sickle cell and a schistocyte.”

Sickle cells typically are larger and more uniform in size than schistocytes. The clinical presentations of sickle cell anemia and microangiopathic hemolytic anemia should be distinct.

Hemolysis is associated with thrombocytopenia, increased bilirubin and LDH, and decreased haptoglobin, so it is helpful to use ancillary tests to help determine the degree of hemolysis.

“Inheritance of any sickle cell hemoglobin will cause a positive Sickledex, so it’s important to realize that a positive Sickledex does not necessarily equal sickle cell disease in the patient, or even that the patient will sickle clinically,” Dr. Chabot-Richards said.

A 44-year-old woman returned to her doctor six months after a routine CBC that showed increased platelets. The

blood smear at follow-up showed increased platelets with a range in size but normal granulation. The woman had no history of thrombotic or hemorrhagic events and no medication history, and she had normal coagulation and iron studies, LDH, and liver function tests.

The patient's hematologist recommended a bone marrow biopsy because of the sustained thrombocytosis. The patient wanted to avoid a biopsy and requested additional pre-procedure workup.

"In this patient, we could do mutation testing on the peripheral blood before going to bone biopsy," Dr. Chabot-Richards said. "We sent this for *JAK2, CALR*, and *MPL* testing." The *JAK2* V617F testing was negative, and reflex testing for *CALR* and *MPL* revealed the patient had a *CALR* 52 base pair insertion.

"Given what we were seeing in the peripheral blood, the sustained thrombocytosis, the *CALR* mutation, we said it's likely essential thrombocythemia, but you cannot make that diagnosis without a bone marrow biopsy to confirm the morphology."

Essential thrombocythemia is a myeloproliferative neoplasm associated with increased platelets. The blood findings usually consist of platelet anisocytosis with small and large forms, possibly hypogranular platelets ("we think of that as a dysplastic finding, but it can be seen in these cases"), possibly circulating megakaryocytic nuclei appearing as dark, very condensed nuclei, and neutrophilia without significant left shift. "They should not have significant dysplastic features, you shouldn't be seeing any increase in blasts, and basophils and the red blood cells should be fairly unremarkable," Dr. Chabot-Richards said.

The 2016 WHO update sets forth the major and minor diagnostic criteria for ET (**Fig. 3**).

Essential thrombocythemia diagnosis requires either all four major criteria, or the first three major and the minor criteria. "If you can't find mutation in *JAK2, MPL*, or *CALR*, but you can find some other clonal marker, you can still make this diagnosis."

Fig. 3. ET diagnostic criteria

Major criteria	Platelets >450 BM biopsy ■ Proliferation of mainly megakaryocytes with increased large, mature forms with hyperlobulated nuclei ■ No increase in granulopoiesis or erythropoiesis ■ Fibrosis MF-0 or very rarely MF-1 Not meeting criteria for another diagnosis Presence of *JAK2, CALR,* or *MPL* mutation
Minor criterion	Presence of a clonal marker or rule out reactive

PV diagnostic criteria

Major criteria	Increased RBC parameters ■ Hb >16.5 or Hct >49% in men ■ Hb >16 or Hct >48% in women ■ Or increased red cell mass Bone marrow biopsy findings ■ Hypercellularity, panmyelosis including prominent erythroid, granulocytic and megakaryocytic proliferation, pleomorphic, mature megakaryocytes with differences in size Presence of *JAK2* mutation
Minor criterion	Subnormal serum erythropoietin level
	Diagnosis requires either all three major or first two major and the minor or Sustained erythrocytosis, major three, and the minor

It's important to distinguish ET from polycythemia vera, which is commonly associated with increased RBCs as opposed to high platelet count (Fig. 3). Diagnosis requires either all three of the major criteria or the first two and the minor, or a patient with sustained erythrocytosis, *JAK2* mutation, and the minor criteria. "You can make this diagnosis without the bone marrow biopsy," Dr. Chabot-Richards said, but "I would not recommend it because the assessment of myelofibrosis is very important for prognosis."

Primary myelofibrosis is a third myeloproliferative neoplasm, one associated with leukocytosis with ultimate progression to cytopenias due to fibrosis. There are two stages: pre-fibrotic and overt. The early phase can be similar to ET, and bone marrow is required for diagnosis.

“We usually do reflex testing for these myeloproliferative-associated mutations,” Dr. Chabot-Richards said. “We will start with a *JAK2* V617F. It’s the most common mutation we see in a polycythemia vera, but it is also seen in 60 percent of cases of ET and primary myelofibrosis.” If the results are negative, they reflex to additional testing based on the clinical picture.

“Typically, we would either go to *JAK2* exon 12, if it looked more consistent with polycythemia vera; that’s positive in an additional four percent of those cases and is not seen in ET and primary myelofibrosis. If it looked more like ET or primary myelofibrosis, we would do *CALR* and *MPL*.” Rarely do they do all such tests on any one patient, she said, and initial molecular testing typically consists only of *JAK2, CALR*, and *MPL*.

If additional prognostic testing is requested, it can be done on blood, though it is more typically done on bone marrow, and it would be sent for next-generation sequencing using a myeloid gene panel. *ASXL1* and *SRSF2* are associated with inferior overall and leukemia-free survival. *EZH2* mutations are associated with inferior overall survival. *IDH1/2* mutations are associated with inferior leukemia-free survival.

“The significance of these is mostly known in primary myelofibrosis. It’s not clear in ET and PV, but it seems reasonable that it would be similar, and preliminary studies have shown that,” she said.

National Comprehensive Cancer Network guidelines recommend that cytogenetic testing be performed on a bone marrow specimen, but peripheral blood testing is acceptable. “This is used to detect clonal abnormalities, and you can see prognostic abnormalities,” Dr. Chabot-Richards said. “Most of the abnormalities here are similar to those we see in any myeloid neoplasm, such as MDS.”

Chronic myeloid leukemia must also be considered with essential thrombocythemia. Patients may show thrombocytosis but should also have absolute basophilia, a leukocytosis with a left shift, and are positive for t(9;22) *BCR-ABL1*.

Reactive thrombocytosis can have many causes. A patient who has thrombocytosis post-surgery or trauma will resolve quickly, in contrast to patients who have, for example, iron deficiency anemia, chronic inflammation, or post splenectomy. “It’s important to figure that out,” she said.

The initial molecular testing can be performed on peripheral blood specimens, though the NCCN recommends bone marrow, Dr. Chabot-Richards said. Peripheral blood testing results may convince the patient and oncologist that bone marrow testing is necessary. “Bone marrow is required for the ultimate diagnosis because the presence of fibrosis is so important.”

A 68-year-old man with myasthenia gravis was being followed for anemia. His CBC showed a white blood cell count of 7.6×10^9/L, with neutrophils slightly decreased for his age and monocytes slightly increased. His peripheral blood smear revealed only 57 percent neutrophils and many large, mononuclear cells.

A review of the chart found that the patient’s WBC had fluctuated between normal and elevated, and his monocyte count ranged between one and 2.2. His anemia fluctuated between mild and moderate. The patient also had thrombocytopenia, ranging from mild to moderate.

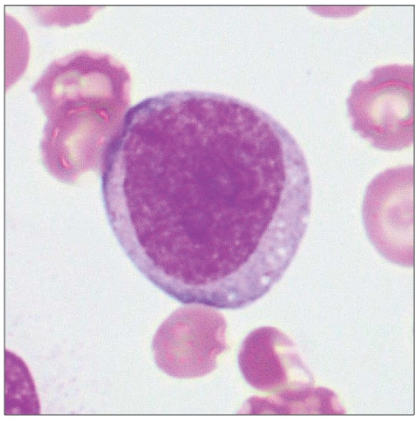
Dr. Chabot-Richards took another look at the blood smear and saw many large cells with convoluted nuclei, consistent with monocytes, and a few neutrophils with nuclear irregularities, including neutrophils with Pelger-Huet-like nuclei. In the lateral edges of the blood smear, there were larger cells with smooth nuclear contours and delicate folds, accounting for less than one percent of cells. She also discovered rare large cells consistent with blasts.

The patient’s blood was sent for flow cytometry. “Based on our side scatter and CD45, we were able to get 23.4

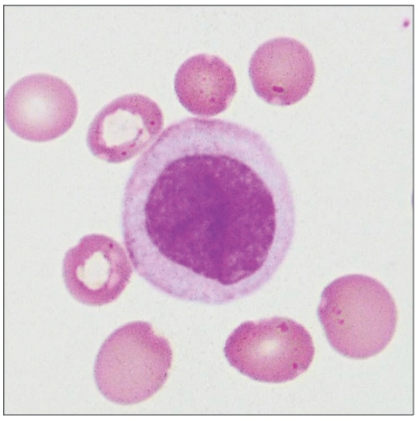
percent monocytes," Dr. Chabot-Richards said. "Monocytes showed normal expression of CD36, CD64, CD13, and CD14." There was aberrant expression of CD56 and abnormally dim HLA-DR.

The diagnosis: chronic myelomonocytic leukemia (CMML), an overlap myelodysplastic/myeloproliferative neoplasm. These neoplasms typically have dysplastic features and a combination of cytopenias and cytoses. "In this disease, patients often have thrombocytopenia. A monocytosis is required for diagnosis," she said. The WHO criteria consist of a persistent peripheral blood monocytosis of $>1 \times 10^9$/L and >10 percent of the WBC count. Patients should have less than 20 percent blasts and equivalents in the blood and bone marrow; a higher percentage would give them a diagnosis of leukemia.

Fig. 4. Blast equivalents in CMML



Monoblasts: Fine, open chromatin, round or minimally irregular nuclei, prominent nucleoli



Promonocytes: Stippled chromatin, variable nucleoli, delicate nuclear folds, light gray cytoplasm

"One thing that can trip people up is this idea of blast equivalents," Dr. Chabot-Richards said. While in most diseases, a myeloblast is a blast, "in this disease, we're going to include some other things."

Promonocytes are blast equivalents in CMML. "It's important to recognize these because while they are blast equivalents, atypical monocytes that are mature are not. It can be difficult to distinguish between the two," she said (**Fig. 4**). Dr. Chabot-Richards typically performs her differential twice in these cases.

"You need to do bone marrows on these patients because often the blast count can be much higher in the bone marrow than it is in the peripheral blood," she said (**Fig. 5**).

Flow cytometry can help to quantify the immature cells. Most of the monocytes in CMML show classical immunophenotype: CD14 positive and CD16 negative. An aberrant phenotype, such as increased CD56 or aberrant expression of CD2, is also common.

The morphologic blast count takes priority, she said. "No matter what your immature blast count is on flow, if it doesn't correlate with your morphology, you should trust your morphology."

Most cases of CMML will have a normal karyotype; when abnormalities are seen they're similar to those seen in MDS. *BCR-ABL1* should be excluded in all cases, and *PDGFRA, PDGFRB, FGFR1*, and *PCM1-JAK2* should be excluded if eosinophilia is present. "The new WHO update recommends that if you have a patient you think has chronic myelomonocytic leukemia, you should do FISH or molecular testing for t(9;22) to completely exclude chronic myeloid leukemia, because it is important to treat those patients with tyrosine kinase inhibitors," Dr. Chabot-Richards said.

Most cases of CMML have mutations on gene panel testing, commonly *TET2*, *SRSF2,* and *ASXL1* (the latter is predictive of aggressive disease). *SETBP1* is strongly associated with a CMML diagnosis but is less common. "If you do find it, it can be strongly supportive of this specific diagnosis," she said.

Fig. 5. Blast-based classification of CMML (WHO 2016)

	% Peripheral blood blasts	% Bone marrow blasts	Other
CMML-0	<2	<5	
CMML-1	2–4	5–9	
CMML-2	5–19	10–19	Auer rods present

Bone marrow biopsy is *required* for final classification—many cases show significantly higher blast counts in marrow.

Other mutations seen are *RUNX1, NRAS/KRAS, CBL,* and *EZH2*. The NPM1 mutation is rare but associated with increased progression to acute leukemia. "Finding a mutation in any gene is not sufficient for diagnosis of CMML."

CMML is subdivided into proliferative and dysplastic types. A WBC >13 × 10^9/L is proliferative, which has a higher monocyte count, higher circulating immature myeloid cells and blasts, and higher LDH, and is more likely to have cytogenetic abnormalities and mutations in *ASXL1*, *JAK2*, and *CBL*. Dysplastic type (WBC <13 × 10^9/L) has lower monocyte and blast counts, lower LDH, and normal cytogenetics, and is more likely to have a mutation in *SF3B1*.

Myelodysplastic syndromes, which share the same cytogenetic abnormalities and mutations with CMML, can be distinguished by a predominance of cytopenias and a lack of monocytosis. "These patients have dysplastic findings in one or more of the lineages, in greater than 10 percent of cells of that lineage," she said. Promonocytes should not be used as blast equivalents in MDS.

Of the myeloproliferative neoplasms, chronic myeloid leukemia must be excluded, and FISH or molecular testing is recommended. "In particular, CML-P190 variant is associated with a monocytosis, so these patients can have similar peripheral blood findings," Dr. Chabot-Richards said. Dysplasia or thrombocytopenia is not typically seen in CML.

Other myeloproliferative syndromes may present with monocytosis. *JAK2, CALR*, and *MPL* mutations may support a diagnosis of MPN. "They're not specific, so you can see *JAK2* mutations in CMML. But if you find them, it might be good to take another look to see if there's anything else abnormal about the patient presentation," Dr. Chabot-Richards said.

All MPNs require bone marrow for correct classification and assessment of fibrosis. "This can help you distinguish between MPN and CMML based on the bone marrow morphology."

Other MDS/MPN overlap syndromes may be confused with CMML. Atypical chronic myeloid leukemia usually has more prominent dysplasia, marked left shift, a lack of significant monocytosis, and *SETBP1* mutations. There is some overlap in diagnostic criteria with juvenile myelomonocytic leukemia, which is rare and usually fairly distinct clinically because most patients are under age three.

In summarizing this case, Dr. Chabot-Richards said, "We always suspect CMML in an adult with monocytosis." The accurate blast count is key for CMML classification, and bone marrow biopsy is required for final classification. Gene mutations are helpful but not sufficient in the absence of other criteria.

Amy Carpenter Aquino is CAP TODAY senior editor. Part one was published in the March issue of CAP TODAY.