Anemia

Evaluation and Diagnostic Tests

Michael J. Cascio, MD[a], Thomas G. DeLoughery, MD, MACP, FAWM[b,c,*]

KEYWORDS

• Red cell indices • Schistocytes • Microcytic • Macrocytic • Cytogenetics • Anemia • Diagnostic testing

KEY POINTS

- Both the red cell indices and blood smear can offer clues to diagnosis and help to guide laboratory testing.
- Classification of anemia by either size of the red cell or mechanism (decreased production or increased loss) can narrow down the differential diagnosis.
- New molecular technologies may offer improved diagnostic sensitivity and specificity.

ANEMIA: DEFINITION

Although anemia is common, the exact cutoff to establish a diagnosis can be elusive. The standard definition is population-based and varies by gender and race. Current hemoglobin cutoff recommendations range from 13 to 14.2 g/dL in men and 11.6 to 12.3 g/dL in women.[1] Data from large population studies suggests that hemoglobin levels for African Americans tend to be 0.8 to 0.7 g/dL lower, perhaps owing to the high frequency of alpha-thalassemia in this population.[2] Another important factor is the trend of hemoglobin. For example, a patient with previous hemoglobin values at the higher end of the normal range, who now presents with a hemoglobin concentration at the lower end of the normal range, can now be considered anemic.

SYMPTOMS AND SIGNS OF ANEMIA

In general, the signs and symptoms of anemia are unreliable in predicting the degree of anemia. Several factors determine the symptomatology of anemia, with time of

The authors report no conflict of interest.

[a] Department of Pathology, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, MC L-471, Portland, OR, 97239, USA; [b] Division of Hematology/Medical Oncology, Department of Medicine, Knight Cancer Institute, Oregon Health and Science University, MC L586, 3181 Southwest Sam Jackson Park Road, Portland, OR 97239, USA; [c] Division of Laboratory Medicine, Department of Pathology, Oregon Health and Science University, Hematology L586, 3181 Southwest Sam Jackson Park Road, Portland, OR 97201-3098, USA
* Corresponding author. Division of Hematology/Medical Oncology, Department of Medicine, Knight Cancer Institute, Oregon Health and Science University, MC L586, 3181 Southwest Sam Jackson Park Road, Portland, OR 97239, USA.
E-mail address: delought@ohsu.edu

Med Clin N Am 101 (2017) 263–284
http://dx.doi.org/10.1016/j.mcna.2016.09.003 **medical.theclinics.com**

onset and overall baseline health of the patient being the most important. Patients who gradually develop anemia over a period of months can tolerate lower hemoglobin owing to the use of compensatory mechanisms. An example would be a patient with sickle cell disease who can tolerate a chronic hemoglobin concentration of 7 g/dL. Because blood delivers oxygen, many of the signs are related to lack of oxygen delivery, chiefly, fatigue and shortness of breath. On physical examination, anemia is manifested by paleness of the mucous membranes and resting tachycardia. One should look for other physical examination clues to a possible source of anemia, such as splenomegaly, guaiac-positive stools, or oral telangiectasia.

COMPENSATION FOR ANEMIA

There are 3 physiologic compensatory mechanisms for anemia. The first is by increasing cardiac output. Because oxygen delivery is cardiac output times hemoglobin, patients with decreased hemoglobin can maintain the same level of oxygen delivery by increasing cardiac output. Therefore, patients with limited cardiac reserve (heart failure, coronary artery disease) will have symptoms at higher hemoglobin concentrations than those with normal cardiac function. Increasing plasma volume is the second compensatory mechanism. This allows the remaining red cells to move around more efficiently owing to decreased viscosity. The increased plasma volume also increases cardiac output and helps to maintain blood pressure. Finally, red cell 2,3-diphosphoglycerate increases, which decreases oxygen affinity for hemoglobin. This results in more oxygen delivery to tissues. The high ambient oxygen tension in the alveoli leads to full oxygenation of hemoglobin despite its decreased oxygen affinity, but at the tissue level this results in more delivery of oxygen.

CLASSIFICATION

There are 2 classification systems for anemia (**Box 1**). The first is based on Wintrobe observations that red cell size can differentiate potential etiologies of anemia. This led to the concepts of "microcytic," "normocytic," and "macrocytic" anemia.[3] Microcytic anemias are those with a mean corpuscular volume (MCV) less than normal (<80 fL). Microcytic anemias reflect defects in hemoglobin synthesis. Lack of iron, either owing to deficiency or sequestration (anemia of inflammation), thalassemia, or sideroblastic anemias (defect of heme synthesis) all can lead to microcytosis.

There are 2 general etiologies of macrocytic anemias (MCV > 100 fL)[4]—red cell membrane defects and DNA synthesis defects. Red cell membrane defects can occur in the setting of liver disease or hypothyroidism. Macrocytic red blood cells (RBCs) in this setting tend to be round on review of the peripheral smear. In contrast, defects in DNA synthesis (such as those seen with megaloblastic anemia or chemotherapy) show a prominent oval macrocytosis. One of the most common causes of macrocytic anemia is the presence of a reticulocytosis. The average size of the reticulocyte (160 fL) can yield an high MCV in the setting of hemolysis.

The difficulty in using red cell size as a means of distinguishing potential etiologies for anemia is that, in many cases, the red cells demonstrate a normal size ("normocytic anemia," MCV 80–100 fL). This may occur during early stages of a process (such as iron deficiency) or when multiple processes occur simultaneously (concurrent iron deficiency and liver disease) and lead to a red cell size within the normal range.

The other classification schema uses the underlying mechanism of anemia (increase in RBC loss or decrease in RBC production). The first branch point is if red cell

Box 1
Classification of anemia

Size

- Microcytic
  - Iron deficiency
  - Thalassemia
  - Sideroblastic anemia
  - Anemia of inflammation
- Macrocytic
  - Round
    - Aplastic anemia
    - Hypothyroidism
    - Liver disease
    - Renal disease
    - Reticulocytosis
    - Thyroid disease
  - Oval
    - Vitamin B_{12} and folate deficiency
    - Chemotherapy
    - Myelodysplastic syndrome
- Normocytic
  - Anemia of inflammation
  - Acute onset hemolysis or blood loss
  - Renal disease

Mechanism

- Increased loss
  - Hemorrhage
  - Hemolysis (immune, microangiopathic, intrinsic red blood cell defects)
- Decreased production
  - Stem cell (myelodysplastic syndrome, acute leukemia)
  - Nutritional (iron, vitamin B_{12}, folate, copper)
  - Toxin/drug
  - Lack of growth factors (renal disease, anemia of chronic disease)
  - Myelophthisic process (metastatic cancer, infection, fibrosis)

production is increased or decreased as determined by the reticulocyte count. If increased, then hemolysis and blood loss are primary considerations. If red cell production seems to be decreased, then basic causes of impaired marrow production should be considered:

- Nutritional: iron, vitamin B_{12}, folate, copper deficiency
- Marrow failure: aplastic anemia, pure red cell aplasia, myelodysplasia, leukemia
- Lack of growth factors: lack of erythropoietin (EPO) owing to chronic renal disease
- Myelophthisic process: cancer, infection

DIAGNOSTIC TESTS

Diagnostic testing should focus on (1) determining whether an anemia is present and (2) identifying the underlying etiology. Basic assays (complete blood count, reticulocyte count, and blood smear) will be reviewed first, with more specific assays covered elsewhere in this article.

Complete Blood Count

The complete blood count is probably one of the most widely performed tests in the world. The complete blood count was originally performed by manual methods, but currently there are 2 main forms of automated technology that are used. Each automated method directly measures the number of cells, the volume of individual cells, and the hemoglobin concentration.

The first automated method was devised by Wallace Coulter in the 1950s and relies on electrical impedance. When RBCs flow through an aperture in a current-conducting solution, the nonconducting RBCs induce a momentary alteration in current impedance between the sensing electrodes. Each impedance pulse correlates with an individual RBC passing through an aperture, whereas the magnitude of the impulse is directly proportional to cell size.

A newer method uses laser light scatter properties. In this assay, RBCs are hydrodynamically focused in a flow cell and a beam of laser light is applied. A photodetector then captures the light scatter emitted from each cell. The photodetector then converts this light scatter to electrical impulses, the number of which is proportional to the number of RBCs passing through the laser. Similar to the impedance method, the amplitude of the light scatter pulse is proportional to RBC size.

Hemoglobin concentration is determined by photospectometry after erythrocyte lysis. Although red cell number, size, and hemoglobin concentration are directly measured, the hematocrit is a calculated value, and can be less reliable when there is inaccuracy in the measurements of the other red cell indices.

Peripheral Blood Smear Assessment

The blood smear can offer valuable diagnostic clues to the etiology of anemia (**Table 1**). The clinician can request a formal blood smear review by the laboratory technologist or pathologist to assess red cell, white cell, and platelet morphology. When initiating such requests, it is important to convey relevant clinical and laboratory findings that may not be available to the pathologist.

Assessment of red cell size is an important part of the peripheral smear evaluation. Normocytic red cells are approximately the same size as the nucleus of a small resting lymphocyte (**Fig. 1**). Red cells that are larger than the lymphocyte nucleus are considered macrocytic and those that are smaller are microcytic. As noted, when a macrocytic anemia is present, it is important to distinguish round and oval macrocytosis owing to differing underlying etiologies.

Microcytosis may be seen in the setting of iron deficiency, hemoglobin H disease, thalassemia minor, sideroblastic anemias, and in some cases of anemia of chronic disease. When evaluating a microcytosis, assessment for hypochromia can be helpful in determining whether the changes may be owing to or in part reflect iron deficiency. In normal red cells, central pallor occupies approximately one-third of the RBC diameter. An increase in the central pallor is indicative of hypochromia, which generally occurs at hemoglobin concentrations less than 10 g/dL and the degree of which correlates with the severity of the anemia (**Figs. 2** and **3**). The differential diagnosis for microcytic hypochromic anemia includes thalassemia, iron deficiency, sideroblastic anemias, and anemia of chronic disease.

Anisocytosis and poikilocytosis refer to the variation of red cell size and shape, respectively. Although a mild increase in anisopoikilocytosis lacks diagnostic specificity, it is important to note whether the RBC abnormality observed is a high frequency or low frequency abnormality. High frequency abnormalities, as the name suggests, are RBC types that occur more often in normal blood films. Examples include

Table 1
RBC morphologic findings

RBC Finding	Mechanism	Clinical Setting
Coarse basophilic stippling	Remnant of ribosomes	Lead poisoning, marrow stress, thalassemia
Bite cells	Hemoglobin denaturation and removal by RES	Oxidative hemolytic anemia
Burr cells	Membrane alteration related to increased lipids, calcium	Artifact, liver disease, uremia, hyperlipidemia
Acanthocytes	Increased cholesterol in RBC membrane	Liver disease, abetalipoproteinemia, McLeod syndrome
Elliptocytes	RBC cytoskeletal defects	Heredity elliptocytosis, iron deficiency
Howell-Jolly bodies	RNA remnants	Splenectomy or functional hyposplenism
Nucleated red cells	Retention of nucleus	Splenectomy, marrow stress, myelopthisis
Schistocytes	Mechanical RBC damage	Microangiopathic hemolytic anemia
Reticulocytosis (shift cells)	High RNA content	Hemolysis, marrow stress
Spherocytosis	Loss of RBC membrane, cytoskeletal defect	Hereditary spherocytosis, autoimmune hemolytic anemia
Target cells	Excess RBC membrane cholesterol, decreased hemoglobin	Thalassemia, hemoglobin C and E disease, obstructive liver disease
Tear drop cells	Extension of RBC cytoplasm	Myelopthisis, fibrosis, extramedullary hematopoiesis

Abbreviations: RBC, red blood cells; RES, reticuloendothelial system.

acanthocytes, echinocytes, target cells, and ovalocytes. In contrast, low frequency abnormalities are rare or seldom seen in blood of healthy individuals. These include schistocytes, tear drop cells, and blister cells, among others. The severity of the poikilocytosis can be estimated based on the relative percent of each abnormal RBC type observed, which are commonly reported by the laboratory in a tiered grading scheme.

Schistocytes, or helmet cells, are red cell fragments that contain 2 or 3 pointed ends (**Fig. 4**). The presence of an increased number of schistocytes implies intravascular damage to red cells. The mechanism of schistocyte formation involves mechanical shearing of the red cell membrane by intravascular deposits, such as fibrin strands or platelet aggregates. Schistocytes can be increased in the setting of disseminated intravascular coagulation, thrombotic thrombocytopenia purpura, hypertension, preeclampsia, mechanical heart valves, and a ventricular assist device.

Spherocytes appear as round, slightly smaller red cells that lack central pallor and have a more deeply red appearance than other red cells. Spherocytes are formed through loss of red cell membrane; this converts the biconcave disk (most surface area for a given volume) to a sphere, which is the least amount of surface area. An increased number of spherocytes is seen in hereditary spherocytosis, hemolytic anemias mediated by immune mechanisms, and in microangiopathic hemolytic anemias (see **Fig. 4**).

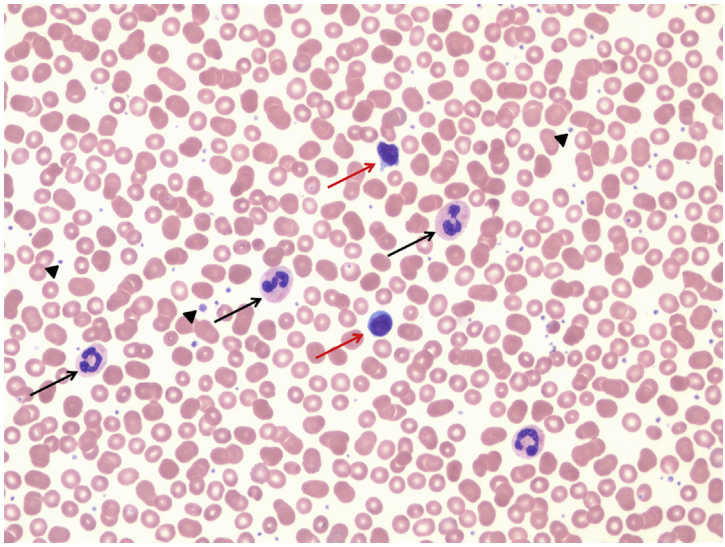

Fig. 1. Normal peripheral blood smear (original magnification, ×400). Red blood cells (RBCs) show a normal central pallor (less than one-third of the diameter of the RBC) and have a size that is roughly equal to the size of the nucleus of a small lymphocyte (*red arrows*). Normal neutrophils (*black arrows*) with segmented nuclei and pink cytoplasm are shown. Platelets with normal size and blue-purple alpha granules are easily identified in the background (*black arrowheads*).

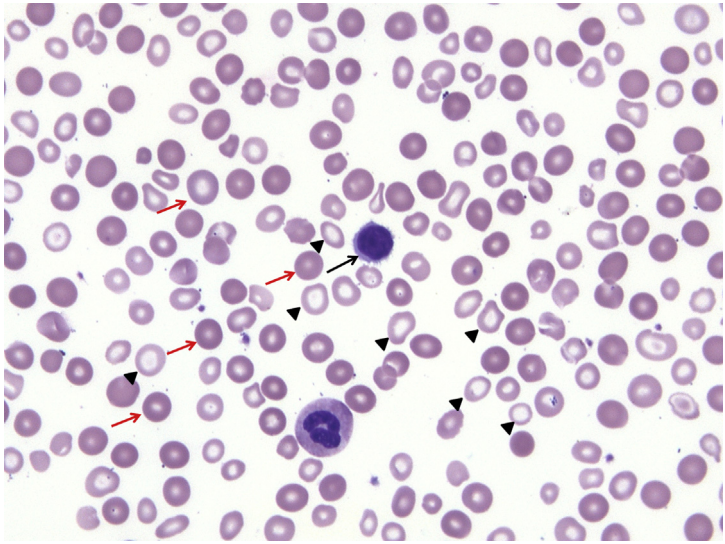

Fig. 2. Microcytic hypochromic anemia (original magnification, ×630). This peripheral blood film was from a patient with iron deficiency anemia. Note the "dimorphic" red cell populations. One population (*black arrowheads*) seems to be smaller than the nucleus of a small lymphocyte (*black arrow*) and shows an increase in central pallor. The second population shows a more normal size and lacks central pallor (less than one-third of the red blood cell diameter, *red arrows*).

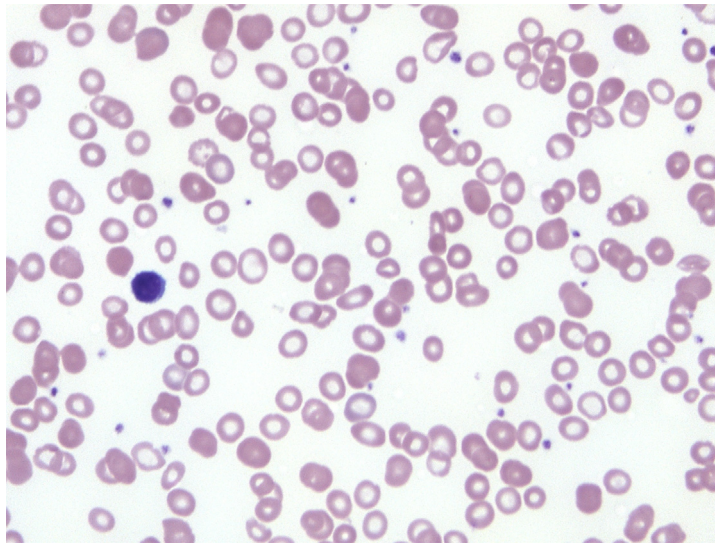

Fig. 3. Hypochromic microcytic anemia (original magnification, ×630). In contrast with Fig. 2, the red blood cells (RBCs) on this smear show a relatively uniform appearance, with "normal appearing" RBCs absent.

Elliptocytes (ovalocytes) are oval to elongated rodlike red cells. A uniform increase in elliptocytes is mainly seen in the setting of hereditary elliptocytosis, which is caused by abnormalities in red cell membrane or cytoskeletal proteins, alpha-spectrin (*SPTA1*), beta-spectrin (*SPTB*), or protein 4.1R[5] (Fig. 5). In iron deficiency, elliptocytes

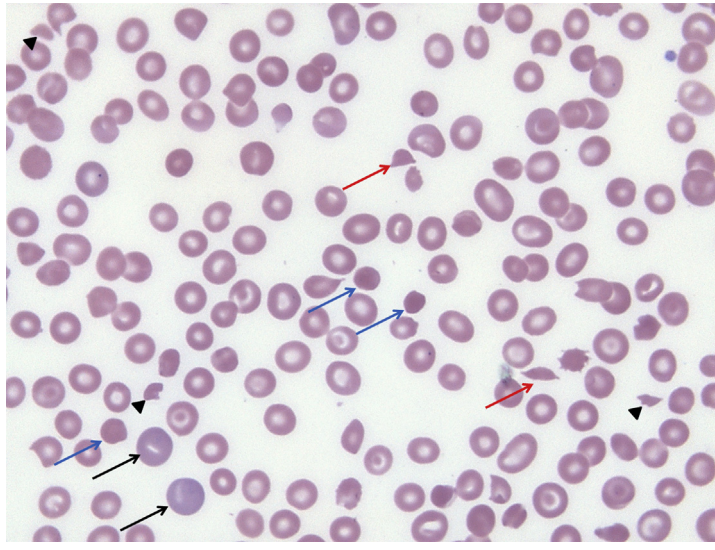

Fig. 4. Microangiopathic hemolytic anemias are characterized by an increase in spherocytes (*blue arrows*), schistocytes (*red arrows*), nonspecific red cell fragments (*black arrowheads*), and polychromatophilic cells (*black arrows*) (original magnification, ×630).

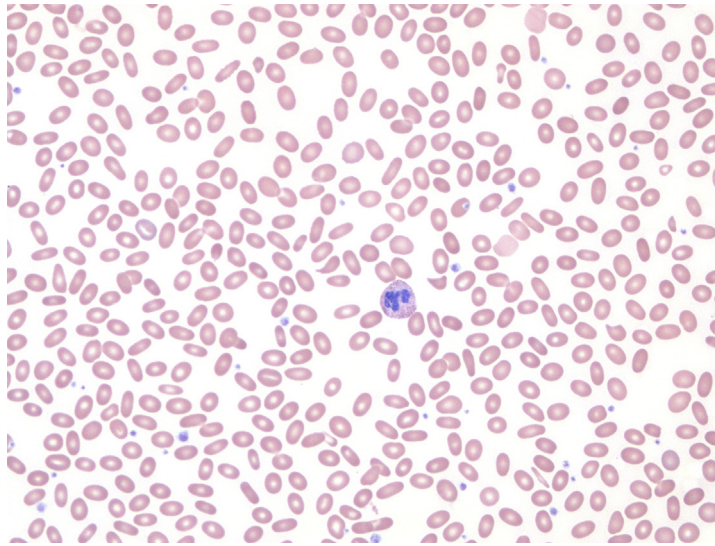

Fig. 5. Elliptocytes are increased in a variety of conditions. Here, a relatively uniform population of elliptocytes is noted incidentally on peripheral smear review during a bleeding diathesis work up. The patient had a positive family history of hereditary elliptocytosis (original magnification, ×400).

are microcytic and more elongate and are termed "pencil" cells. A dramatic increase in anisopoikilocytosis with hypochromic microcytes can help to support a diagnosis of iron deficiency. Ovalocytes can be seen in the setting of hereditary conditions such as southeast Asian ovalocytosis or in acquired cases, such as myelodysplastic syndrome (MDS). Southeast Asian ovalocytosis results from mutations in *SLC4A1* and occurs in a geographic distribution that parallels that of malaria endemic regions. The presence of this mutation confers resistance to *Plasmodium falciparum* and *Plasmodium vivax*.[5]

Drepanocytes (sickle cells) are bipolar red cells with characteristic points at each end (**Fig. 6**). These cells are characteristic of the sickling syndromes, such as Sickle cell disease or trait and hemoglobin SC disease. Quantitative assessment of sickle cells on peripheral smear review is of dubious clinical value, with no correlation between the degree of disease severity and the number of drepanocytes observed.

Target cells are formed owing to excess red cell membrane (**Fig. 7**). On blood smear review, they have a characteristic "bull's eye" appearance owing to hemoglobin occupying the central portion of the cell. Target cells occur when there is excess red cell membrane compared with hemoglobin level. In liver disease, target cell formation is owing to altered lipid metabolism, whereas in the setting of setting of thalassemia, the abnormality is owing to lack of hemoglobin production. Clinical findings, MCV, and red cell count can help to differentiate between the two.

Teardrop cells (dacrocytes) have an extended cytoplasmic projection resembling a tail. Increased numbers of tear drop cells are most often seen in the setting of marrow myelophthisis (such as metastatic carcinoma), marrow fibrosis, and extramedullary hematopoiesis, but also can be found in cases of severe iron deficiency.

Spur cells (acanthocytes) have a few, irregularly spaced and variably sized projections on their surface (**Fig. 8**). Additionally, the central pallor of the RBC is absent and the red cell has a "contracted" or dense appearance. Acanthocyte formation is related

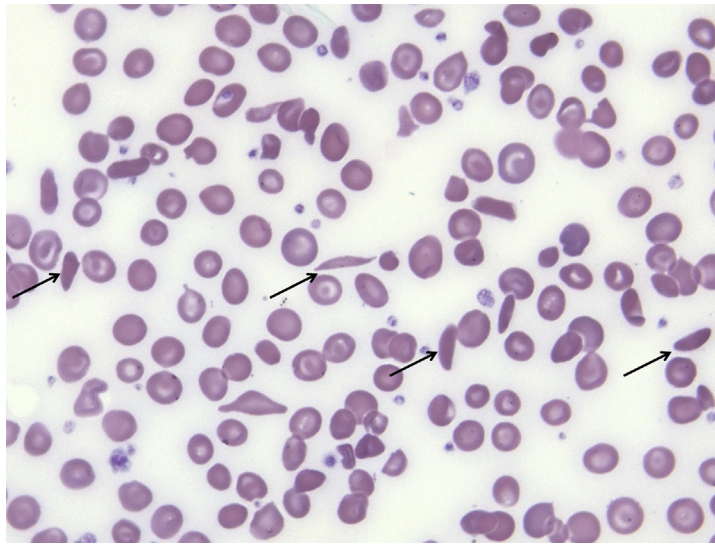

Fig. 6. Drepanocytes (sickle cells) are denoted by black arrows (original magnification, ×630).

to defects in the lipid or protein composition in the red cell membrane, most commonly seen in the setting of severe liver disease. Other less common causes include abetalipoproteinemia, malnutrition, McLeod phenotype (lack of Kx antigen), and neuroacanthocytosis syndrome.

Burr cells (echinocytes) are red cells with short, evenly spaced, uniform projections and preserved central pallor (see **Fig. 8**). Probably the most common cause of Burr

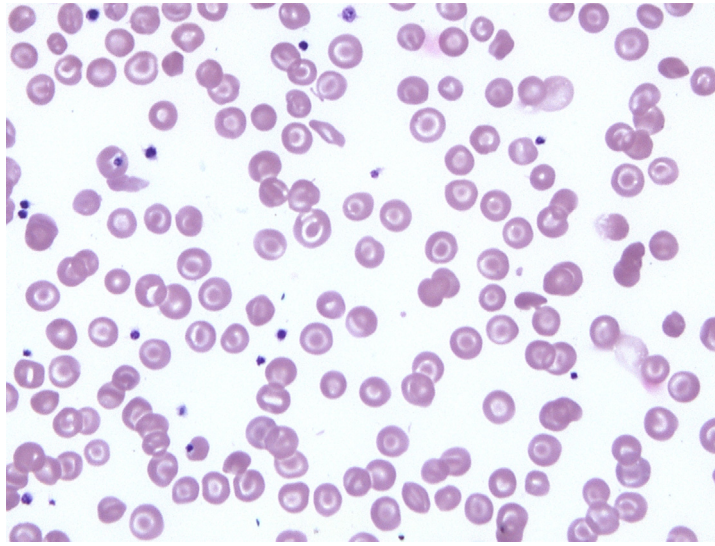

Fig. 7. Innumerable target cells are present in this patient with hemoglobin C disease (original magnification, ×630).

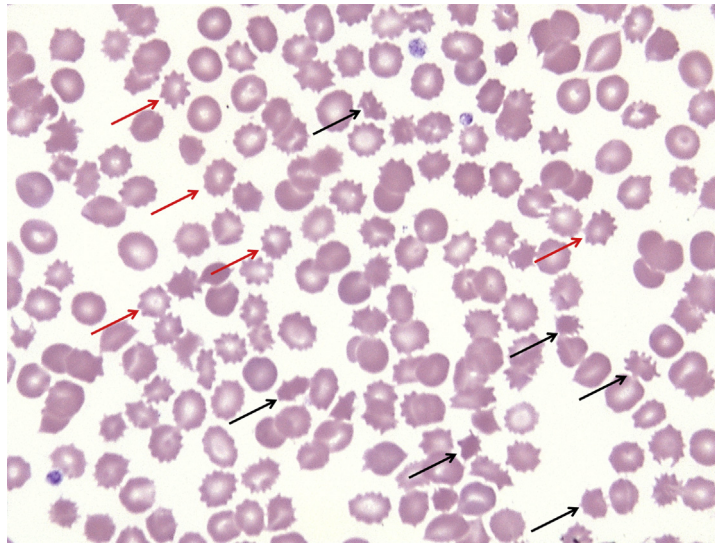

Fig. 8. Acanthocytes (*black arrows*) and Burr cells (*red arrows*) in a patient with end-stage liver disease owing to hepatitis C virus infection (original magnification, ×630). Note the irregularly spaced, "sharp" projections and loss of central of the acanthocyte versus the more "blunt" and regularly distributed projections and retained central pallor of the Burr cell.

cells is artifact related to prolonged storage before smear preparation. Pathologically, Burr cells are seen in end-stage kidney disease (uremia) and obstructive liver disease.

Bite cells (degmacytes) have semicircular defects in the edge of the red cell membrane that resembles a "bite." During oxidative stress, hemoglobin precipitates out of solution to form insoluble aggregates (Heinz bodies) that are then removed by the reticuloendothelial system of the spleen and liver. Bite cells and blister cells are seen in the setting of glucose-6-phosphate dehydrogenase deficiency and drug-induced oxidant hemolysis (Fig. 9).

When evaluating for a macrocytic anemia, the recognition of polychromatophilic red cells can be an important finding in determining the cause of red cell macrocytosis. Polychromatophilic cells are macrocytic, lack central pallor, and have a blue-gray appearance (Fig. 10). Their appearance is a function of their immaturity, with their distinctive color attributable to the presence of residual polyribosomes not found in mature RBCs. An increase in polychromatophilic cells is seen under conditions of marrow stress or hemolysis and can be a useful marker of a raised reticulocyte count. Identification of reticulocytes, and thus, the calculation of the reticulocyte count, requires special staining protocols with fluorescent or supravital dyes (ie, methylene blue).

Nucleated red cells (nRBCs) are not normally present in the peripheral blood (see Fig. 10). nRBCs can be seen in several situations and usually indicate the presence of severe hemolysis, hemoglobinopathies, a myelophthisic process, or severe physiologic stress. Because the spleen normally functions to remove nRBCs from the circulation, postsplenectomy patients will demonstrate an increased number of nRBCs in addition to significant anisopoikilocytosis and Howell-Jolly body formation.

Rouleaux is manifest in the thin part of the smear by the stacking of greater than 3 red cells, resembling a "stack of coins" (Fig. 11). This is an in vitro phenomenon related

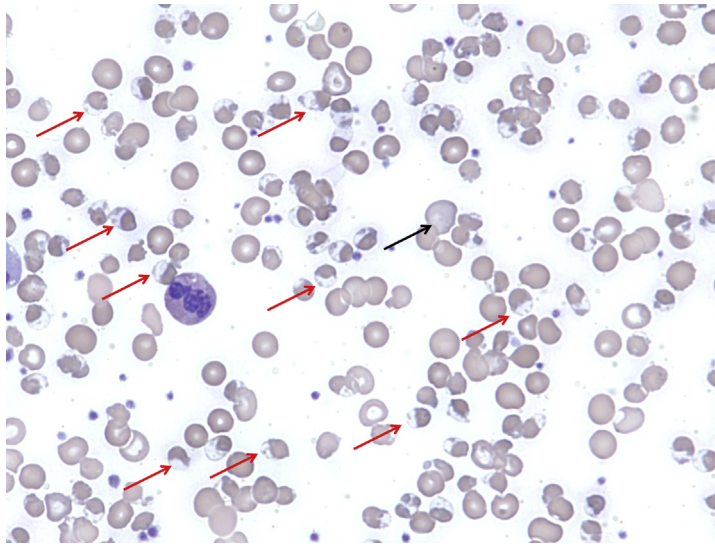

Fig. 9. Blister cells are seen in settings of drug or medication induced oxidative injury, usually in patients with glucose-6-phosphate-dehydrogenase deficiency. Note the presence of numerous "blister" cells (*red arrows*), as characterized by the puddling of hemoglobin at 1 aspect of the cell with the other aspect having a "cleared-out" look surrounded by a thin rim of red membrane (original magnification, ×600). A few polychromatophilic cells are present (*black arrow*).

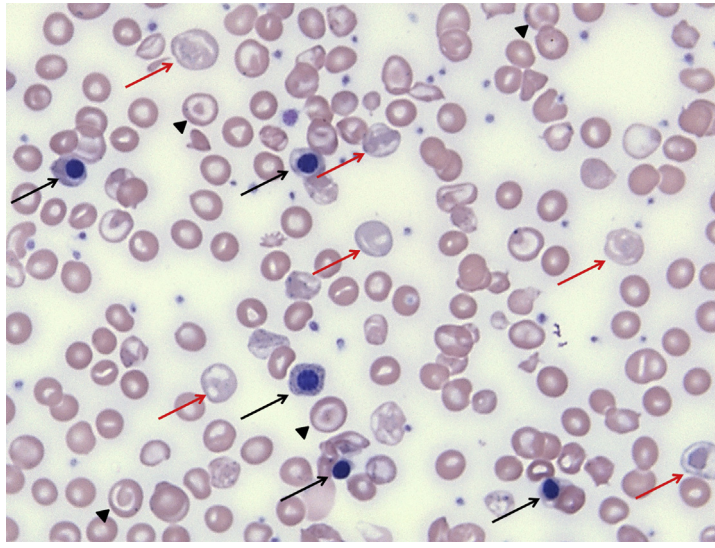

Fig. 10. This peripheral blood smear demonstrates numerous nucleated red blood cells (*black arrows*), polychromatophilic cells (*red arrows*), and target cells (*black arrowheads*; original magnification, ×630). The patient had beta thalassemia/hemoglobin E disease.

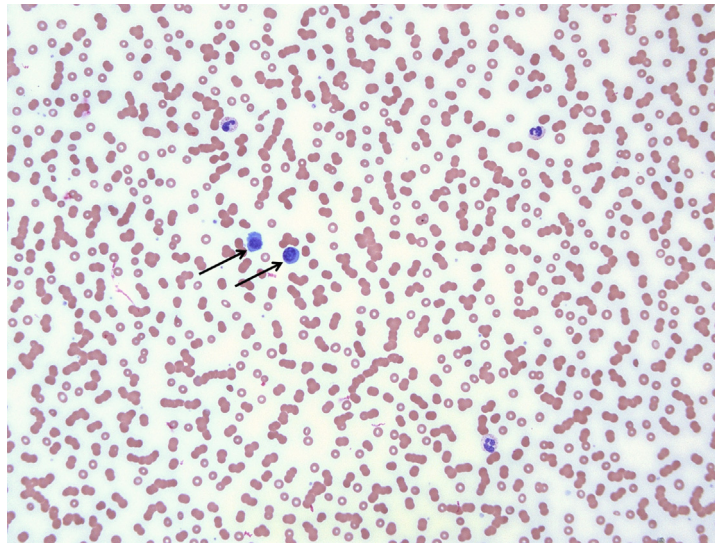

Fig. 11. Rouleaux is an in vitro phenomenon characterized by stacking of greater than 3 red blood cells and is mainly seen in the setting of increased serum protein levels, typically in patients with a severe infectious/inflammatory process or a monoclonal protein spike. This peripheral blood smear demonstrates an increase in Rouleaux formation in a patient with plasma cell myeloma (original magnification, ×50). Rare circulating plasma cells are identified (*black arrows*).

to an high protein concentration, which functions to disrupt the zeta potential on the red cell surface that normally causes the RBCs to repel one another. Rouleaux can occur in the setting of increased acute phase reactant proteins (ie, fibrinogen) in inflammatory states or in the presence of a monoclonal paraprotein.

Autoagglutination manifests as rounded aggregates or clumps of RBCs that form owing to the presence of an antibody. This in vitro phenomenon occurs most commonly in the setting of an immunoglobulin (Ig)M autoantibody and is referred to as "cold agglutinins." Although many cold agglutinins may not have clinical significance, from a laboratory perspective, their presence may interfere with the ability of the automated analyzer to accurately determine RBC count or MCV.

Howell-Jolly bodies are single small, dense basophilic inclusions located at the periphery of the red cell. Howell-Jolly bodies represent nuclear remnants (DNA). Because Howell-Jolly bodies are usually removed by the spleen, their presence indicates a hyposplenic state (anatomic or functional).

Coarse basophilic stippling manifests as punctate basophilic inclusions in the red cell. These inclusions are composed of precipitated ribosomes and RNA and are indicative of impaired hemoglobin synthesis. Although classically associated with lead or other heavy metal poisoning, in clinical practice it is more commonly seen in conditions with increased red cell turnover and altered hemoglobin synthesis, especially hemoglobinopathies, dyserythropoietic states, or any process that leads to marrow stress. Fine basophilic stippling is observed in polychromatophilic cells and is a clinically insignificant artifact related to an air-drying artifact of the peripheral smear.

Reticulocyte Count

The reticulocyte count is a measure of the production of new red cells by the marrow. Because the average lifespan of a red cell is 120 days, approximately 1% of red cells

will be removed from the circulation each day. To maintain steady state, the marrow needs to constantly produce new RBCs. Reticulocytes are immature, nonnucleated RBCs that circulate in the peripheral blood for 1 day before losing their RNA and becoming mature RBCs.

The traditional method of measuring the reticulocyte count is a manual method that uses supravital stains (such as methylene blue) to highlight the reticulum (RNA) network of this immature red cell fraction. The technologist then enumerates the number of reticulocytes and expresses this value as a percent of total RBCs. The main difficulty with this test is that it depends on the hematocrit. Thus, a reticulocyte count of 1% in a patient with a hematocrit of 45 would "increase" to 2% if the hematocrit decreased to 22.5. Therefore, the raw number needs to be corrected for the hematocrit by multiplying the value by patient's hematocrit divided by 45 (corrected reticulocyte count = retic % [Hct%/45]). So, in the above example, the corrected reticulocyte count would be 2% times (22.5/45) equals 1%.

The number of reticulocytes can be measured directly by most automated analyzers by staining the remnant RNA with a fluorescent dye. This is known as the absolute reticulocyte count. Advantages of automated methods include improved turnaround time, measurement precision, and lack of need to adjust for hematocrit.

The usefulness of the reticulocyte count is in assessing the marrow response to anemia. If the reticulocyte count is elevated, then either blood loss or hemolysis is suggested. "Normal" reticulocyte counts are indicative of production causes of anemia. Very low reticulocyte counts (<0.1% or 10,000/μL) are seen in aplastic anemia or pure red cell aplasia.

RED CELL INDICES: OLD AND NEW

Originally, 3 of the red cell indices were derived or calculated (**Table 2**): (1) MCV, (2) mean corpuscular hemoglobin concentration (MCHC), and (3) mean corpuscular hemoglobin (MCH). On most new analyzers MCV, hemoglobin, and red cell count are directly measured and MCHC and MCH are calculated. Because the MCHC and MCH tend to trend with the MCV, these indices are rarely used anymore.

With more widespread use of new automated complete blood count analyzers, there are now a wider array of newer "indices" available.[6] With the ability to rapidly identify reticulocytes these machines can determine both reticulocyte MCV and reticulocyte MCHC, and from this derive an MCH (CHr/Ret-He). Most studies of these newer indices have been in patients receiving EPO who develop "functional" iron deficiency where the delivery of iron to the developing red cell cannot keep up with demand. The CHr/Ret-He falls with the onset of iron deficiency and is the first to increase with iron supplementation. However, the CHr/Ret-He also can be reduced in thalassemia; therefore, hemoglobinopathies should be excluded before it is used to assess iron status.

Table 2
RBC indices

Index	Normal Values	Comment
MCV	80–100 fL	RBC size
Mean corpuscular hemoglobin concentration	33.8–34.2 m/dL	Increased in spherocytosis
Mean corpuscular hemoglobin	28.5–32.3 pg	Trends with MCV

Abbreviations: MCV, mean corpuscular volume; RBC, red blood cell.

BONE MARROW EXAMINATION

For some causes of anemia a bone marrow evaluation is required. A common issue that arises is determining when a bone marrow examination should be performed in the evaluation of anemia. In general, a bone marrow examination should be considered if:

- Circulating immature cells are identified on peripheral smear (blasts, etc);
- Severe pancytopenia (decreased numbers of all cell types);
- Very low reticulocyte counts (<0.1%);
- Circulating nucleated RBCs;
- Evidence of marrow infiltration (leukoerythroblastic blood picture);
- Evaluation of a monoclonal paraproteins; and
- Unexplained severe anemia, especially if there is suspicion of myelodysplasia.

The aspirate part of the procedure involves withdrawing the liquid marrow. From the aspirate, smears are made for morphologic review (Fig. 12) and material can be sent for flow cytometry, cytogenetics (karyotyping and fluorescence in situ hybridization studies), molecular diagnostics, and microbiologic studies. A core biopsy should also be obtained for accurate assessment of marrow cellularity (Fig. 13). The core biopsy also provides the opportunity to identify disease processes that may show patchy involvement of the marrow space, such as in the case of some lymphomas and granulomatous infectious/inflammatory infiltrates.

CYTOGENETICS AND MOLECULAR TESTS

Cytogenetic and molecular studies are a useful adjunct in the workup of a patient with unexplained anemia. To achieve optimal results, good quality aspirate material should

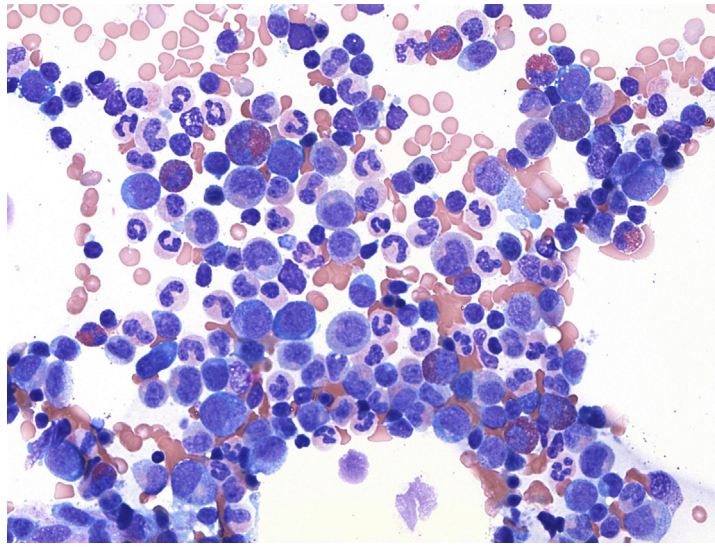

Fig. 12. Normal bone marrow aspirate (original magnification, ×400). This aspirate shows the presence of a mixture of maturing granulocytic and erythroid precursors. Well prepared aspirate smears with adequate spicules should be obtained when considering a diagnosis of myelodysplastic syndrome.

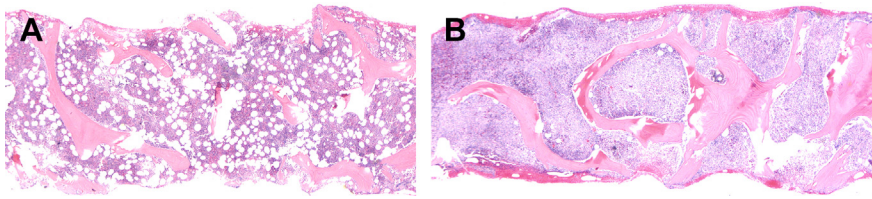

Fig. 13. (*A*) Normal bone marrow core biopsy (stain: hematoxylin and eosin; original magnification, ×25). The normal adult marrow shows a cellularity of 30% to 70%, which is estimated by comparing the proportion of cells to adipocytes. The core biopsy is important for determining marrow cellularity and the presence of any focal infiltrates such as granulomas, lymphoma, or metastatic cancer. (*B*) Bone marrow core biopsy demonstrating cellularity of greater than 95% in a patient with myelodysplastic syndrome (stain: hematoxylin and eosin; original magnification, ×25).

be obtained and with minimal peripheral blood hemodilution. Additionally, the treating clinician should communicate diagnostic considerations to the pathologist or the laboratory for optimal triage of the specimen and selection of appropriate studies.

Classical cytogenetic studies involve the culture of cells obtained from marrow aspirates and subsequent assessment of the metaphase karyotype. In most instances, routine karyotyping is complemented by the use of fluorescence in situ hybridization (FISH). Fluorescent probes are designed to label certain genes or chromosome regions. Depending on probe design, FISH can be used to detect chromosomal deletions, duplications, rearrangements, and translocations. After hybridizing the FISH probe(s) to interphase cells, up to 200 nuclei are observed by a technologist and the presence of any abnormalities are noted and scored as a percent of all nuclei. For malignancies such as plasma cell myeloma, acute myeloid leukemia, and B-lymphoblastic leukemia, the use of FISH has allowed for improved detection of abnormalities that are not commonly seen in karyotypic analysis, allowing for better risk stratification and optimal therapeutic management.

Increasingly, molecular genetic studies are being routinely used in the clinical setting. Sequencing technologies such as allele-specific oligonucleotide polymerase chain reaction or reverse transcriptase polymerase chain reaction have been used to identify specific recurrent genetic alterations. Common examples include the use of *BCR-ABL* reverse transcriptase polymerase chain reaction to diagnose and/or monitor patients with chronic myeloid leukemia and the use of allele-specific oligonucleotide polymerase chain reaction to detect *KIT* D816V mutations in systemic mastocytosis.

Next-generation sequencing (NGS) is a massively paralleled sequencing platform that allows a multitude of specific genes or even entire exomes to be sequenced and analyzed. Increasingly, laboratories are designing large NGS panels to detect a wide array of gene mutations commonly seen in hematologic malignancies and in solid tumors. Although many NGS panels focus on detecting mutations seen in myeloid and lymphoid malignancies, there is increasing use in the diagnosis of nonneoplastic diseases, such as inherited hemolysis and hemoglobinopathies.

TESTING FOR SPECIFIC CAUSES OF ANEMIA

Renal Disease

The juxtaglomerular apparatus in the afferent arterioles of the kidney produce EPO, the key hormone responsible for inducing the growth and production of erythroid

precursors in the bone marrow (**Box 2**). The anemia of renal disease is owing to lack of EPO. The level of renal function at which patients become anemic can vary. Classically, anemia is not seen until the creatinine clearance is less than 30 mL/min. However, in the setting of concurrent inflammation or advanced age, anemia can be seen even with creatinine clearance in the 60 mL/min range. Certain medications can decrease EPO production, with angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers being the most common.[7]

Anemia of Inflammation

The anemia of inflammation, also known as the anemia of chronic disease, is a diagnosis of exclusion. In anemia of chronic disease, iron stores are adequate, but there is impaired iron delivery to the developing red cell. EPO production is suppressed. Therefore, the finding of an insufficiently increased EPO level in the setting of anemia with adequate iron stores is strongly suggestive of anemia of chronic disease. In the

Box 2
Testing for specific causes of anemia

Renal disease

- Creatine/blood urea nitrogen
- Erythropoietin level

Anemia of chronic disease

- Erythropoietin
- Ferritin

Nutritional deficiencies

- Iron: ferritin
- Vitamin B_{12}: methylmalonic acid
- Folate: homocysteine
- Copper: serum copper, ceruloplasmin

Thalassemia

- Hemoglobin electrophoresis
- DNA sequencing

Sickle cell disease

- Sickle solubility test
- Hemoglobin electrophoresis

Hemolysis (general screening)

- Haptoglobin
- Lactate dehydrogenase
- Indirect bilirubin
- Reticulocyte count

Myeloma

- Serum protein electrophoresis and immunofixation
- Serum free light chain analysis

future measure of hepcidin levels—the molecular mediator of anemia of chronic disease—will offer direct testing.

Endocrine Disease

Several endocrine diseases can lead to anemia with the etiology related to decreased red cell production. Hypogonadism is an important cause of anemia in men. Testosterone sensitizes erythroid precursors to the effects of EPO. This provides the rationale for why postpubertal males have a 10% to 15% greater hemoglobin concentration, hematocrit, and RBC counts than women.[7] Hypothyroidism can lead to a normocytic or macrocytic anemia.

Iron Deficiency

Over time, a negative iron balance owing to blood loss or increased demand will lead to a reduction in total body iron stores in the reticuloendothelial system and the bone marrow. Three stages in this process have been described. The iron depletion stage shows a decrease in iron stores without a decrease in serum iron levels or hemoglobin concentration outside of the normal range. Serum ferritin is low in this stage. The second stage is characterized by the detection of abnormal iron serologies, such as reduced transferrin saturation, increased total iron-binding capacity, and increased zinc protoporphyrin. The third and final stage demonstrates a hemoglobin concentration of less than the lower limit of the normal range.

Although there are a variety of tests to assess iron stores, studies have shown the most efficient test is the serum ferritin. A serum ferritin level greater than 100 ng/mL essentially rules out iron deficiency.[8] Other "classic" tests such as serum iron levels and transferrin saturation, have low predictive value. Increased total iron binding capacity is specific, but not sensitive for early stages of iron deficiency.

Iron assessment can also be performed on bone marrow aspirate material. Adequate bone marrow spicules must be present to assess for storage iron, which is contained in macrophages. Some investigators suggest a minimum of 7 particles be present to establish the absence of storage iron.[8] In addition to assessment of iron stores, evaluation for increased ring sideroblasts (erythroid precursors containing iron particles that encircle greater than two-thirds of the diameter of the nucleus) can also be performed. An increase in ring sideroblasts can signify a number of different pathologies, such as drugs/toxins (alcohol), heavy metal poisoning (lead), and MDSs.

Other Nutritional Deficiencies

Vitamin B_{12} deficiency

Vitamin B_{12} deficiency produces a megaloblastoid macrocytic anemia, which manifests in the peripheral blood with hypersegmented neutrophils and in the marrow with megaloblastoid maturation of granulocytic and erythroid lineages. There is increasing recognition of the difficulty in using serum vitamin B_{12} levels to determine tissue deficiency. Currently, measurement of methylmalonic acid is recommended. Excess methylmalonic acid is produced in the absence of vitamin B_{12} and is more sensitive and specific for vitamin B_{12} deficiency than direct measurements of serum vitamin B_{12}.[4]

Folate deficiency

Similar to vitamin B_{12} deficiency, folate deficiency manifests as a megaloblastoid macrocytic anemia and shows identical morphologic features. Both serum and red cell folate concentration lack diagnostic specificity and, like vitamin B_{12} deficiency, the use of metabolite assays are more accurate.[4] Lack of folate leads to elevation in

serum homocysteine levels. Because vitamin B_{12} deficiency can also lead to elevated homocysteine, methylmalonic acid levels should be incorporated into the workup for folate deficiency.

Copper

Determining copper status is relatively straightforward. Measurement of serum copper and its carrying protein, ceruloplasmin, can aid in determining whether a copper deficiency is present. In most laboratories the ceruloplasmin assay has a faster turnaround time and may be more reflective of copper deficits.[9]

Thalassemia

Thalassemias are inherited genetic disorders that lead to impaired production of hemoglobin. They can range in severity from just a decreased MCV to very severe anemia. Most common types are alpha and beta thalassemia. The hallmark of all thalassemia is a microcytosis owing to decreased production of hemoglobin with a concurrent increase in RBC count. There are many prediction rules based on MCV and red cell count with the most popular being the Mentzer index (MCV/RBC count). A Mentzer index of less than 13 is thought to favor a diagnosis of thalassemia over iron deficiency, but this tends to lack sensitivity and specificity. More complex algorithms have also been derived and improve accuracy.

The diagnosis of beta thalassemia trait is made by hemoglobin electrophoresis. In this assay, hemoglobin is separated by size and charge to assess the presence and quantity of the different hemoglobin species. In beta-thalassemia the levels of a minor hemoglobin component—hemoglobin A2—is increased. In contrast with hemoglobin A, which is composed of 2 alpha chains and 2 beta chains, hemoglobin A2 is composed of 2 alpha chains and 2 delta chains. Increased hemoglobin A2 is a compensatory process owing to decreased production of beta chains.

Alpha thalassemia trait cannot be diagnosis by electrophoresis because there is no increase in hemoglobin A2. Often it is a diagnosis of exclusion in a patient with microcytosis who has normal iron stores and normal hemoglobin electrophoresis. To establish the diagnosis, DNA testing can be performed to determine the presence or absence of the alpha chain gene deletions.

Sickle Cell Disease and Other Hemoglobinopathies

A rapid screening test for sickle cell is the sickle solubility test. In this assay, whole blood is lysed with a reducing agent. If a sickling hemoglobin is present, an insoluble precipitate is produced. Because this is a screening, one cannot differentiate between sickle cell disease, sickle cell trait, or one of the less common sickling hemoglobin species. Definitive diagnosis is made by hemoglobin electrophoresis. Hemoglobin electrophoresis is the standard screening test for hemoglobinopathies. Clues to the presence of hemoglobinopathies are hemolysis, cyanosis, or erythrocytosis. Increasingly, DNA sequencing to find specific mutations is being used to make a precise diagnosis.[10]

Hemolysis

The diagnosis of hemolysis is a 2-step process. The first is to assess for the presence of a hemolytic anemia and the second it to establish the etiology. Screening for hemolysis uses a variety of tests, which often must be interpreted in unison and with knowledge of the patient's medical history.[11]

Haptoglobin is a protein that acts as a scavenger of free hemoglobin, thereby protecting the body from its toxic effects.[12] Low haptoglobin levels are sensitive for

hemolysis, but are not specific, because severe liver disease, transfusions, and regular exercise can lead to low levels. An additional cause of absent haptoglobin levels is ahaptoglobinemia. This is an inherited condition that results in total lack of haptoglobin production. This is a relatively common condition that is found in 1:1000 whites and up 4% of African Americans.[13]

Lactate dehydrogenase is an intracellular enzyme found in abundance in the red cell. Although lactate dehydrogenase is sensitive for hemolysis, an increased level can be found in many other disorders, especially liver disease.

Indirect bilirubin increases as the breakdown of heme overwhelms the liver's ability to form conjugated bilirubin and excrete it into the bile. Liver disease can also increase levels and an increased level of indirect bilirubin can only be interpreted to imply hemolysis in the setting of a normal direct bilirubin level. Outside of fulminate hemolysis it is rare to see an indirect bilirubin greater than 4 mg/dL.

As noted, the reticulocyte count will be increased in hemolysis. Sensitivity may be an issue, because some patients will have normal reticulocyte counts owing to nutritional deficiencies, lack of EPO, or destruction of red cell precursors.[14]

Once the presence of hemolysis is established, the next step is to determine the cause. Acquired causes of hemolysis are most often owing to autoimmune disease, mechanical destruction, toxins, or paroxysmal nocturnal hemoglobinuria. The direct antibody test (Coombs test) helps to determine the presence of an antibody-mediated cause, such as might be seen in the setting of autoimmune causes. Inherited etiologies for hemolysis may involve defects in the RBC membrane/cytoskeletal proteins, hemoglobin, or intracellular enzymes. Often the blood smear provides clues to the presence of membrane or cytoskeletal defects, such as those seen in hereditary spherocytosis or elliptocytosis. Hemoglobin electrophoresis is useful for identifying hemoglobinopathies, whereas specific assays are needed to identify enzymatic defects.

Plasma Cell Myeloma

A rare but important cause of anemia, especially in older patients, is plasma cell myeloma. An high index of suspicion for this possibility should be had when evaluating anemic patients with back pain or renal disease. Serum protein electrophoresis with immunofixation and serum free light chain analysis should be obtained when evaluating for myeloma. Assessing for light chain disease is important because 10% of myelomas only excrete light chains.

Myelodysplastic Syndrome

MDS is a group of clonal bone marrow failure disorders that result in anemia and varying degrees of leukopenia and thrombocytopenia. MDS occurs in predominantly older patients, but may occur in younger individuals exposed to cytotoxic chemotherapy for childhood malignancies. Laboratory clues for MDS include a macrocytic anemia with nonelevated reticulocyte count (hypoproliferative anemia). Other cytopenias may be present. A morphologic review of the peripheral blood smear may reveal an oval macrocytosis without an increase in polychromatophilic cells. Neutrophils may show dysplastic features including nuclear hypolobation (unilobed or pseudo-Pelger Huet nuclei) or hypogranular cytoplasm (**Fig. 14**). Platelets may also show dyspoeitic features, including the presence of large or giant forms or those with absent granules.

Bone marrow examination is a requisite in the workup of a patient with suspected MDS. Adequate and well-prepared aspirate smears are of paramount importance, because this allows the pathologist to assess for diagnostic dyspoietic changes in the granulocytic, erythroid, and megakaryocytic lineages and enumerate blasts (see **Fig. 13**; **Figs. 15** and **16**). Cytogenetic studies, including FISH for MDS-related

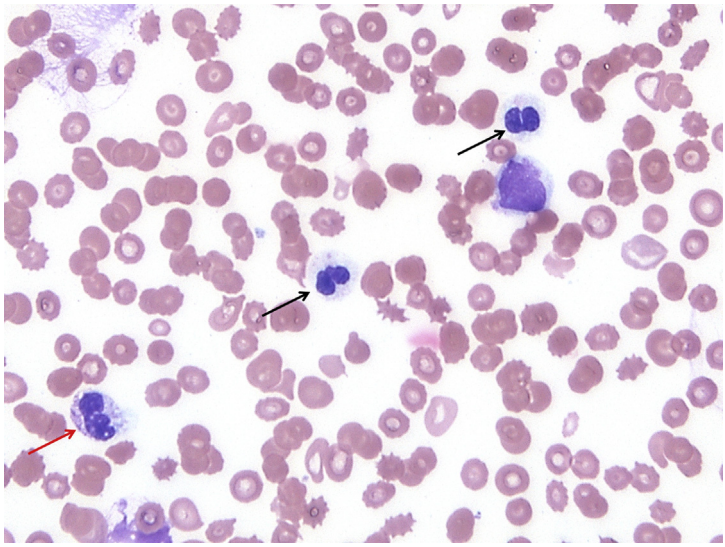

Fig. 14. Dysplastic neutrophils in the setting of acute myeloid leukemia with myelodysplasia-related changes (*black arrows*; original magnification, ×630). These polymorphonuclear leukocytes (PMNs) show bilobed nuclei with "water clear" hypogranular cytoplasm. This is contrast with the pink cytoplasm seen in normal-appearing PMNs (*red arrow*). Identification of such features on peripheral blood film can help to direct the workup and prompt earlier consideration of bone marrow examination.

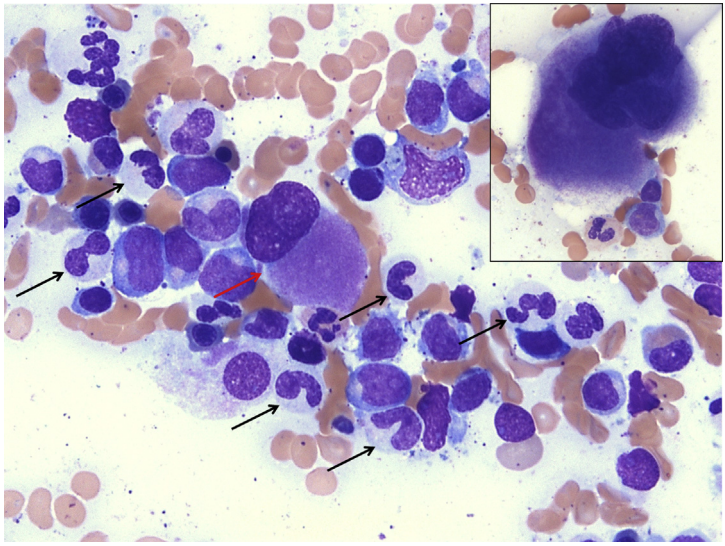

Fig. 15. Multilineage dysplasia in patient with myelodysplastic syndrome (original magnification, ×630). A small megakaryocyte with a hypolobate nucleus (*red arrow*) in a background of granulocytic dysplasia manifest as hypogranular neutrophils (*black arrows*). A normal megakaryocyte is shown for comparison (*inset*; original magnification, ×630).

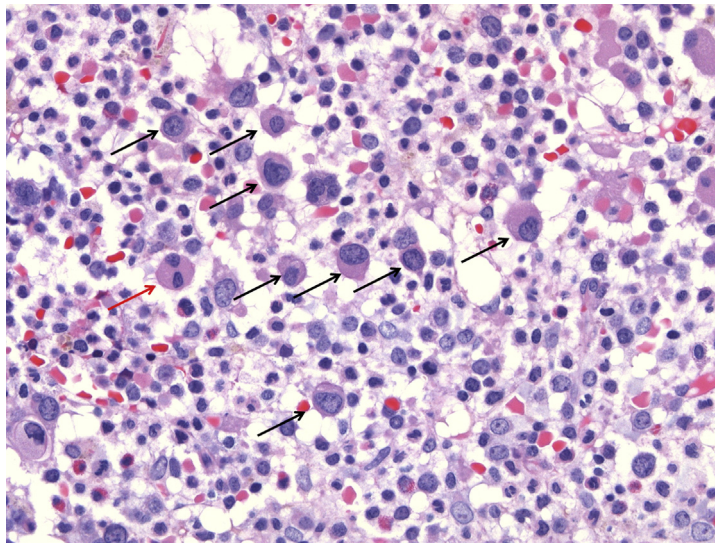

Fig. 16. At greater magnification, megakaryocytes are increased and show prominent dysplastic findings, including small size and hypolobate nuclei (*black arrows*). Rare megakaryocytes also show separated nuclear lobes (*red arrow*), another diagnostic feature of myelodysplasia (stain: hematoxylin and eosin; original magnification, ×630).

abnormalities, are extremely useful for confirming the presence of a clonal disorder, particularly in cases with subtle dyspoietic changes on morphologic examination. NGS panels for genes frequently mutated in myeloid neoplasms are becoming commonly used in routine clinical practice. Similar to cytogenetic studies, pathogenic gene mutations can help to confirm the presence of a clonal disorder and serve as a useful adjunct in cases that show a normal karyotype and lack abnormalities by FISH analysis.

Box 3
Rational approach to anemia

Basic tests

Complete blood count with indices

Reticulocyte count

Ferritin

Methylmalonic acid

Homocysteine

Creatinine/blood urea nitrogen

If older or back pain add

Serum protein electrophoresis with immunofixation

Serum-free light chain analysis

If neurologic disease present add

Copper

A Rational Approach to Anemia

By combining clues from the patient history and initial complete blood count one can plot a rational approach to the anemic patient. The reticulocyte counts should be obtained to determine the presence of hemolysis. Given how common iron deficiency is and the lack of specificity of the MCV for this diagnosis, serum ferritin should be tested in every patient. Further specific testing is guided by the results of these initial studies **Box 3**.

REFERENCES

1. Cappellini MD, Motta I. Anemia in clinical practice-definition and classification: does hemoglobin change with aging? Semin Hematol 2015;52(4):261–9.
2. Beutler E, Waalen J. The definition of anemia: what is the lower limit of normal of the blood hemoglobin concentration? Blood 2006;107(5):1747–50.
3. Brugnara C, Mohandas N. Red cell indices in classification and treatment of anemias: from M.M. Wintrobes's original 1934 classification to the third millennium. Curr Opin Hematol 2013;20(3):222–30.
4. Green R, Dwyre DM. Evaluation of Macrocytic Anemias. Semin Hematol 2015; 52(4):279–86.
5. Da CL, Galimand J, Fenneteau O, et al. Hereditary spherocytosis, elliptocytosis, and other red cell membrane disorders. Blood Rev 2013;27(4):167–78.
6. Piva E, Brugnara C, Spolaore F, et al. Clinical utility of reticulocyte parameters. Clin Lab Med 2015;35(1):133–63.
7. Pratt MC, Lewis-Barned NJ, Walker RJ, et al. Effect of angiotensin converting enzyme inhibitors on erythropoietin concentrations in healthy volunteers. Br J Clin Pharmacol 1992;34(4):363–5.
8. DeLoughery TG. Microcytic anemia. N Engl J Med 2014;371(14):1324–31.
9. Halfdanarson TR, Kumar N, Li CY, et al. Hematological manifestations of copper deficiency: a retrospective review. Eur J Haematol 2008;80(6):523–31.
10. Harteveld CL. State of the art and new developments in molecular diagnostics for hemoglobinopathies in multiethnic societies. Int J Lab Hematol 2014;36(1):1–12.
11. Ruiz EF, Cervantes MA. Diagnostic approach to hemolytic anemias in the adult. Rev Bras Hematol Hemoter 2015;37(6):423–5.
12. Shih AW, McFarlane A, Verhovsek M. Haptoglobin testing in hemolysis: measurement and interpretation. Am J Hematol 2014;89(4):443–7.
13. Delanghe J, Langlois M, De BM. Congenital anhaptoglobinemia versus acquired hypohaptoglobinemia. Blood 1998;91(9):3524.
14. Liesveld JL, Rowe JM, Lichtman MA. Variability of the erythropoietic response in autoimmune hemolytic anemia: analysis of 109 cases. Blood 1987;69(3):820–6.