Evaluation of Macrocytic Anemias

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Macrocytic anemia, defined as a mean cell volume (MCV) $\geq$100 fL in adults, has a narrow differential diagnosis that requires evaluation of the peripheral blood smear as well as additional laboratory testing taken in conjunction with clinical information that includes patient history and physical examination findings. This review is an update on the approach to a patient with macrocytic anemia with attention paid to the differentiation of megaloblastic and non-megaloblastic macrocytic anemias. Critical to the determination of the diagnosis is the judicious use of laboratory testing and the evaluation of those findings in conjunction with the patient medical, surgical, and medication history.
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Of all the red blood cell indices in routine clinical use, the mean cell volume (MCV) has the most widespread utility. This was not always the case. When Wintrobe first promulgated the red blood cell indices,[1] the MCV was calculated rather than measured directly. With the advent of electronic cell counting by electrical impedance and later by light scatter, accurate and highly precise direct measurements of MCV became available[2] and the MCV became a convenient touchstone for most algorithms used for anemia diagnosis. In the evaluation of anemias in clinical practice, a common way of developing a differential diagnosis is to separate them, in the first instance, according to size as measured by the MCV, part of the complete blood count (CBC) as measured by an automated hematology analyzer. Typically, anemias are divided into microcytic (MCV $<$80 fL), normocytic (MCV = 80–100 fL), and macrocytic (MCV $\geq$100 fL). Each of these categories of anemia includes conditions with a wide variety of etiologies but with some considerable overlap between categories.

It is always important to assess the MCV in relation to age-appropriate reference ranges since the normal range for MCV is generally lower in children, except during the neonatal period up to 6 months of age, when it is higher. During pregnancy, there is a physiological increase in MCV of about 4 fL. Also, apparently unexplained macrocytosis has been reported among the elderly, although the macrocytosis may represent an evolving myelodysplasia.[3] Examination of the blood smear to estimate red blood cell size is fraught with difficulty, since the estimation must be based on an area or linear diameter instead of an actual volume measurement. To obtain an approximate impression of cell size, it is helpful to compare red cells to a small lymphocyte nucleus since both should normally have the same diameter.

CAUSES OF MACROCYTOSIS—MEGALOBLASTIC

There are many causes of macrocytic anemias, but this group of anemias is generally subdivided into megaloblastic anemias and non-megaloblastic anemias. Figure 1 shows an algorithm for the evaluation of macrocytic anemias.

Classically, megaloblastic anemias are defined by morphologic characteristics noted on evaluation of the peripheral blood smear. These characteristics include the presence of oval shaped (as opposed to spherical) macrocytic erythrocytes (macroovalocytes) and the presence of hypersegmented neutrophils. Nuclear segmentation of neutrophils is assessed microscopically by enumeration wof the average number of nuclear lobes that are either distinct or connected to a neighboring lobe by only a linear chromatin band. A hypersegmented neutrophil is defined as a neutrophil with six or more lobes.[4] However, the presence of 5% or more of neutrophils containing five or more lobes is generally considered indicative of hypersegmentation and represents plausible reason to investigate the patient for evidence of an underlying megaloblastic condition. Hypersegmented neutrophils may be seen alone without the presence of macroovalocytes as hypersegmentation has been noted to be the first morphologic change seen in patients with megaloblastic anemia, as well as the last morphologic change to disappear after suitable treatment.[5,6] While the rapid appearance of hypersegmented

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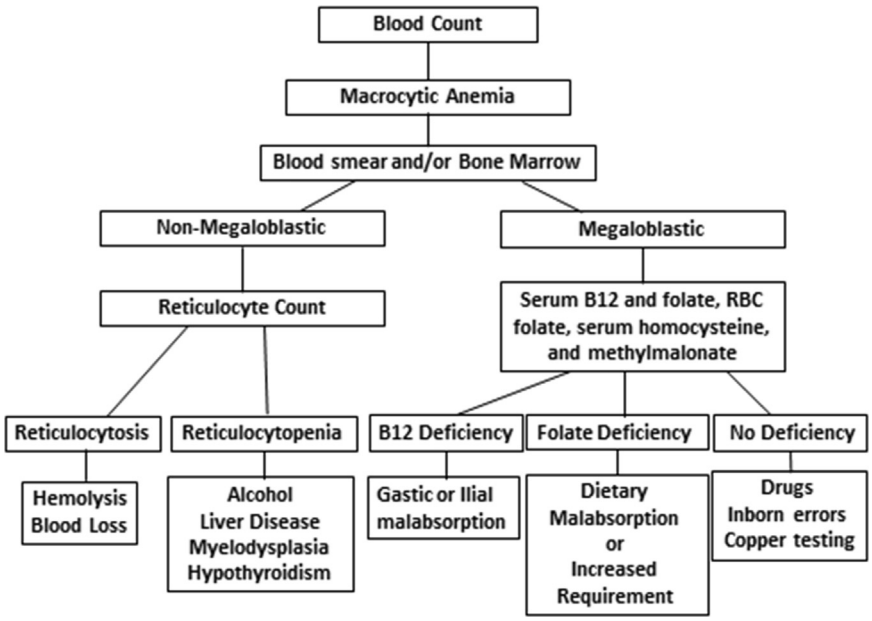

Figure 1. Algorithm for the evaluation of patients with macrocytic anemia. Adapted and revised from Green R., Laboratory Medicine 1999;30:595-99.

neutrophils may be explained by the relatively short life span of circulating granulocytes, their late disappearance does not have any convenient explanation. The finding of these cytologic features in the peripheral blood smear of a patient being evaluated for a macrocytic anemia is sufficient reason to further investigate the presence of underlying megaloblastosis. However, the finding of hypersegmented neutrophils is not pathognomonic of megaloblastic anemia and has been reported in iron deficiency anemia.[7] Another useful clue from the blood count that favors a megaloblastic cause of macrocytosis is the finding of a raised red blood cell distribution width (RDW). This provides a quantitative measure of anisocytosis. Since there is usually a marked variation in the size of red blood cells in B_{12} or folate deficiencies owing to the progressively more severe effects of nutrient deficiencies on red blood cell production as well as the poikilocytosis that results from both ineffective erythropoiesis and the hemolysis that occurs in megaloblastosis, the RDW is usually markedly elevated when there is megaloblastic macrocytosis. In fact, even when the bone marrow is producing macrocytic red blood cells, the MCV may fall within the normal range for some time, because of the 4-month longevity of circulating red blood cells. Megaloblastic anemia is a result of a defect in DNA synthesis and repair usually caused by a deficiency or perturbation of thymidine synthesis. This results in a mismatch substitution of uracil in place of thymidine in maturing hematopoietic progenitor cells. In the bone marrow, this results in a disruption of normal nuclear development with the effect of a lagging of nuclear behind cytoplasmic maturation. The progeny of these abnormal precursors are the oval macrocytes and hypersegmented neutrophils described above. When the underlying cause of the megaloblastic process is a deficiency of either vitamin B_{12} or folate, these morphologic changes are accompanied by biochemical changes, including increases in methylmalonic acid (MMA) in the case of B_{12} deficiency and homocysteine (HC), in the case of either B_{12} or folate deficiency.[8] Deficiencies of either folic acid or vitamin B_{12} can result in megaloblastic anemia, as both are required for the synthesis of thymidylate for DNA synthesis.

Other general biochemical consequences of megaloblastic hematopoiesis result from the ineffective hematopoiesis that occurs. These include increased serum levels of lactic dehydrogenase and bilirubin and a disruption of normal iron reutilization for hemoglobin synthesis, resulting in raised serum iron, ferritin, and transferrin saturations.[9] In general, folate deficiency results from insufficient intake, whereas B_{12} deficiency is more often the result of malabsorption. However, this paradigm has shifted since the introduction of mandatory folic acid fortification of the diet in North America and elsewhere and megaloblastic anemia caused by folate deficiency is now rare in those countries practicing folic acid fortification. As a consequence, B_{12} deficiency is now much more common than folate deficiency as a cause of metabolic disruption indicative of a possible underlying megaloblastic condition.[10,11] Overall, the prevalence of B_{12} deficiency increases with increasing age. The large Framingham study reported that greater than 12% of healthy elderly people had low serum B_{12} levels.[12]

In countries that do not practice mandatory folic acid fortification, which currently includes all European countries, the situation is different and folate deficiency remains a leading cause of megaloblastic anemia, particularly among the target groups consisting of the elderly, the poor, and malnourished alcoholics. Folate stores in the body are limited and last only for months, in contrast to B_{12} in which stores may last for years after cessation of intake or absorption.[9,13]

When dealing with a macrocytic anemia, it is critical to consider and confirm or exclude B_{12} and folate deficiencies, because if present, these conditions are correctable but if missed, they can lead to serious and sometimes irreversible complications, particularly in the case of B_{12} deficiency.

LABORATORY TESTING

Evaluation of megaloblastic anemia requires measurement of plasma or serum levels of vitamin B_{12} and folate as well as red cell folate.[14] A B_{12} level of <200 pg/ml ($\sim$150 pmol/L)* is strongly suggestive of $_{B12}$ deficiency and generally points to an underlying malabsorptive disorder. A B_{12} level of >400 pg/mL ($\sim$300 pmol/L) generally rules out B_{12} deficiency. However, spuriously normal levels of $_{B12}$ may occur in some patients with pernicious anemia if they have high levels of circulating antibodies to intrinsic factor. This is caused by interaction of the intrinsic factor antibodies with the intrinsic factor binder that is used in most B_{12} assays.[15] B_{12} levels of 200–400 ng/L ($\sim$150–300 pmol/L) are considered borderline. Borderline B_{12} levels should be complemented by additional more sensitive tests for measuring metabolite levels that rise in B_{12} deficiency.[8] Elevated MMA levels are widely considered to be the single most sensitive and specific test for identifying B_{12} deficiency and low MMA levels are strongly indicative of normal B_{12} status.[8,9,16]

Because B_{12} deficiency is usually caused by a failure of either the gastric or the ileal phase of absorption of the vitamin, in the past routine investigation of a patient with possible B_{12} deficiency included testing for B_{12} malabsorption. Since the Schilling test for B_{12} absorption became obsolete several years ago, no replacement test for B_{12} absorption has been validated clinically.[9] Consequently the only available test for diagnosing pernicious anemia is the test for the presence of circulating anti-intrinsic factor antibodies. While the test has a very high specificity with few false positive results, unfortunately it has poor sensitivity, being positive in only 60% of patients with pernicious anemia.[9,17,18]

Regarding testing for possible folate deficiency, a plasma or serum level of less than 4 μg/L (9 nmol/L)* is indicative of folate deficiency. While levels above 4 μg/L are considered normal, there is some evidence that borderline levels (4–8 μg/L or 9 nmol/L) associated with high plasma levels of homocysteine represent evidence of biochemical deficiency with incipient tissue effects leading to megaloblastic change in the marrow. On the other hand, borderline levels with normal homocysteine levels are not considered deficient.[19] However, homocysteine is typically increased in both B_{12} and folate deficiency, so that elevations in this metabolite alone do not help to distinguish folate from B_{12} deficiency.[8] Yet another measure of B_{12} status has come into use. Measurement of the fraction of the B_{12} in plasma that is bound to the plasma B_{12}-binding protein transcobalamin (TC), has, for some time, been considered to provide a more reliable indicator of functional B_{12} status because it is this component of circulating B_{12} that is responsible for cellular delivery and uptake of the vitamin.[20] However, methods to quantify this small amount of plasma B_{12}, termed holotranscobalamin (holoTC) or "active" B_{12}, comprising only around 20% of the total, were at first imprecise and have only been introduced for more widespread use over the past decade.[21,22] Overall, use of holoTC measurement as a single indicator of B_{12} status in place of total B_{12} or MMA has not received widespread acceptance for clinical diagnosis. However, because of longstanding problems with other indices of B_{12} status assessment used alone, there has been a tendency to recommend combined use of analytes, such as total B_{12} with either holoTC or MMA.[23] This approach has recently been refined by Fedosov in the form of a combined indicator of B_{12} status (cB_{12}) that incorporates B_{12}, holoTC, MMA, and homocysteine.[24]

As with the serum folate, red blood cell folate is typically low in folate deficiency but may also be low in B_{12} deficiency. Red blood cell folate testing, therefore, should be interpreted in conjunction with plasma B_{12} and folate testing. The major value of measuring red blood cell folate lies in the fact that it is not influenced by recent dietary intake of folate, whereas serum folate is. Regarding the differentiation between folate and B_{12} deficiencies as a cause of macrocytic anemia, it is important to consider elements of the patient's clinical history and presentation. The most noteworthy difference between B_{12} and folate deficiencies is that, whereas the symptoms of folate deficiency are primarily hematologic, patients with B_{12} deficiency additionally often have neurologic signs and symptoms. It is now well known that folate deficiencies can result in a number of long-term consequences, including cardiovascular, developmental, neurologic, and immunologic; some of these long-term effects are attributable to hyperhomocysteinemia.[6,25]

CAUSES OF MEGALOBLASTIC ANEMIA NOT RELATED TO B_{12}/FOLATE DEFICIENCIES

Apart from deficiencies of B_{12} and folate, other causes of megaloblastic anemia are shown in Table 1. These include a variety of drugs that that either interfere with DNA synthesis or with the absorption, metabolism or processing of one or both vitamins. Methotrexate and other antifolates can induce functional folate deficiency and result in megaloblastic changes.[26]

Likewise, purine and pyrimidine nucleoside analogues that interfere with DNA can cause megaloblastic changes. Several other drugs including folate analogues used to treat microbial and parasitic infections, anticonvulsants, metformin, sulfasalazine, and the anesthetic nitrous oxide can also cause macrocytosis.[27] In children, inherited disorders, including inborn errors of metabolism, can be causes of macrocytosis, including megaloblastic changes. Detailed

Table 1. Pathologic Causes of Macrocytosis (MCV >100 fL)

Non-megaloblastic	Megaloblastic
Increased reticulocytes/reticulocytosis	Folate deficiency
Hemolytic anemia	Cobalamin deficiency
Erythropoietin treatment	Drugs
Alcohol	Inborn errors of metabolism
Liver disease	Thiamin responsive megaloblastic anemia
Myelodysplastic syndrome	
5q- syndrome	
Congenital dyserythropoietic anemia (CDA I)	
Aplastic anemia	
Hypothyroidism	
Drugs	
Diamond-Blackfan anemia	
Fanconi anemia	
Copper deficiency	

NOTE. Causes are separated by presence of macrocytosis with megaloblastic changes *v* macrocytosis without megaloblastic changes. Table derived from Green.[52]

descriptions of these disorders lie beyond the scope of this publication, but there are a number of excellent reviews that deal with these disorders.[28–30] Megaloblastic anemia may rarely also be associated with other micronutrient deficiencies. Usually, these occur in conjunction with an inborn metabolic error, such as thiamine-responsive megaloblastic anemia in which there is a defect in RNA ribose synthesis.[31]

Just as not all macrocytic anemias are related to B_{12} or folate deficiencies, not all B_{12} and folate deficiencies are macrocytic. Masking of these megaloblastic anemias is often attributable to a concomitant iron deficiency or thalassemia, conditions that typically give rise to microcytosis or microcytic anemia. Similarly, partially treated megaloblastic anemias may show only transient macrocytosis.[6,32] In these situations, while the macrocytosis is masked, if a megaloblastic condition is present, then hypersegmentation of neutrophils will still be evident in the blood smear, and megaloblastic features will also be present in the bone marrow, in both the granulocytic and erythroid series. In such cases of complex anemias, after treatment with folate or B_{12}, a fall in the MCV will be noted, sometimes even into the microcytic range.

In the past, a bone marrow examination was carried out to confirm that a macrocytic anemia was megaloblastic and likely due to either B_{12} or folate deficiency. However, this is no longer deemed necessary. Since blood tests usually suffice to identify or exclude deficiency of either of these vitamins as a cause of the macrocytic condition, current standards of practice restrict the need to perform a bone marrow biopsy to only certain indications. This applies in patients who have normal B_{12} and folate status indicators suggesting that there is some other underlying cause of the macrocytosis or in patients in whom lab testing is equivocal, and therefore a bone marrow aspirate/biopsy may be necessary in order to further investigate the macrocytic anemia. Also, in patients given B_{12} and/or folate in whom there is either no response or a suboptimal hematologic response to treatment, examination of the bone marrow is justified and may be indicated. A hematologic response to treatment with folate or B_{12} consists of a rise in hematocrit of 5% or more or a fall in MCV of 5 fL or more, regardless of the initial MCV.[33]

Evidence of megaloblastic change in the bone marrow consists of several elements. These features are shown in Figure 2. Changes in nuclear chromatin may be seen in bone marrow blood precursor cells of all series but may not uniformly affect all the cells in those series. In other words, megaloblastic forms may be seen alongside normoblastic precursors. In addition, gradations in the degree of megaloblastic change may be evident. For this reason, careful examination of the marrow aspirate is often necessary to identify more subtle degrees of megaloblastosis. The term "megaloblastoid" has sometimes been applied to describe cells that are difficult to classify as either "normoblastic" or "megaloblastic". This term is best avoided as it is a construct that is used to describe a condition of uncertainty. The possible explanations for the discrepant appearances of precursors in the same marrow include the possibility of differential susceptibility of some hematopoietic clones to limiting nutrient supply as well as temporal variations in the nutrient supply that may occur as a result of short bursts of temporary remission of the megaloblastic condition that may arise from partial correction of the deficiency due either to intermittent supply or discontinuous treatment with the missing nutrient.

In a classical megaloblastic marrow, erythroid precursors typically show arrest of nuclear chromatin maturation

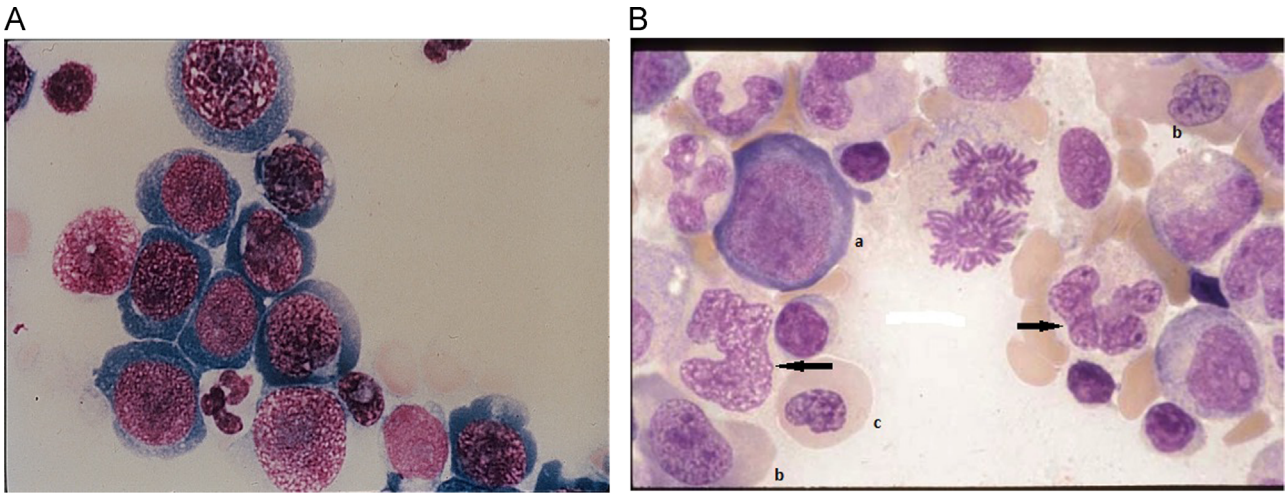

Figure 2. Photomicrograph of bone marrow aspirate smear from a patient with megaloblastic anemia caused by vitamin B_{12} deficiency. (A) Erythroid precursors showing apparent maturational arrest with predominance of early basophilic (non-hemoglobinized) forms. Nuclear chromatin shows an "open" uniformly fine stippled immature appearance. (B) Mixed erythroid and granulocytic precursors showing "giant" forms (solid arrows) and megaloblastic erythoid precursors at varying maturational stages: (a) proerythroblast; (b) polychromatophilic erythroblasts; (c) late erythroblast. A mitotic granulocytic precursor is present.

with normal cytoplasmic maturation (normal hemoglobinization of the cytoplasm); this finding is often referred to as nuclear cytoplasmic dyssynchrony (Figure 2A). Myeloid precursors also show immature chromatin with the presence of large band forms ("giant bands") (Figure 2B). Hypersegmented neutrophils may also be seen in the marrow. Megakaryocytes may show hypersementation of nuclear lobes with 16 or more lobes present. These cytologic findings in the marrow, while typical of megaloblastosis arising from B_{12} or folate deficiency, are also seen in other megaloblastic processes and may at times be seen in myelodysplastic syndromes. Furthermore, late-stage erythroblasts underdo premature programmed cell death in megaloblastic marrow,[34] and the ensuing karyorrhectic process can contribute further to some of the morphological features of a megaloblastic marrow that may give rise to difficulty in differentiating a megaloblastic from a myelodysplastic process.

NON-MEGALOBLASTIC MACROCYTIC ANEMIAS

Evaluation of the peripheral smear typically is not very helpful in determining the cause of non-megaloblastic macrocytic anemias. An exception is the finding of polychromasia. Polychromasia indicated by the presence of an increased proportion of immature non-nucleated erythrocytes, identified by their large size and blueish-gray hue, in the peripheral blood is suggestive of increased numbers of reticulocytes. This finding is typically confirmed either by supravital staining with methylene blue or brilliant cresyl blue or by flow cytometry using a fluorescent dye-like acridine orange. Both methods stain the residual RNA present in reticulocytes and are used in determining reticulocyte counts. Since they are almost twice as large as mature erythrocytes, reticulocytes, when present in increased numbers, raise the MCV. As a very rough rule of thumb, for every 1% increase in the reticulocyte percentage, there will be an increase of 1 fL in the MCV. Increased peripheral blood reticulocytes are a feature of increased marrow erythroid activity. Clinically, the presence of macrocytosis with increased polychromasia and reticulocytosis is indicative of high red blood cell turnover caused either by blood loss or hemolysis. If in the evaluation of macrocytosis, reticulocytosis is found, testing for hemolytic anemia should be undertaken, including seeking confirmation of increased red cell destruction by measuring serum bilirubin and lactate dehydrogenase (LDH). If red blood cell destruction is confirmed, then a full investigation of the underlying cause of the hemolysis should be performed. Hemolytic anemias may be either normocytic or macrocytic and the test panels and algorithms that should be followed lie beyond the scope of this review. Other conditions that can lead to reticulocytosis with increased MCV include hypoxia/chronic obstructive pulmonary disease[35] and recent acute blood loss.

Alcohol abuse is a common cause of macrocytic anemias.[36] In a study from hospitalized patients in New York City published in 2000, alcohol, with or without liver disease, was second only to medications/drugs as a cause of macrocytosis.[37] More recently, in a study from India, alcohol abuse was found to be the most common cause of secondary macrocytosis.[38] Alcohol causes non-megaloblastic macrocytic anemia, in addition to being a contributing factor in megaloblastic macrocytic anemia. In this situation, macrocytosis will often resolve with cessation of alcohol intake.[39] The megaloblastic component is typically associated with poor general nutrition, and

particularly folate deficiency.[40] Non-megaloblastic macrocytic anemia among alcoholics is also frequently related to alcoholic liver disease.

Liver disease, associated either with cirrhosis or acute liver damage, results in macrocytic anemia. However, macrocytosis can be seen in chronic alcohol users even before anemia develops. Between one and two thirds of all patients with chronic liver disease have macrocytosis.[41] Notably, the macrocytosis in liver disease is usually uniform and round, with the blood count showing a normal RDW.

In addition to drugs that directly affect DNA synthesis leading to megaloblastic anemia, other medications noted to cause macrocytosis include anticonvulsants (valproic acid, phenytoin), sulfonamides, (trimethoprim/sulfamethoxazole), metformin, cholestyramine, numerous chemotherapy medications, anti-retrovirals, triamterene, methotrexate, sulfasalazine, and nitrous oxide.[9,27,37,42] Treatment with recombinant erythropoietin (eg, in renal disease) can lead to macrocytosis either through causing reticulocytosis or through an independent mechanism, and is sometimes responsive to folic acid administration; in these situations, the folate deficiency may be related to loss of folates during hemodialysis. Macrocytosis might also be related to acceleration in the program of erythroid maturation or may be caused by a state of relative folate deficiency induced by a speeding up of the cell cycle.[43]

The anemia of hypothyroidism is either normocytic or mildly macrocytic. A recent study from Iran did not demonstrate a significant difference in MCV between hypothyroid patients and patients with normal thyroid function tests.[44] However, in the evaluation of macrocytic anemia, it is still prudent to consider hypothyroidism in the workup.

Several primary hematological diseases may present with macrocytic anemia. Some patients with aplastic anemia have macrocytosis, but in the clinical practice of hematology and hematopathology, myelodysplastic syndrome (MDS) is a more common cause of macrocytic anemia. Typically, MDS, a group of clonal disorders, present with cytopenias. When anemia is one of the cytopenias, the MCV typically is raised, usually in the range of 98–104 fL. Macrocytosis in MDS is often associated with other features of MDS, including cytopenias affecting either one or both of the other two major cell lineages with dysplastic features sometimes seen on the blood smear, including nuclear hypolobation and hypogranularity of granulocytes. The consideration of MDS should arise when other etiologies of macrocytic anemia fail to elucidate a cause, particularly in older patients with other hematologic features, in whom a bone marrow evaluation is necessary to exclude or confirm this diagnosis. Isolated unexplained macrocytosis may occur in older individuals. This may be due to an evolving MDS.[3] Being a clonal disorder, the bone marrow in established MDS demonstrates dysplasia in one, two, or all three cell lines, and may show the presence of increased numbers of blasts when the MDS evolves into an acute leukemia. Additionally, megaloblastic changes may be seen in the marrow in MDS, most commonly in the erythroid precursors. A particular form of MDS, the 5q- syndrome, in which there is deletion of the long arm of chromosome 5, is classically associated with macrocytosis.[45]

In patients with the rare congenital dyserythropoietic anemia (CDA) type I, macrocytosis is present, together with dysplastic changes in the erythroid precursors.[46] Patients with Diamond-Blackfan anemia may be macrocytic, and it is important to consider this diagnosis in anemic children with MCV above normal for age.[47]

A more recently recognized cause of macrocytic anemia is copper deficiency. Copper deficiency has been diagnosed more frequently recently due to the increased number of surgeries being performed for morbid obesity. Bariatric surgery can result in malabsorptive disorders, including poor absorption of copper, iron, and B_{12}.[48] Deficiency of copper can result in either macrocytic, microcytic, or normocytic anemia.[49] As malabsorption of one or more of these nutrients can result in either a microcytic anemia (iron), macrocytic anemia (B_{12}), or a mixed picture (two or more of these nutrients), the evaluation of anemia in post-bariatric surgery patients should include testing for deficiencies of all of these nutrients. Copper deficiency following bariatric surgery may be particularly problematic because the dysplastic morphologic changes that occur in the marrow make it difficult to distinguish from MDS. Erythroid precursors with cytoplasmic vacuoles, a finding seen in MDS, can also be seen in copper deficiency. Ring sideroblasts have also been observed in these patients.[48,49] If there is concomitant B_{12} deficiency, megaloblastic changes can also occur in the marrow, further complicating interpretation of the marrow findings.

On occasion, the MCV may be elevated due to patient conditions unrelated to marrow production of erythroid cells. Cold agglutinins can cause the red blood cells to clump and appear larger to the automated instrument. Although automated blood counting instruments are usually calibrated to ignore large clusters, red blood cell doublets may be measured and give rise to spurious macrocytosis. Hyperglycemia associated with a low fluid status in patient can show macrocytosis when the blood is diluted on testing, resulting in false macrocytosis.[50] Also, extreme leukocytosis can result in a false elevation of MCV, since leukocytes can also be sized in the red blood cell channel of automated blood counting systems.

CONCLUSION

The finding of macrocytic anemia includes a finite differential diagnosis. The evaluation should begin with evaluation of the peripheral blood smear and determining if the anemia most likely is megaloblastic or non-megaloblastic. Evaluation of the patient's history, including medication use, is critical. There are several strategies that can be adopted toward the evaluation of a patient

with macrocytic anemia. These approaches differ in respect to detail but are essentially variations on a general thematic approach. All are designed to establish, as efficiently and economically as possible, what the cause of the anemia is and to institute appropriate treatment wherever possible.[7,51]

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