Laboratory diagnosis of anemia: are the old and new red cell parameters useful in classification and treatment, how?

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SUMMARY

Introduction: Anemia is a global problem affecting the population in both developing and developed countries, and there is a debate on which hemoglobin level limit should be used to define anemia in general population and particularly in the elderly. We present herein a laboratory approach to diagnosing the possible causes of anemia based on traditional and new erythroid parameters. In this article, we provide practical diagnostic algorithms that address to differential diagnosis of anemia. Based on both morphological and kinetic classifications, three patterns were considered: microcytic, normocytic, and macrocytic.

Methods: Main interest is on the clinical usefulness of old and new parameters such as mean cell volume (MCV), red blood cell distribution width (RDW), hypochromic and microcytic erythrocytes, immature reticulocyte fraction (IRF), and some reticulocyte indices such as reticulocyte hemoglobin content and mean reticulocyte volume. The pathophysiologic basis is reviewed in terms of bone marrow erythropoiesis, evaluated by reticulocyte count (increased or normal/decreased) and IRF. The utility of reticulocyte indices in the diagnosis of iron-deficient erythropoiesis (absolute or functional) and in monitoring of response to treatment in nutritional anemia (iron and cobalamin) was also investigated.

Results: For each parameter, the availability, the possible clinical applications, and the limitations were evaluated. A discussion on intraindividual biological variation and its implication on the usefulness of conventional reference intervals and in longitudinal monitoring of the patients was also reported.

Conclusion: Red cell parameters and reticulocyte indices play an essential role in differential diagnosis of anemia and in its treatment. More efforts are needed in harmonizing parameters whose results are still too different when produced by different analyzers.
INTRODUCTION

Anemia is when blood hemoglobin (Hb) concentration is below the lower limit of the reference interval stated for age, sex, race, and altitude. The commonly accepted lower limits for adult population are the WHO criteria suggested by an expert committee nearly 50 years ago: 130 g/L in men and 120 g/L in women, without the distinction between age and race (1). The definition of anemia has attracted interest in recent years because epidemiologic studies suggest that anemia may be associated with poor prognosis in many different diseases, particularly among aged people. A recent large population survey based on WHO criteria (NHANES-III) (2) showed that nearly ten percent of men and woman older than 65 years were anemic. These percentages rose to 26% in males and 20% in females older than 85. It is not clear whether the difference in lower limits, justified in androgen-dependent age, should be continued after 65 years of age. Many of these subjects were apparently healthy, and in most cases, clinical investigations did not uncover a specific cause of anemia. These results suggest that somewhat lower limits than ‘normal’ might be used in the elderly. Nevertheless, the too easy acceptance of mild anemia as physiologic in the elderly runs the risk of ignoring an underlying disease. There is a debate on which hemoglobin lower limit should be used to define anemia in general population and particularly in the elderly (3). Two different, relatively recent, large databases (NHANES-III and Scripps-Kaiser) (4, 5) in which the hemoglobin determination was carried out with standardized automated methods obtained a good agreement and new lower limits are proposed (6). It would seem that these limits (5% of normal distribution) are 137 g/L in white men (20–59 years) and 132 g/L for men after the age of 60; the corresponding value for women is 122 g/L independently of age. In Afro-Americans, these limits are lower: 129 g/L in younger men and 127 g/L in men older than 60, while the corresponding value for women is 115 g/L at all ages.

For many practical approaches, a decrease in hematocrit (Hct) is considered equivalent to a decreased hemoglobin concentration, but this simplification is not always correct. All hematology impedance-based analyzers falsely overestimate Hct in hypochromic red cells. In this last condition, the use of Hct rather than the more accurate measured Hb can overestimate the diagnosis of anemia in subjects with iron deficiency (7). Also, the optical-based instruments with isovolumetric sphering provide a falsely elevated Hct when they analyze sickle red cells that cannot be sphered. A typical artifactual dissociation with all the automated analyzers between Hb result (usually correct) and Hct (underestimated) is the presence of red blood cell (RBC) agglutinates. Because the upper volumetric threshold to consider cells as RBC is between 200 and 300 fL according to the analyzer, the large RBC clumps are not counted as RBC. This causes a spuriously low RBC count and low Hct. In contrast, Hb is measured after RBC lysis and is unaffected by agglutinins. As a consequence, MCHC is abnormally high, usually greater than 360 g/L. Moreover, also Hb can be erroneously overestimated, although more rarely, in subjects with severe hypertriglyceridemia or receiving an intravenous administration of fat emulsions, or with high WBC counts, due to the excessive turbidity.

OLD ERYTHROCYTE INDICES

Maxwell Wintrobe 80 years ago proposed the anemia classification based on the mean cell volume (MCV) obtained by Hct/RBC ratio from the measurement of spun Hct and manual hemocytometric RBC count. The MCHC was calculated as Hb/Hct ratio, where Hb was also based on manual measure (8). Of these two indices, which allowed to classify the anemia as microcytic, normocytic, and macrocytic based on MCV value and hypochromic, normochromic or hyperchromic based on MCHC, only MCV has survived as key parameter for the classification of anemia with automated hematology analyzers (Figures 1–3). With the data collected on a cell-by-cell basis, modern instruments generate a histogram of erythrocytes size distribution. From this histogram, an index of heterogeneity referred to as red cell distribution width (RDW) can be determined. This is almost always expressed as percentage coefficient of variation and, less frequently, as standard deviation. The possibility of a quantitative, nonsubjective measurement of an anisocytosis index has reawakened interest. Bessman et al. (9) in the early 1980 proposed a classification of anemia based on both MCV and RDW: homogeneous (with normal RDW) and heterogeneous (with

increased RDW) erythrocyte population. The former includes hypoproliferative anemia, marrow aplasia, and thalassemia heterozygosity; the latter includes nutritional anemias (iron, cobalamin, and folic acid deficiency) and sideroblastic anemia. Although this approach was largely accepted and RDW was added to routine analysis in many laboratories, numerous exceptions began to be observed. There is a wide distribution of RDW values within a given disease, whose usefulness in differential diagnosis has decreased, but its utility as a general marker of abnormality has been maintained. A further complication derives from the difference in reference intervals obtained with analyzers from different manufacturers (10–12). This is explained by the different algorithms used to cut the tails of distribution, which is needed to eliminate extreme values often due to artifacts.

**NEW RED CELL PARAMETERS**

Some hematology analyzers can quantitate the percentage of hypochromic, microcytic, and more rarely...
hyperchromic erythrocytes (Table 1). In iron-deficient erythropoiesis, a greater fraction of RBC is hypochromic rather than microcytic, and the microcytic/hypochromic ratio shows the best diagnostic efficiency in the differential diagnosis with beta-thalassemia trait (14, 15). Many other discriminant algorithms have been recently proposed including conventional and new RBC parameters (15, 16). The results were in general better than the traditional discriminant functions, but the performance of any index seems to depend on the geographical origin of the population in which it is applied (17). Recent studies have shown that hypochromic erythrocytes are useful in identifying iron-restricted erythropoiesis in anemic patients treated with erythropoiesis-stimulating agents (ESAs), particularly anemia of the chronic kidney disease or in hemodialyzed patients. The response to ESAs is strictly dependent on iron availability and is limited by iron deficiency that can be absolute or functional (i.e., limitation of bone marrow erythropoietic activity by the inability to mobilize the sufficient iron from body storage sites) (18–20). The presence of hyperchromic cells is useful in the diagnosis of spherocytosis either by hereditary or by immune hemolysis (21, 22). The main limit in the use of these parameters is due to the fact that they are affected by temperature and storage time. In fact, erythrocytes in the samples stored at room temperature tend to progressively swell, with a consequent reduction in cellular hemoglobin concentration and an increase in hypochromic and decrease in hyperchromic RBC%.

EVALUATION OF BLOOD SMEAR

All the red blood cell indices, although useful, are the representation of the mean and overall dispersion of the erythroid cellular population and provide little information about specific red blood cell shapes or the presence of minor populations of abnormal cells. Examination of blood smear for some specific shapes as reported in Figures 2 and 3 can provide a valuable information to aid in the diagnosis of the underlying disease.

RETICULOCYTE AND IMMATURE RETICULOCYTE FRACTION

In addition to red cell indices and morphological criteria, anemia may be classified by kinetic approach, that is, the degree of bone marrow response evaluated by the reticulocyte count. The reticulocyte count is clinically important both for the pathophysiological classification of anemia (due to an inadequate production of erythrocytes by the bone marrow, in which case there is a decreased number of reticulocytes, or to an excessive loss or the destruction of erythrocytes, in which there is an increase in reticulocyte count) and for the early identification of the normalization of erythropoiesis by the marrow after therapeutic intervention (iron, cobalamin, folic acid, ESAs, etc.), after spontaneous or pharmacologically induced aplasia of the marrow, or following bone marrow transplantation. However, the imprecision of the manual microscopic method (coefficient of variation (CV) between 68.6% at low concentration and 16% at high level) (23) makes it almost useless mainly in severe reticulocytopenia. It does not allow for the observation of small but significant variations during the early recovery of erythropoietic bone marrow activity, nor does it clearly define the difference between normal and
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low reticulocyte levels. Automated analyzers represent a revolution for this cell type using dyes to bind reticulocyte RNA and flow cytometers to perform rapid and objective counts. The possibility to analyze tens of thousands of cells per sample has reduced imprecision (CV between 25% at low concentration and 3.0% at high counts) (24) (M. Buttarello, personal observations). Furthermore, using an absolute reticulocyte count (expressed as the number of cells per unit of volume: x 10^9/L) rather than proportion no longer requires correction for reduced hemoglobin concentrations. A little complication derives from the differences in the reference intervals that are strictly method dependent (lower limit of the 95% interval between 19 and 30 x 10^9/L and upper between 85 and 130 x 10^9/L) (24). What has created an additional interest about automated reticulocyte analysis is the availability of a new parameter called immature reticulocyte fraction (IRF) based on reticulocytes RNA content (25). Reticulocytes originate from orthochromatic erythroblasts following ejection of the nucleus, and they gradually mature, partly in the marrow (3 days on average) and partly in the peripheral blood (1 day). Reticulocytes gradually lose their RNA and ultimately become RNA-free red cells, while some RNA-rich, more immature reticulocytes are found in relatively narrow proportion in the peripheral blood of the healthy subjects. There are, however, various expressions according to the analyzer used, and thus, the reference intervals are different (low level between 0.012 and 0.20 and high level between 0.14 and 0.40) (26, 27). Independently by the way on as it is produced, the IRF is an early and sensitive index of erythropoiesis; in fact, immature reticulocytes appear in a larger proportion when red cell production increases. The IRF has a weak but significantly positive correlation with the absolute reticulocyte count, indicating that it is an additional useful parameter to evaluate the erythropoietic activity. The greatest clinical usefulness, especially in the classification of anemia based on marrow response, is found using two-dimensional matrices of IRF vs. the absolute reticulocyte count (25, 26). With covariance analysis (ANCOVA), it is possible to identify some well-differentiated behaviors in certain areas of the matrices: (i) In reticulocytopenia, there is no covariance, both in marrow aplasia and in early erythropoietic response; (ii) in normal or mild reticulocytosis, there are two subsets with positive covariance corresponding to the healthy subjects and to accelerated erythropoiesis; (iii) for marked reticulocytosis, the covariance is negative, suggesting a gradual deceleration of erythropoiesis (28). Therefore, IRF and reticulocyte count may vary in a concordant or independent way according to the erythropoietic conditions and can be hypothesized the IRF as an index of acceleration and the absolute reticulocyte count as a quantitative measure of the effectiveness of erythropoiesis (28, 29). This parameter is therefore useful in distinguishing (i) anemia characterized by an increase in erythropoiesis, like acquired hemolytic anemias or the loss of blood, which produces an increase both in total reticulocytes and in IRF; (ii) anemias due to the reduced marrow production (i.e., chronic renal disease) in which both values are found to be decreased, and (iii) anemia of acute infections or myelodysplastic syndromes in which there is a dissociation between total reticulocyte count (reduced or normal) and the IRF which can be increased (30–33). Other uses include monitoring the therapy efficacy in nutritional anemia (e.g., cobalamin, folates, and iron) because the increase in IRF precedes the increase in total reticulocyte count by several days. In subjects with iron-deficient anemia treated with iv iron, it increased at day 1 and continued to increase until reaching the maximum value at day 5 (34). This value was correlated with the erythropoietin concentration at the beginning of therapy (M. Buttarello, data not published). The combination of reticulocyte count >80 x 10^9/L with a reticulocyte count/IRF ratio >7.7 is considered useful for the screening of trait and mild hereditary spherocytosis (35).

RETICULOCYTE INDICES

The latest generation of hematology analyzers provides some reticulocyte indices analogous to the equivalent RBC indices (Table 1). Among these, the most promising from a clinical point of view are the hemoglobin content of reticulocyte and the mean reticulocyte volume. The hemoglobin content, which directly reflects the synthesis of hemoglobin in marrow precursors, is a measure of adequacy of iron availability (34, 36–38). This parameter is important because its reduction indicates iron-deficient erythropoiesis, even in conditions in which traditional
biochemical markers such as ferritin and transferrin saturation are inadequate (e.g., in inflammations or anemia from a chronic disease), and besides, it is useful for monitoring early response to intravenous iron therapy because it increases significantly after only 48–72 h (Figure 4) (34, 36). Exceptions are heterozygotes for beta-thalassemia whose reticulocyte hemoglobin content is found to be always reduced independently of iron stores (39). Low values of this index are indicative even in functional iron deficiency which appears in patients treated with erythropoietin (38). Few studies are available on the clinical usefulness of mean reticulocyte volume. In subjects with depleted iron stores, this index increases rapidly following iron therapy and decreases equally as rapidly with the development of iron-deficient erythropoiesis. The reticulocyte volume decreases dramatically and reticulocytes are smaller than the circulating RBCs, in nutritional macrocytosis after therapy with vitamin B12 and/or folic acid (Figure 5) (37). The main limit of the use of these indices is the difficulty to compare numeric results obtained from the analyzers of different manufacturers (the lower limit of the 95% interval between 91 and 100 fL and the upper limit between 111 and 120 fL) (40).

**INTRAINDIVIDUAL BIOLOGICAL VARIATION**

One source of variability of clinical laboratory results (besides preanalytic and analytic variations) is the intraindividual variation around the homeostatic setting point (41). Several studies have investigated this biological variation and the results are similar even if carried out at different time points and in different geographical areas (42–44). The reported values varied between 1.9 and 2.8% for Hb and Hct; between 0.6 and 1.3% for MCV; and between 5.8 and 9.5% for reticulocytes. From these values, an index of individuality as ratio of intraindividual to interindividual coefficient of variation can be calculated. For the above-mentioned parameters, it is between 0.19 and 0.42 for Hb, between 0.17 and 0.27 for MCV, and between 0.18 and 0.30 for reticulocytes, according to the different studies. A low index of individuality (<0.5) indicates that conventional reference intervals may be of little usefulness, especially when deciding if the change observed in a subject is clinically significant.
remarkable. Useful in serial monitoring of physiologic or pathologic conditions is the critical difference (called also reference change value: RCV), which defines the percentage change that should be exceeded (given the analytic and intraindividual biological variations) so that there is a significant difference between two consecutive measurements. The significant percentage changes (for probabilities of 95%) for the named red cell parameters are (43, 44) as follows: between 6.22 and 6.82% for Hb and Hct, between 2.35 and 3.12% for MCV, and between 36.7 and 41.7% for reticulocytes. Differences depend on biological variation (42–44) and on the analytical variability of analyzers, and these differences are smaller for instruments with smaller imprecision.

CONCLUSIONS
Red cell parameters and reticulocyte indices play an essential role in the differential diagnosis of anemia and in its treatment. More efforts are needed in harmonizing parameters whose results are still too different when produced by different analyzers. Moreover, it should be remembered that despite the essential role of automation, microscopic control of pathologic samples remains indispensable.

COMPETING INTERESTS
The author has no competing interest.


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