Automated Blood Cell Counts

State of the Art

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Abstract

The CBC count and leukocyte differential count (LDC) are among the most frequently requested clinical laboratory tests. These analyses are highly automated, and the correct interpretation of results requires extensive knowledge of the analytic performance of the instruments and the clinical significance of the results they provide. In this review, we analyze the state of the art regarding traditional and new parameters with emphasis on clinical applications and analytic quality. The problems of some traditional parameters of the CBC count, such as platelet counts, some components of the LDC such as monocyte and basophil counts, and other commonly used indices such as red cell volume distribution width and platelet indices such as mean platelet volume and platelet distribution width are considered. The new parameters, evaluated from analytic and clinical viewpoints, are the available components of the extended differential count (hematopoietic progenitor cells, immature granulocytes, and erythroblasts), the immature reticulocyte fraction, the reticulocyte indices, the fragmented RBCs, and the immature platelet fraction.

During the last 2 decades, automated blood cell counters have undergone a formidable technological evolution owing to the introduction of new physical principles for cellular analysis and the progressive evolution of software. The results have been an improvement in analytic efficiency and an increase in information provided, which, however, require ever more specialized knowledge to best discern the possible clinical applications. In addition to the traditional parameters of the CBC count and leukocyte differential count (LDC), the more complete analyzers are able to provide much more information, both quantitative, such as the extended differential count (EDC), and qualitative. The latter is represented by flags that indicate technical problems (eg, malfunction, analytic interference) and, above all, cells that are normally absent from peripheral blood such as blasts, atypical lymphocytes, immature granulocytes (IGs), and nucleated RBCs (NRBCs).

For some consolidated parameters, such as WBC and RBC counts, hemoglobin concentration, or mean corpuscular volume (MCV), analytic performance is generally excellent.[1] For others, in particular, certain components of the LDC and reticulocyte or platelet counts, especially at low concentrations, performance is less satisfactory.[2,3]

Further considerations are necessary regarding the possible clinical use of new analytic parameters that are available only with automated analyzers but that have not yet reached their full potential. The immature reticulocyte fraction (IRF), reticulocyte indices such as mean reticulocyte volume (MCVr) and mean reticulocyte hemoglobin content (CHr), fragmented RBC (FRBC) count, and the immature platelet fraction (IPF) are among these.

Other parameters such as the RBC distribution width (RDW) and platelet indices, such as the mean platelet volume

(MPV) and platelet distribution width (PDW), must be used with caution. Despite being available for several years, they are still not standardized as RDW or are influenced by preanalytic variables such as the time between sampling and analysis or the physical principles used by individual instruments, as for platelet indices. The aim of this review was to evaluate the state of the art of traditional parameters of the CBC count and LDC and analyze the possible clinical applications of recently introduced parameters provided by modern hematologic instruments **Table 1**.[4-44]

Analytic Performance

Analytic performance is traditionally evaluated by imprecision, inaccuracy, and clinical sensitivity.[45,46] Imprecision (or random error) is important in interpreting the results obtained from patient samples, even if it is not directly perceived by clinicians, because the results for a single individual can be influenced by analytic and individual biologic variability. Thus, improvement beyond certain limits of analytic precision for parameters with high biologic variability adds only minimal advantage to clinical use. Inaccuracy (or systematic error) has as a consequence the different placement of the results with respect to established cutoff values (upper or lower limits of the reference interval or decision threshold, useful for clinical decision making). The consequences are a decrease in sensitivity or specificity of a test based on the direction of the shift.

Various methods have been proposed to define the analytic goals for imprecision and inaccuracy, from those based on the opinions of clinicians to those that refer to daily variation of distribution of results with respect to an established decision threshold, including those based on components of biologic variability.[47-50] Each of these has advantages and limits. We have applied the goals obtained using the components of biologic variability because these are present in the literature for the parameters of the CBC count and LDC.[51-53]

Table 1
New Parameters: Proposed Clinical Applications and Technical Limitations

Parameter	Availability*	Proposed Clinical Applications	Limitations	References
Hematopoietic progenitor cells	XE 2100	Surrogate for CD34 stem cell quantitation before peripheral harvesting	Reduced availability; measurement depends on time between sampling and analysis; high imprecision	4, 5
Immature granulocytes	XE 2100	Diagnosis of bacterial infections in appropriate clinical setting	Reduced availability	6-8
Nucleated RBCs	Sapphire; Pentra 120 DX; LH 750; ADVIA 2120; XE 2100	Diagnosis of hematologic diseases; prognostic factor in patients from surgery department or undergoing stem cell transplantation; evaluation of the efficacy of transfusion therapy in thalassemic syndromes	Higher performance on fluorescence-based methods	9-14
Immature reticulocyte fraction	Sapphire; Pentra 120 DX; LH 750; ADVIA 2120; XE 2100	Classification of anemias; monitoring the efficacy of therapy in nutritional anemia; early identification of marrow regeneration (after bone marrow transplantation or chemotherapy); verify aplastic anemia; timing for stem cell collection	Not standardized; reference intervals method-dependent; higher sensitivity in fluorescence-based analyzers	15-21
Reticulocyte indices				
Mean reticulocyte hemoglobin content	ADVIA 2120; XE 2100	Diagnosis of iron-deficient erythropoiesis (absolute or functional); monitoring response to iron supplements; monitoring erythropoietin treatment during dialysis	Reduced availability	22-27
Mean reticulocyte volume	Pentra 120 DX; LH 750; ADVIA 2120	Diagnosis of iron-deficient erythropoiesis; early monitoring of response to treatment in nutritional anemia; early signs of erythropoietic recovery following bone marrow transplantation; evaluation of erythropoietin abuse in sports	Not standardized; reference intervals method-dependent	17, 28-32
RBC fragments (schistocytes)	ADVIA 2120; XE 2100	Diagnosis and monitoring of microangiopathies	Reduced availability; not standardized; definition based only on size and hemoglobin content	33-35
Reticulated platelets	XE 2100	Differential diagnosis of thrombocytopenia; prediction of total platelet recovery after chemotherapy or stem cell transplantation; risk index of thrombosis in patient with thrombocytosis; timing for prophylactic platelet transfusion; evaluation of platelet turnover	Reduced availability; not standardized	36-44

* Sapphire, Abbott, Abbott Park, IL; Pentra 120 DX, ABX-Horiba, Montpellier, France; LH 750, Beckman Coulter, Hialeah, FL; ADVIA 2120, Siemens Diagnostics, Tarrytown, NY; XE-2100, Sysmex, Kobe, Japan.

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According to this approach, in the monitoring of patients, which is the most restrictive condition, the maximum allowed imprecision must be less than half of the within-subject variability, whereas the inaccuracy must be less than one quarter of the group biologic variation (defined as within- plus between-subject variation). These 2 goals can be combined to calculate the total allowable error.[54]

Table 2 compares the analytic goals obtained with this approach with the state of the art (total current error) obtained from the literature.[1,55] The performance is satisfactory for the majority of parameters such as total WBC count, RBC count, hemoglobin concentration, MCV, and neutrophil and lymphocyte counts. The results are acceptable for other parameters, such as reticulocyte and eosinophil counts, but are far from optimal for monocyte and basophil counts.

For platelet counts, it is necessary to distinguish performance at normal or moderately reduced concentrations, where it is generally good,[56,57] from counts in severe thrombocytopenia, where performance is still not optimal. In severely thrombocytopenic patients, the accuracy of platelet counts is fundamental because the count is used to decide if the patient needs a platelet transfusion. Studies suggest that the threshold for prophylactic transfusion in patients without additional risk factors could be lowered from a platelet concentration of $20 \times 10^3/\mu L$ to $10 \times 10^3/\mu L$ ($20 \times 10^9/L$ to $10 \times 10^9/L$).[58-60] Other authors[61,62] have suggested that in patients without fever or bleeding, there may be even lower values. However, the utilization with confidence of these new thresholds requires knowledge of the limitations in precision and accuracy of the analyzers at these count levels. A comprehensive multicentric study on patients treated with chemotherapy and with a platelet concentration less than $20 \times 10^3/\mu L$ ($20 \times 10^9/L$) showed that optical methods are no better than impedance and that most analyzers tend to overestimate the count (between 1.2 and 3.5 $\times 10^3/\mu L$ [1.2-3.5 $\times 10^9/L$]) when compared with the reference immunologic method.[2] In this case, the method that has the best agreement with the reference is that based on the use of monoclonal antibodies (MoAbs) anti-CD61 and available on Abbott Cell-dyn 4000 (Abbott Diagnostics, Santa Clara, CA) and Sapphire analyzers (Abbott, Abbott Park, IL).[2]

Clinical sensitivity is defined as the ability to distinguish between normal and pathologic samples in terms of quantitative anomalies and, above all, for qualitative alterations such as the presence of immature or atypical cells and for significant morphologic anomalies of RBCs. According to this definition, sensitivity is usually excellent. In fact, despite the need for further improvement, the combined use of quantitative abnormalities and flags, in association with simple demographic data for the patient, allows for the construction of decision-making algorithms with a false-negative rate of less than 3%,[63] and rarely missing potentially important abnormalities. However, when evaluating the reliability of morphologic flags to indicate the presence of specific anomalies, the results are less satisfying, and good sensitivity but modest specificity can be observed. These limits have led some authors to hypothesize their elimination because they could cause unnecessary microscopic revision, or, worse, they could induce observer bias of later microscopic analysis.[64]

Table 2
Analytic Goals and State of the Art

Parameter	Analytic Goal (TAE, %)	State of the Art (TE, %)
CBC count		
Leukocytes	16.5	5.4-8.8
Erythrocytes	3.75	1.5-1.8
Hemoglobin	4.0	1.2-1.9
Mean cell volume	2.23	2.0-2.4
Platelets	6.32	5.2-9.8
Leukocyte differential count		
Neutrophils	23.4	3.06-7.0
Lymphocytes	15.0	4.0-11.9
Monocytes	14.8	13.4-58.7
Eosinophils	26.0	16.0-37.3
Basophils	15.7	35.5-155.5
Reticulocyte count	13.0	8.9-41.3

TAE, total allowable error[54]; TE, total current error.[1,55]

Leukocyte Differential Count

The LDC consists of the quantification of the various WBC populations present in peripheral blood. Even though they derive from the same progenitor cell and interact with one another, each population can be considered relatively independent in terms of maturation, function, and control mechanism. It is, therefore, fundamental to express the results in absolute values.[65] The differential count should respond to 2 principal needs: (1) the search for quantitative abnormalities in morphologically normal WBC populations (eg, in the diagnosis of infectious or allergic diseases and for monitoring cytotoxic or myelotoxic therapies), which requires high levels of precision and accuracy; and (2) the search for morphologic abnormalities, ie, the identification of immature or atypical cells for diagnostic or monitoring purposes, which requires a high level of clinical sensitivity.[66]

The traditional microscopic method based on the count of 100 cells has 3 types of error: statistical error, distributional error owing to unequal distribution of cells in the smear, and error in identifying cells related to the subjective interpretation of the examiner. The most important error is statistical because it is invariably related to the total number of cells analyzed.[67]

This method, therefore, suffers from imprecision, poor accuracy, and reduced clinical sensitivity. The automated counters performing LDCs analyze thousands of cells per

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sample and can produce morphologic and quantitative flags, which have significantly reduced error and allow for reliable absolute counts at low and high concentrations. Expressing WBC populations in absolute values has many uses, from noting the increase in lymphocytes in lymphoproliferative diseases or viral infections, to the increase in eosinophils in parasitosis and allergic diseases, to the increase in neutrophils seen in infections and acute inflammation. The absolute count is even more useful for monitoring neutropenia during chemotherapy or after bone marrow transplantation. In the case of monocytes, only an absolute count can discern monocytopenia and study its causes or associations (eg, marrow aplasia, hairy cell leukemia, HIV infection, megaloblastic anemia).

Several problems must still be resolved, such as the analytic quality of the count of certain populations, monocyte counts, for example (which nevertheless vary on different counters),[55,68] and basophil counts,[55,69] which are the most difficult population to count, to the point at which in cases of suspected basophilia it is necessary to resort to manual counts. Automatic counters, in fact, tend to underestimate the counts during true basophilia. Moreover, when elevated basophil counts are produced, they must be examined with caution because they can be artifacts due to the presence of abnormal cells such as blasts, plasma cells, and lymphoma cells.

Extended Differential Count

The EDC is the counting of other cell types in addition to the 5 leukocyte populations normally present in peripheral blood, a possibility offered by some analyzers. Currently, the cell types included in the EDC are immature or atypical cells such as blasts, IGs, atypical lymphocytes, hematopoietic progenitor cells (HPCs), and NRBCs.[70] The principal aims of the EDC are to further reduce the need for microscopic revision, to obtain more precise and accurate counts for rare populations with respect to microscopic count, and to allow for differential counts on material with a more complex cell composition, such as marrow blood.

In the past, some hematologic analyzers performed counts of additional WBC populations, including the "large unstained cells" of Technicon-Siemens instruments (Siemens Diagnostics, Tarrytown, NY). The main problem with these counts is the lack of specificity because there is no univocal relationship between these populations and their individual cellular counterparts. The large unstained cells, for example, can alternatively be constituted of blasts, atypical lymphocytes, plasma cells, or, simply, by peroxidase-negative neutrophils. Some manufacturers have developed methods that are sufficiently specific and sensitive for the identification and quantification of certain cell types included in the EDC: HPCs, IGs, and NRBCs.

Hematopoietic Progenitor Cells

The optimal apheresis time point to obtain a sufficient number of peripheral blood stem cells (PBSCs) for transplantation is based on the count of these cells after mobilization using hematopoietic growth factor and chemotherapy. A cutoff varying from 10 to 20 CD34 cells/μL is used to determine the time to harvest.[71,72] The recommended method for stem cell counts is fluorescence flow cytometry with MoAb anti-CD34[71]; this, however, is a time-consuming and expensive procedure and requires skilled personnel.

With the SE-9000 and, more recently, with the XE-2100, Sysmex (Kobe, Japan) proposed an alternative method to use as screening for the HPC count that is quick, does not require MoAbs, and can be used together with the CBC count and LDC. The imprecision of this method is concentration-dependent, with a coefficient of variation (CV) of 24.7% for values near 30 HPC/μL and of 64% for values lower than 15 HPC/μL.[4] The comparison with the method using the anti-CD34 MoAb has furnished acceptable results (r between 0.64 and 0.83).[4,5] This measurement strongly depends on the time between sampling and analysis, with a reduction of up to 50% after 3 hours from collection.[5] Given the time limits for analysis, HPC counts have their maximum clinical use in 2 situations[4]: (1) when HPCs are not detectable after mobilization (in which case it is useless to perform counts with the MoAb method) and (2) when HPC counts are greater than 30/μL because in this case, it is possible to harvest without performing cytofluorimetric quantification for CD34 cells. When the HPC count is between 0 and 30 cells/μL, CD34 enumeration is required.

Immature Granulocytes

The measurement of the immature cells of the myeloid series, specifically "band" cells, is considered clinically useful for the diagnosis of infections, especially neonatal sepsis.[73,74] Even though a morphologic definition of these cells exists, it is not universally accepted.[75] Interobserver variability of the results is so high as to produce different reference intervals,[76] which makes this parameter useless; it is, therefore, not recommended for use in daily clinical practice.[77]

Other immature cells such as metamyelocytes, myelocytes, and promyelocytes, all included in the IG compartment, are better defined morphologically and are identified together with the multicolor flow cytometry method and MoAbs.[78] Because their increase has been proven potentially useful in diagnosing neonatal sepsis,[6] they constitute an alternative to a band cell count.

The IGs, normally absent from peripheral blood, are increased also in other conditions such as bacterial infections, acute inflammatory diseases, cancer (particularly with marrow metastasis), tissue necrosis, acute transplant rejection, surgical and orthopedic trauma, myeloproliferative diseases,

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steroid use, and pregnancy (mainly during the third trimester). In these cases, the increase in IGs is accompanied by an increase in neutrophils, which are freed from the marginal pool and bone marrow. In some subjects, especially elderly people, neonates, and myelosuppressed patients, the increase in neutrophils may be absent, and, in other conditions, such as sepsis, there can even be neutropenia. In these situations, the increase in IGs (>2%), even if isolated, can be useful for identifying an acute infection, even when not suspected.[7] Microscopic IG counts have limits of imprecision and lack clinical sensitivity because these components are usually found in low concentrations (<10%). The Sysmex XE 2100 automated analyzer can count IGs while performing the LDC, with notably lower imprecision (CV near 7%).[7] Accuracy, when obtained from comparison with microscopic examination or flow cytometry with MoAb methods, is also high (r between 0.78 and 0.96).[7,79] Published studies agree that IG counts have a high specificity for infectious conditions (from 83% to 97%) but are accompanied by low sensitivity (between 35% and 40%).[7,8] This low sensitivity means that this count is not indicated as a screening test for infection, even though a significant association exists between elevated IG counts and positive blood cultures.

Nucleated RBCs

Erythroblasts are normally present only in neonatal peripheral blood at low concentrations. They may be present at high concentrations in neonatal hemolytic disease. Studies indicate an increased concentration in premature neonates and neonates affected by hypoxia in the perinatal period.[9,80,81]

NRBCs can, however, be present in numerous conditions, even in adults: thalassemic syndromes, myeloproliferative diseases (specifically, myelofibrosis), bone marrow metastases of solid tumors, extramedullary hematopoiesis, and all of the conditions of hematopoietic stress (eg, septicemia, massive hemorrhage, and severe hypoxia).[9,10] In these situations, their presence is correlated with the severity of the prognosis. In hospitalized patients after general or cardiothoracic surgery or with other nonhematologic disease, the mortality rate was 21.1% for patients with NRBCs, whereas it was 1.2% for patients without. The mortality increased with increasing concentrations of NRBCs.[11] The persistence of NRBCs in the peripheral blood of subjects undergoing stem cell transplantation has been shown to be a poor prognostic factor, and even in this situation the mortality rate increases with the NRBC concentration (100% mortality among patients with an NRBC concentration of more than 0.2×10^9/L).[12] In other cases, the concentration is useful to evaluate the efficacy of transfusion therapy, as with thalassemic syndromes in which it is advisable to maintain an NRBC concentration of less than 5/100 WBCs.[13] Therefore, it is useful not only to identify the presence of NRBCs, but also to estimate the NRBC count.

Traditional microscopic counts, aside from having the typical precision and accuracy limitations of rare cell populations, are performed only by specific request (counting in known clinical situations) and when the sample is specifically flagged. In other situations, these cells can be overlooked. In addition to missed identification, there is an overestimation of the WBC count, and, if the LDC is performed, lymphocytes are also overestimated. In fact, nearly all automated analyzers place NRBCs partially or entirely within the lymphocyte population. Presently, 3 of the most sophisticated analyzers (Abbott Sapphire, Beckman Coulter LH 750 [Beckman Coulter, Hialeah, FL], and Siemens ADVIA 2120) are able to perform the NRBC count by default on all samples for which the LDC is requested. For other analyzers (Table 1), the determination of this parameter must be specifically programmed.

Published results indicated excellent performance in terms of precision (CV <10%) and accuracy, even when the latter is evaluated by comparison with microscopic or flow cytometry and MoAb methods (r between 0.90 and 0.99).[82-86] Detection limits, depending on the analyzer, are between 1 and 2 NRBCs/100 WBCs.[14]

Immature Reticulocyte Fraction

Heilmeyer[87] was one of the first to propose a classification of reticulocytes based on maturation as judged by the quantity of reticulofilamentous particles as seen under a microscope after staining with brilliant cresyl blue. Despite the potential usefulness of a classification based on reticulocyte maturation as an index of marrow erythropoietic activity, this did not have clinical application because the results were not reproducible. Later, it was demonstrated that the reticulum is composed of protein and ribosomal RNA.[88] The introduction of cytometric methods that use dyes that selectively bind RNA and, therefore, are able to generate reproducible signals proportional to the nucleic acid content has reproposed the reticulocyte maturation index.

The term immature reticulocyte fraction was introduced to indicate the less mature reticulocyte fraction.[89] There are, however, various expressions according to the analyzer used. Some divide the reticulocytes into 3 distinct populations and others into only 2 based on RNA content; thus, the reference intervals are different and the comparison of samples analyzed with different techniques can be problematic.[15] Independent of the way in which it is produced, the IRF is an early and sensitive index of erythropoiesis. The greatest clinical usefulness, especially in the classification of anemias based on marrow response, is found using 2-dimensional matrices of IRF vs the absolute reticulocyte count.[90] A particularly useful application during reticulocytopenia is the early identification of marrow regeneration in patients undergoing bone marrow

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transplantation or chemotherapy. This condition with marked reticulocytopenia is characterized by the reappearance of reticulocytes with high RNA content **Figure 1**. In particular, in autologous and in allogeneic transplantation, an increase in the IRF predicts the success of the transplantation even before the increase in absolute neutrophil and total reticulocyte counts.[16,17]

This parameter is useful in distinguishing anemias characterized by increased marrow erythropoiesis, as in acquired hemolytic anemias or the loss of blood that produce an increase in total reticulocytes and in the IRF, from anemias due to reduced marrow activity (ie, chronic renal disease), in which both values are decreased, and from situations such as acute infections and myelodysplastic syndromes in which there is a dissociation between total reticulocyte counts (reduced or normal) and the IRF, which is increased. Other uses include monitoring the efficacy of therapy in nutritional anemias (eg, B_{12}, folates, and iron) because the increase in IRF precedes the increase in total reticulocyte count by several days and the prediction of the increase in peripheral CD34 cells to evaluate optimal timing for stem cell collection following mobilization.[18-21] The ongoing problems regarding the generalized use of this index are linked to the varying analytic sensitivity of different analyzers, which is higher in counters using fluorescence methods, and to the difficulty in comparing results obtained by different models or from counters from different manufacturers.

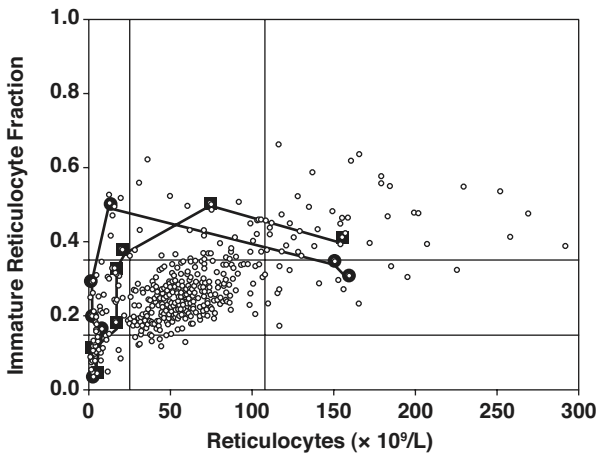

Figure 1 Biparametric plot. Relationship of immature reticulocyte fraction (IRF) and absolute reticulocyte count. The vertical and horizontal lines represent the reference intervals. Superimposed are the trends for 2 patients with an early increase in the IRF after bone marrow transplantation.

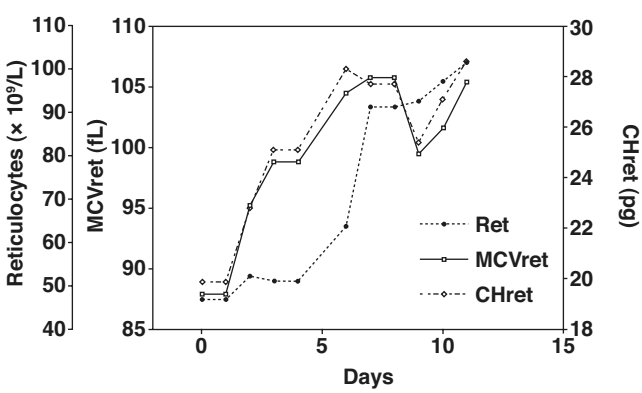

Figure 2 A case of iron deficiency treated with daily intravenous iron. Note the early and parallel response of the mean reticulocyte volume (MCVret) and mean reticulocyte hemoglobin content (CHret). Ret, reticulocyte count.

Reticulocyte Indices

The latest generation of hematologic analyzers provides some reticulocyte indices analogous to the equivalent RBC indices. Among these, the most promising from a clinical viewpoint are the CHr and the MCVr. The CHr, which directly reflects the synthesis of hemoglobin in marrow precursors, is a measure of the adequacy of iron availability.[22] On the one hand, this parameter is important because its reduction indicates iron-deficient erythropoiesis, even in conditions in which traditional biochemical markers such as ferritin and transferrin are inadequate (eg, in cases of inflammation or anemia from chronic disease),[23] and, on the other hand, it is useful for monitoring early response to intravenous iron therapy because it increases significantly after only 48 hours **Figure 2**.[24] Exceptions are heterozygotes for β-thalassemia whose CHr is always reduced independent of iron stores.

Low values of CHr are indicative of iron-deficient erythropoiesis in patients undergoing dialysis[25-27] and even in functional deficits, which appear in patients treated with erythropoietin.[91] CHr is considered the most reliable index of iron deficit and iron-deficiency anemia, even in pediatric populations.[92]

Few studies are available on the clinical usefulness of MCVr. 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.[28,29,93] MCVr decreases and reticulocytes are smaller than the circulating RBCs found in macrocytosis after therapy with vitamin B_{12} and/or folic acid.[29,30] The MCVr multiplied by the number of reticulocytes gives the values of hematocrit-reticulocytes used to evaluate possible abuse of erythropoietin in sports.[31] It has also been noted that a sudden increase in MCVr/MCV ratio was one of the earliest signs of erythropoietic response after bone marrow transplantation.[17,30] Therefore, CHr and MCVr have many overlapping clinical uses.

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Presently, the main limit to the use of these indices is related to the small number of the instruments that can perform them. CHr is only available on the Siemens ADVIA 2120 analyzer, and an equivalent index called the reticulocyte hemoglobin equivalent is available on the XE analyzers manufactured by Sysmex.[94] The MCVr produced by various instruments presents important problems of standardization, which makes it difficult to compare numeric results obtained from analyzers of different manufacturers. In a parallel evaluation, median and reference intervals, respectively, were as follows: 102 and 91-111 fL for the ABX Pentra (ABX-Horiba, Montpellier, France), 108 and 98-120 fL for the Beckman Coulter LH-750, and 106 and 100-114 fL for the ADVIA 120.[32]

RBC Distribution Width

From the RBC volume distribution histogram, modern analyzers calculate an index of heterogeneity known as the RDW, almost always expressed as a percentage coefficient of variation and, less frequently, as the SD. The usefulness of the anisocytosis obtained from the measurement of RBC size (diameter) has been recognized ever since the work of Price-Jones[95]; however, the difficulty in obtaining this parameter limited its application. The possibility of a quantitative, nonsubjective measurement of this index has reawakened interest in many researchers. Bessman et al,[96] in the early 1980s, proposed a classification of anemia based on MCV and RDW. In addition to microcytic, normocytic, and macrocytic, this classification further divides the RBC population into homogeneous (with normal RDW) and heterogeneous (with increased RDW). The former include hypoproliferative anemia, aplasia, and thalassemia heterozygosity; the latter comprise nutritional anemias—deficiencies in iron, B_{12}, and folic acid and sideroblastic anemia. There was large acceptance of this classification, and the RDW was added to routine analysis in many laboratories. Nevertheless, numerous exceptions began to be observed, such as an increase in the RDW in patients with anemia due to chronic infections and at least half of heterozygotes for thalassemia, and, conversely, normal values were seen in approximately 15% to 20% of iron-deficient anemias.[97,98]

There is a wide distribution of RDW values within a given disease, which has diminished its usefulness in differential diagnosis, but its importance as a general marker of abnormality has been maintained.[99] A further complication derives from the method of calculation of the RDW. Under the same name of RDW there are indices that are expressed in entirely different ways: CV percentage for the most part (Abbott, ABX, Beckman Coulter, and Siemens) and also as a direct measurement of the width of the distribution (Sysmex). Even in cases in which the RDW is expressed in the same manner, the reference intervals calculated from healthy subjects differ when calculated by analyzers from different manufacturers **Table 3**[100,101] and, at times, even with different models from the same manufacturer. This is explained by the different algorithms used to "truncate" distribution, which is indispensable for eliminating extreme values often due to artifacts. To make the results obtained from different analyzers comparable, the International Council for Standardization in Haematology has suggested a statistical method for the analysis of cell volume distribution based on fitting a reference log-normal distribution and checking the goodness of fit.[102,103] These proposals have not yet produced results, and, thus, any consideration of clinical use of RDW (eg, diagnosis, differential diagnosis, or monitoring after therapy) must be evaluated by the comparison with reference values established for each model of analyzer.

Schistocytes (FRBCs)

Schistocytes are circulating FRBCs formed as a consequence of mechanical damage. They can be found in the peripheral blood of patients affected by various diseases: from cardiovascular disorders (eg, prosthetic valve and endocarditis) to microangiopathies (eg, thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, disseminated intravascular coagulation, and after stem cell transplantation). Among these, schistocytes in microangiopathies need immediate diagnosis and treatment, and the identification and quantification of schistocytes represents an important diagnostic criterion.[33] FRBC quantification was also proposed for the definition of a grading system for stem cell transplantation–associated microangiopathy[104,105] and for monitoring over time.

However, schistocytes can also be observed in healthy subjects, with differences in upper limits according to different studies: 0.10%,[34] 0.20%,[35] 0.27%,[33] and 0.60%.[106] FRBCs are usually evaluated by the microscopic method, and

Table 3
RDW Reference Intervals in a Parallel Study With Five Analyzers*

Analyzer	Median	2.5th Percentile	97.5th Percentile
Abbott CD 4000	11.6	10.7	13.8
ABX VEGA retic	14.4	12.9	17.8
Bayer ADVIA 120	13.4	12.1	15.0
Beckman Coulter Gen S	13.0	11.9	15.3
Sysmex SE 9500 ret	13.3	12.3	14.9

RDW, RBC distribution width.
* 220 healthy subjects.[101]

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the observed differences can depend on a lack of standardization of the morphologic definition of schistocytes and on high imprecision in counting because of their low concentration. A CV between observers of 50% was reported for a schistocyte concentration of 10%.[107]

Two recently commercialized analyzers (Siemens ADVIA 2120 and Sysmex XE-2100) offer the possibility of direct, nonsubjective quantification of FRBCs, on a routine and an urgent basis. The former uses the RBC/PLT (platelet) channel on which the schistocytes correspond to particles with volume smaller than 30 fL and with a refractive index greater than 1.40[35] (to differentiate them from large platelets); the latter uses the reticulocyte channel, and the schistocytes are gated from the RBC area as the smallest events with low RNA content.

In both analyzers, FRBCs are identified only on the basis of size and hemoglobin content, independent of their shape; therefore, other particles such as small RBCs or even membrane fragments can be included in the count. Published studies show good correlation between the automated and microscopic methods (r from 0.73 to 0.95), even though there is a general tendency toward overestimation.[34,35,108,109] The imprecision is lower than in the visual method and is concentration-dependent, with CVs of 1.42% and 6% for schistocyte concentrations of 13% and 2.1%, respectively.[108]

The sensitivity for diagnosis of microangiopathy depends on the selected threshold and is excellent (between 91.8% and 100%), but, according to the type of analyzer, the specificity is lower (from 20% to 52.2%).[34,35] In consideration of their high negative predictive value, the automated methods can be useful for screening purposes (when clinically appropriate), but a microscopic examination to confirm schistocyte presence is needed for positive results.

Platelet Indices

Circulating platelets are very different in size, metabolism, and functional activity. The largest are more reactive and produce a greater quantity of thrombogenic factors.[110,111] Automated counters provide platelet counts and generate the MPV and a measure of their size variability (PDW). The great dispersion of platelet volumes (log-normal distribution) depends on the process of platelet production, by fragmentation of cytoplasm of megakaryocytes and proplatelet formation.

Platelet volume seems to be correlated with megakaryocyte ploidy, even though the exact mechanism is not completely known. The increase of MPV in conditions with increased platelet turnover is probably mediated by several cytokines (interleukins 6 and 11 and thrombopoietin) that affect megakaryocyte ploidy and result in the production of larger and more reactive platelets.[112,113] Whether platelets recently released from bone marrow are larger and tend to shrink as they age remains controversial.

In healthy subjects, there is a nonlinear inverse correlation between MPV and platelet concentration: MPV tends to decrease in subjects with higher platelet counts.[114] This relationship is such that the platelet mass is relatively constant within a large interval of platelet counts. The MPV reference intervals should, therefore, be expressed as a function of platelet concentration. This wide dispersion of normal values limits the usefulness of MPV as a screening test to clinical conditions characterized by extreme values such as some hereditary thrombocytopenias (eg, Wiskott-Aldrich syndrome, in which there are decreased values, and Bernard-Soulier syndrome, in which values are increased). In the differential diagnosis of acquired thrombocytopenia, we can distinguish forms with increased MPV (of peripheral origin with increased platelet production and normal megakaryocyte function: immunologic thrombocytopenic purpura and disseminated intravascular coagulation) from those with normal or decreased MPV (in which there is a defect in platelet production: acute leukemia, bone marrow aplasia, and chemotherapy or radiation therapy).[36,115]

The MPV is useful also for monitoring recovery in thrombocytopenias because of an early increase with respect to the platelet concentration,[115] even though not all analyzers can provide this parameter in cases of severely low platelet counts. Because an increase of the MPV is a known marker of platelet activation, several investigations have been performed to verify if this increase is associated with a risk of thrombotic diseases.[116-121] The results have been controversial.

An increase in the MPV is considered an independent risk factor for myocardial infarction in patients with coronary disease[116] and for death or recurrent vascular events after an acute myocardial infarction.[117] Other studies have shown an increase of MPV in patients with acute ischemic stroke, but the association between elevated values and stroke outcome is a matter for debate.[118,119] Elevated MPV values have been reported in subjects with type 2 diabetes, particularly in subjects with vascular complications,[120] but, in contrast, another recent work has shown that there is no difference in MPV between healthy control subjects and patients with diabetes with or without vascular complications.[121]

In healthy populations, there is a direct relationship between MPV and PDW; this relationship is maintained in idiopathic thrombocytopenic purpura and chronic myeloid leukemia, in which both are increased. This does not occur in hypoplastic anemias or megaloblastic anemia or during chemotherapy, in which the MPV decreases with an increasing PDW. The PDW can also be useful in differentiating reactive thrombocytosis from the essential type, especially when it is combined mathematically with the MPV and platelet count to obtain a discriminant function.[122]

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The recommended anticoagulant for a CBC determination including platelet indices is K_2 or K_3-EDTA.[123] When blood comes in contact with EDTA, platelets rapidly change shape from disks with diameters of 2 to 4 μm to spheroids covered with filamentous extensions. The platelet spherical transformation is initially isovolumetric, but within 1 or 2 hours, the volume progressively changes to reach an equilibrium condition, even if not definitive. As a consequence, the MPV increases (from 7.9% within 30 minutes to 13.4% over 24 hours)[124] if measured by the impedance method or decreases by nearly 10% when measured by the optical method, probably owing to the dilution of the cytoplasmic content with a decrease of the refractive index. Various attempts to mathematically correct for this phenomenon have failed owing to the unpredictable behavior of individual samples in terms of intensity and time to equilibrium.

With the use of EDTA, the MPV is, therefore, not a very reliable index.[125,126] The same considerations hold true for PDW, which in certain counters can be influenced by platelet concentration—the analysis of platelet size distribution becomes problematic in thrombocytopenic samples. The lack of standardization and the dependency of results on preanalytic variables and on the measurement method used requires different reference intervals[100] and allows for poor comparison of clinical studies carried out in nonstandard conditions. As a result, despite the many articles published regarding the possible clinical usefulness of platelet indices, in daily practice, they must still be considered little more than experimental.

Reticulated Platelets and Immature Platelet Fraction

Newly released platelets are more reactive than mature platelets and contain RNA. Owing to this similarity with reticulocytes, they were called reticulated platelets.[127] The number of reticulated platelets is related to thrombopoiesis, increasing with increased production and decreasing when production declines. In animal models, it has been observed that reticulated platelets remain in the bloodstream for approximately 24 to 36 hours, during which there is a progressive degradation of RNA and a decrease in volume.[128] With the use of flow cytometers and fluorescent dyes that can bind RNA, it is possible to count reticulated platelets, yet based on the fluorochrome used and the counting conditions, the published reference intervals can vary greatly (from <3% to 20% of the total platelet count).[129,130] Despite evident standardization problems (eg, lack of a reference method and control material), there are numerous potential clinical applications of this parameter for diagnosis and monitoring.

It is most useful for distinguishing thrombocytopenia due to peripheral platelet destruction or acute blood loss, in which the percentage of reticulated platelets is increased, from forms of marrow insufficiency (eg, marrow hypoplasia or aplasia and cytotoxic chemotherapy), in which the percentage is no different from that in control samples[36]; the reported sensitivity and specificity are more than 95%.[131] The increase in reticulated platelets is, thus, an early indicator of platelet destruction in patients with immune thrombocytopenic or thrombotic thrombocytopenic purpura.[37] Following chemotherapy, the increase of reticulated platelets occurs 1 to 3 days before total platelet recovery.[132] A reticulated platelet value of 7.7% was reported as the best threshold in the diagnosis of immune thrombocytopenic purpura and in the recovery phase after chemotherapy, with a sensitivity of 86.8% and a specificity of 92.6%.[38] This parameter has proven to be more reliable than the MPV in predicting marrow recovery.[36,38] For PBSC or allogeneic bone marrow transplantation, the increase in the IPF precedes the increase in the total platelet count on average by 4 to 4.5 days.[39,40,133] The possibility of predicting platelet regeneration a few days after an increase in immature platelets makes it possible to reduce prophylactic platelet transfusion in patients undergoing PBSC transplantation or receiving chemotherapy.[39,41,42]

An increase in reticulated platelet values might reflect increased thrombotic risk in thrombocytosis, both reactive and that caused by chronic myeloproliferative diseases.[43] Moreover a low percentage of reticulated platelets observed in hepatic cirrhosis seems consistent with decreased bone marrow function, so that it can be hypothesized that the low platelet count associated with this pathology is not due only to an increase in splenic sequestration.[44] The insufficient standardization and the need for fluorescence flow cytometry with a specially dedicated staff have limited this test to a few specialized laboratories. The Sysmex XE 2100 hematologic analyzer, with dedicated software and fluorescent dyes, is able to count reticulated platelets together with the reticulocytes, indicating them as the IPF percentage, thus making this parameter available to general clinical laboratories in real time. This measurement is stable in EDTA-treated samples stored at room temperature for at least 12 hours. The imprecision is concentration-dependent (between 4.9% and 22%), and the reference interval for healthy adult populations is between 1% and 8%.[37-39]

Conclusions

The technological evolution as applied to hematology analyzers has provided new opportunities, ie, reticulocyte indices, and has certainly contributed to making other parameters more reliable, such as reticulocyte and platelet counts. Moreover, it is possible to extend the differential count beyond the 5 normal WBC populations. The possibility of

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determining the fraction of immature platelets by using a simplified method opens the door to new applications. It is also desirable that, as with the high standardization for basic CBC parameters, a continued effort be made for the parameters (ie, RDW, IRF, MCVr, and MPV) for which results provided are still too different when produced by different analyzers. To reach these goals, cooperation between long-standing (ie, International Council for Standardization in Haematology and the National Committee for Clinical Laboratory Standards, now the Clinical and Laboratory Standards Institute) and recent (International Society of Laboratory Hematology) organizations interested in hematologic standardization and the manufacturers is fundamental. It should be remembered that despite the essential role of automation in the modern hematology laboratory, microscopic control of pathologic samples remains indispensable, so much so that in certain cases, it alone is diagnostic.[134] Moreover, knowledge of the limits of the specific analyzer in use is of paramount importance for the correct interpretation of results. These considerations require that clinical laboratories performing hematologic diagnostics have personnel with specific training and profound knowledge in laboratory hematology.

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