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REVIEW ARTICLE

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Diagnostic pitfalls and challenges associated with basic hematological tests

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Abstract

Several generations of automated hematology analyzers are currently being used to determine a wide range of hematological parameters. As their results form the basis of many medical interventions, it is required that they undergo analytical validation. Samples flagged as being pathological or non-diagnostic require re-testing in a different mode, revision, or additional diagnostic workup (e.g. microscopic smear). In order to avoid mistakes, close cooperation and continuous communication are needed between laboratory and medical staff. To address this, in this review we discuss the most frequent errors and pitfalls associated with the preanalytical and analytical phases of basic hematological tests. While not all diagnostic pitfalls are avoidable, this guidance regarding potentially problematic diagnostic situations will allow for their quick verification at the laboratory stage. An awareness of the causes of errors and of the existence of pitfalls can lower the costs of analytical procedures by minimizing the need to repeat analyses of potentially pathological samples, and have a positive impact on patient safety. In addition, reducing the potential for laboratory errors can improve the accuracy of medical diagnoses and avoid unnecessary treatment.

Key words: complete blood count, diagnostic pitfalls, hematological parameters, laboratory errors

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Introduction

Although the most important diagnostic component of hematological disorders is medical examination, according to the rules generally applicable in internal medicine a significant role in establishing the diagnosis and implementation of treatment is nevertheless played by laboratory tests.

Analytical results are therefore the basis of many medical interventions, and it is of paramount importance that they are free from laboratory errors which can be a defect occurring at any stage of the laboratory cycle [1]. Laboratory errors can take place in the preanalytical, analytical, or postanalytical phase, i.e. from the moment of ordering the tests to reporting their results and interpreting them. The preanalytical phase includes ordering the test, collecting the material, identifying the patient and the sample, and transporting, storing, fractionating and/or pre-processing the sample. The analytical phase comprises the sample processing procedure directly associated with the assessment of the analyzed parameter, while the postanalytical phase consists of the reporting of results and their analysis by the physician. The evidence indicates that the pre- and postanalytical phases are more likely sources of error than the analytical steps [2].

Results discordant with the true clinical condition of a patient can be caused by incorrect collection of laboratory material, such as insufficient blood volume or blood sampling from cannulas, as well as by the use of the wrong anticoagulant or excessive storage of material from collection to processing. As a consequence of laboratory errors, blood resampling may be necessary in order to avoid incorrect diagnoses [1]. Therefore, to avoid unexpected

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laboratory pitfalls, it is important for laboratory staff to pay attention to such data as patient age and sex, to check the results of each sample, and to compare them to results obtained for the same patient in previous analyses; the diagnostician should also be aware of the properties of the reagents used and the principles of their measurement. In addition, close cooperation is needed between laboratory and medical staff.

The most basic test performed in a hematological laboratory is the complete blood count (CBC) with differential white cells count. Recent technological progress in hematological analyzers has greatly increased the range of parameters that can be estimated [2]. However, despite the high degree of automation in modern hematological laboratories, and the consequent decrease in analytical errors, some factors related to blood sampling procedures or principles of measurement for example can still distort the results of tests. Such problems are typically referred to as 'hematology pitfalls'. Therefore, it is very important to know which of these may be encountered by laboratory staff, and how they can be avoided.

This review presents the most common pitfalls encountered primarily in the pre-analytical and analytical phases of the determination of basic hematological parameters.

Laboratory pitfalls in preanalytical phase

Although each laboratory method must be standardized to yield results that are both reproducible and comparable between laboratories, errors related to the preanalytical phase account for up to 70% of all laboratory errors [3]. The preanalytical phase of a laboratory diagnostic test is influenced by many factors, including any activities performed by the patient before blood sampling. Blood samples should always be collected at the same time of day to avoid diurnal variations of the parameter tested. As a general rule, samples should be collected between 7am and 9am, and at least eight hours after the last meal, and, if possible, before taking medications [4]. The exact time of blood collection should be marked on the test order. It should be remembered that CBC results can also be influenced by other factors including age, gender, pregnancy, inflammatory diseases, time of day, alcohol intake and medications. Correct labeling of the tube is very important as well.

Factors that can lead to preanalytical errors include:

- errors in preparing the patient for the test;
- missing or incorrect patient data, including incorrect tube labeling;
- use of the wrong anticoagulant;
- use of the wrong type or size of tube;
- incorrect collection or excessive storage of material from collection to testing, or insufficient mixing of the sample with the anticoagulant;
- improper sample transportation and storage;
- the presence of factors interfering with diagnostic reagents such as hemolysis, jaundice, lipemia or presence of lupus anticoagulant;
- lack of communication between laboratory and medical staff.

Anticoagulants and collecting material

There is no universal anticoagulant for all blood tests, but the most commonly used anticoagulant in hematological tests is ethylenediaminetetraacetic acid (EDTA). Biochemical tests are mostly performed on serum, and molecular tests with EDTA. Citrate is used especially for coagulation tests, while heparin is commonly used in cytogenetic analyses. Some anticoagulants cover the inner wall of the test tube, while others may be added to the tube as a solution [5–8].

To avoid diagnostic errors, it is important to select an appropriate anticoagulant and to ensure that the correct blood volume is drawn. It is also important to check the expiry date of the tube before blood collection. In expired anticoagulant tubes, there is the risk of disrupting the proportion between anticoagulant and blood sample, which can lead to a false result.

EDTA

Three different sub-types of EDTA are in common use: Na_2EDTA, K_2EDTA and K_3EDTA [5]. Of these, K_2EDTA (edetate dipotassium dihydrate) is recommended by the International Committee for Standardization in Hematology (ICSH) [5–8] as the anticoagulant of choice for hematological tests. It is also routinely used in blood banks for blood group testing and Rh typing, or for antibody screening. Being a calcium chelating compound, the presence of K_2EDTA can interfere with some ion tests, e.g. zinc or magnesium binding. Therefore, it is very important to achieve an optimal ratio between the volumes of blood and EDTA in the test tube. Fresh human whole blood samples, anticoagulated with K_2EDTA (or K_3EDTA), should be used and processed within 4–8 h after blood sampling if stored at room temperature. If samples are refrigerated, hematological parameters are stable for longer [9]. An inadequate EDTA volume may lead to false results of red blood cell (RBC) parameters and potential clotting, while excessive EDTA volume may result in changes in erythrocyte morphology and the formation of echinocytes in the peripheral blood smear due to hypertonic constriction [5].

However, EDTA is not an ideal anticoagulant for platelet (PLT) evaluation. In the presence of EDTA, the PLTs change shape from discoid to spherical within about 60 minutes of blood collection and stabilize within about three hours, leading in turn to disturbances in mean platelet volume (MPV) and overestimation of MPV. Another diagnostic problem related to PLTs is EDTA-induced pseudothrombocytopenia caused by the presence of EDTA-dependent anti-platelet

antibodies in the serum of some individuals, which bring about platelet aggregation and thus a false low count value. It should be remembered that PLTs aggregation does not occur immediately after blood collection; only a slight reduction may be noted by the analyzer within the first few minutes. A more significant decrease in PLTs count is observed within three hours of blood collection. Therefore, if pseudothrombocytopenia is suspected, blood should be collected to the tube using another anticoagulant, usually citrate. However, in some cases, the PLTs count determined immediately after blood collection can be slightly lower in citrate than in an EDTA sample [5]. This can be caused by the blood being diluted by the citrate, which is a liquid anticoagulant (see below) or by accidental centrifugation of the sample for PLTs evaluation [6]. It is important to underscore that a simultaneous determination of the PLTs count from blood drawn on EDTA and citrate should be performed in order to exclude, or confirm, the existence of pseudo EDTA-dependent thrombocytopenia.

Heparin

Heparin acts mainly by creating a bond with antithrombin III, which can interfere with some antibody-antigen reactions. In order to obtain high-quality heparinized plasma samples and to avoid fibrin formation, it is recommended to use lithium heparin at a final concentration of 10–30 USP units per 1 mL of blood. This concentration leads to effective anticoagulation. Higher concentrations of heparin are no more efficacious, and have no effect on a range of the most commonly-requested blood parameters [7]. Tubes containing sodium or lithium heparin are commonly used for blood gasometry, ionized calcium tests, cytogenetics and plasma analysis in clinical chemistry. Heparin is, however, unsuitable for some tests such as coagulation or Wright's stained blood smears, as it can cause staining artifacts (i.e. the smear may become too blue), which affect blood smear examination [8].

Sodium citrate

Sodium citrate is a standard anticoagulant for blood coagulation tests, such as activated partial thromboplastin time (APTT) and prothrombin time (PT), as well as for the classic Westergren erythrocyte sedimentation rate (ESR). Trisodium citrate forms complexes with calcium ions, and stabilizes the labile coagulation factors V and VIII. Sodium citrate solutions are typically used in two concentrations, 3.2% and 3.8%, which are available in buffered or not-buffered liquid forms. The tubes containing the citrate are calibrated to maintain a blood-to-citrate ratio of 9:1 for both the abovementioned concentrations of citrate. These are recommended for coagulation tests [10]. The stability of the citrate samples is satisfactory only up to three hours after blood collection. During analysis, a correction factor of 1.17 must be applied to account for citrate dilution of the blood sample [5, 11]. In addition, as sodium citrate dilutes the blood sample, it is generally unsuitable for most other hematological tests.

In addition to the choice of anticoagulant, it is also important to choose the right type of test tube for the type of blood test. To avoid contamination by anticoagulants during material collection, peripheral blood for different types of tests should be drawn in the correct order. The recommended order of blood collection for various tests is [12]:

- tube for bacteriology;
- tube for coagulation tests with sodium citrate — always as a second tube (when no tube for bacteriology was collected, a non-additive tube should be used first);
- tube with clot activator, or tubes without anticoagulant for chemistry, immunology and serology tests;
- tube with lithium heparin for cytogenetics or gasometry;
- tube with EDTA for CBC;
- tube with acid-citrate-dextrose (ACD, ACDA or ACDB) for human leukocyte antigen (HLA) tissue typing, paternity testing and DNA studies;
- tube with sodium fluoride for glucose test;
- tube for Westergren erytrocyte sedimentation rate (ESR).

The main consequences of incorrect blood collection are hemolysis, bacterial contamination, and platelet aggregation. Of these, hemolysis seems to be the most common problem, usually occurring at the preanalytical stage. Hemolysis *in vitro* may be induced by several factors, and may be aggravated by forced aspiration of blood into vacuum tubes. The aspiration method is believed to be a safer sampling method, especially for patients during chemotherapy, or when venipuncture is difficult, when a vacuum approach could increase the chance of false hemolysis. Lippi et al. [13] reported a significant increase of serum potassium concentration and lactate dehydrogenase (LDH) activity after the collection of blood into vacuum tubes compared to aspiration ones. In addition, excessive time from blood collection to test performance can also lead to hemolysis and the artificial elevation of serum potassium concentration. However, both the aspiration and the vacuum method can result in significant microhemolysis in samples [13].

The probability of hemolysis is further increased by using an under-gauge needle (23G or smaller), or taking a blood sample from an intravenous cannula or central line. Additionally, excessive pressure in the syringe when drawing blood into the tube results in the destruction of a number of RBCs and an underestimation of their count. Other common errors include the collection of a blood sample before complete drying of the disinfectant agent used on the skin, or too intensive mixing of the tubes with collected blood. In blood from pediatric patients, hemolysis can also be caused by the use of an oversized tube or syringe (10–20 mL) [12].

In vitro hemolysis, occurring as a result of incorrect blood collection, is characterized by reduced RBCs count and lowered hematocrit (HCT) value with normal

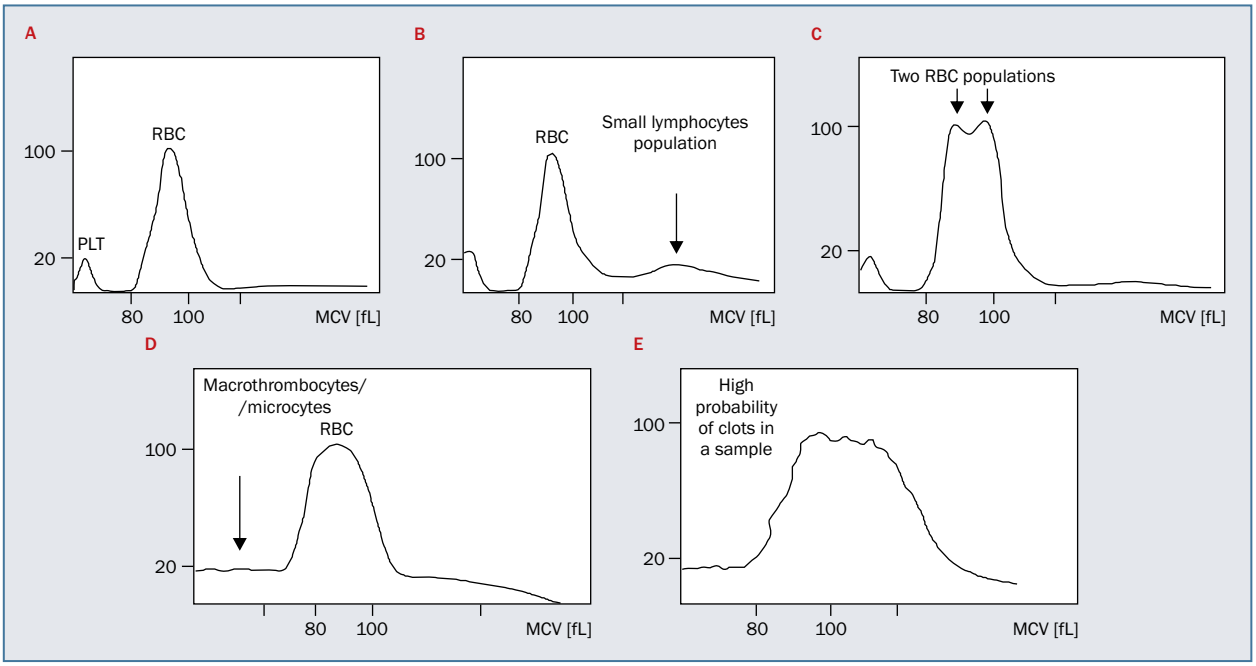

Figure 1. Examples of red blood cells (RBC) histograms: **A.** Normal histogram, normocytosis; **B.** Histogram in micro- and macocytosis; **C.** Histogram distorted by macrothrombocytes, clots, microclots, hemolysis; **D.** Histogram distorted by cold agglutinins; **E.** Lack of separation of cells into individual populations; PLT — platelets ; MCV — mean platelet volume

hemoglobin (HGB) concentration. Such changes can result in incorrect estimation of certain parameters, such as mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). It should be also remembered that *in vivo* hemolysis is indicated by the presence of reticulocytosis and true fragments of RBCs (FRBCs). In contrast, in *in vitro* hemolysis, damaged RBCs are counted by the analyzer as FRBCs ('pseudo' FRBCs) [14, 15].

Transport and storage

Transport and storage of the collected materials also play important roles in the analytical process. The ICSH recommends that samples should be stored at 4°C [16]. The stability of hematological parameters depends on the type of test, method, and the technology of hematological analyzers and reagents used for analysis. Therefore, it is recommended to follow the instructions provided by the manufacturer. Blood samples should be transported in special containers which ensure the right temperature. The recommended time between blood collection and test performance must not be exceeded, as excessive transport time, especially when the ambient temperature is higher than 22°C, can lead to false test results [17].

Diagnostic pitfalls in analytical phase (CBC parameters and microscopic smear)

Modern automatic hematological analyzers employ a range of technologies to determine CBCs parameters, including impedance, spectrophotometry, optical methods and flow cytometry [18, 19].

In addition, laboratory pitfalls observed during the analytical phase may concern many aspects of cell morphology. Impedance-based analysis can simultaneously assess RBCs and PLTs count in one detector.

Figure 1A demonstrates the normal RBCs histogram. However, high leukocytosis with the presence of a small lymphocyte population can falsify RBCs count (Figure 1B). The presence of two RBCs peaks at the histogram indicates two populations of erythrocytes, which may occur after blood transfusion (Figure 1C). The presence of cryoglobulins, macrothrombocytes (giant PLTs), high leukocytosis and low plasma volume (hypovolemia) can result in an overestimation of RBCs count. In the case of the presence of a population of macrothrombocytes, a clear separation of the RBCs from the PLTs population is not seen on the histogram, and the curve corresponding to macrothrombocytes is shifted above the baseline. Additionally, the presence of microcytes is classified by the analyzer as PLTs (Figure 1D). The RBCs count can be lowered by the presence of blood clots or microclots in the tube which may be induced by the cold agglutinins or *in vitro* hemolysis due to the presence of artifacts, e.g. fragments of damaged cell. The histogram shows a lack of separation of the cells into individual populations (Figure 1E). Current automatic hematological analyzers are equipped with the option of heating the sample to 37°C, which allows the influence of cold agglutinins to be excluded. Additionally,

Table I. Most common causes of false increases or decreases in selected parameters associated with complete blood count (based on [20–22])

Parameter	↑ false increase	↓ false decrease
Red blood cells (RBC)	Cryoglobulins	Clots or microclots, hemolysis, cold agglutinins, blood dilution
Hematocrit (HCT)	Cryoglobulins, hyperglycemia	Clots or microclots, hemolysis, autoagglutination of erythrocytes, excess of EDTA in tube
Hemoglobin (HGB)	Carboxyhemoglobin, high leukocytosis, cryoglobulinemia, hyperbilirubinemia, hyperlipidemia	Clots and microclots
Platelets (PLT)	Hemolysis in presence of very small erythrocytes (microcytes), samples contaminated by physical or biological factors (artifacts), too intensive mixing of samples	EDTA-related pseudothromocytopenia, platelet aggregation, platelet degranulation or even their complete degradation, presence of large macrothrombocytes, too long time from blood collection to analysis (MPV ↑), blood dilution, clots and microclots
White blood cells (WBC)	Cryoglobulins, hyperlipidemia, or presence of erythroblasts, very large PLTs and platelet aggregates	Clots or microclots, blood dilution, excessive degradation of some WBCs, agglutination of WBCs in presence of EDTA

EDTA — ethylenediaminetetraacetic acid; MPV — mean platelet volume

underestimations of RBCs count can also result from blood dilution caused by the drawing of blood from the drip infusion site or an increase of patient intravascular liquid volume (hypervolemia) [20].

In automated analyzers, HCT is calculated as the sum of each RBCs volume passing through the detector of the analyzer in a given time [20]. A falsely elevated HCT value may be caused by similar factors to those responsible for the increase of RBCs counts, as well as hyperglycemia above 600 mg/dL [18]. In contrast, a false lowering in HCT value can be caused by excess EDTA in the tube, autoagglutination of erythrocytes, or the presence of a clot or hemolysis in the tube [20] (Table I).

The cyanmethemoglobin method is recommended by the ICSH for the measurement of HGB concentration, a basic diagnostic parameter in any CBC [19, 20]. However, falsely-elevated HGB concentrations can result from the presence of more than 10% carboxyhemoglobin, high leukocytosis, cryoglobulinemia, hyperbilirubinemia and hyperlipidemia, while underestimated HGB values can be associated with the presence of clots and microclots in the tube [20]. In cases of major intravascular hemolysis, mechanical hemolysis associated with artificial heart valves or hemolytic anemias associated with blood transfusions, the presence of free HGB concentration in plasma may be elevated enough to affect HGB measurement by the analyzer. If free plasma HGB concentration is above 200 mg/L, the only reliable parameter is RBCs count [21] (Table I).

Nucleated red blood cells (NRBCs) are not only found in the blood under pathological conditions, they can also be observed under physiological conditions, such as after major hemorrhage or in newborns. They are counted in the appropriate channel of the analyzer under the influence of the lysing fluid, which disintegrates the erythroblast membrane without disturbing the cell nucleus. Specific fluorescent markers labeling nucleic acids can be used to avoid counting NRBCs as PLTs or, if they are large enough, as WBCs [21–23].

Reticulocytes can be separated from mature RBCs, WBCs, and NRBCs by means of frontal scattering light and a fluorescent signal. However, in nearly 9% of cases, the number of reticulocytes can be falsely overestimated in automatic analyzers [24]. Reticulocyte count has been found to be associated with the presence of parasites such as malaria and drug-induced autofluorescence. Reticulocyte count can also be falsely lowered due to the presence of FRBCs in the sample, among other causes, although such disturbances can be detected by modern automatic analyzers which employ alarm alerts and flagging algorithms.

Overestimated PLT values are observed in cases of hemolysis (FRBCs presence) and in the presence of very small erythrocytes (microcytes), which are counted as PLTs instead of RBCs by automatic analyzers. In addition, some physical factors, or bacteria and fungi in the blood as biological contaminants, can also be counted by the analyzers as PLTs, resulting in a falsely elevated count. In contrast, PLTs counts can be underestimated due to EDTA-related pseudothromocytopenia, spontaneous platelet aggregation, platelet satellitism, the occurrence of platelet degranulation or degradation, and the presence of large macrothrombocytes counted as RBCs instead of PLTs (Table I).

A false decrease in platelet count due to PLTs aggregation is a common phenomenon, and is often caused by excessive time from blood collection to analysis; this may result in an overestimation of MPV. In contrast, excessive mixing of the sample can lead to PLTs degradation and thus a decrease in MPV [18]. Additionally, as mentioned above, the influence of anticoagulant on MPV values depends on the method used; for example, MPV may be slightly overestimated when an impedance method is used. Therefore, a light scattering-based method is recommended to check the accuracy of determination of all PLTs parameters [5, 25].

As in the case of EDTA-related thrombocytopenia discussed above, the presence of EDTA can lead to agglutination of WBCs and so to a falsely-lowered WBCs number [20]. WBCs values may be increased by the presence of cryoglobulins, hyperlipidemia, erythroblasts and very large PLTs and platelet aggregates, which can be counted by the analyzer as WBCs [21]. In contrast, falsely lowered WBCs values are mainly caused by the presence of blood clots, blood dilution, or excessive degradation of some WBCs, but also by the occurrence of pseudo-neutropenia deriving from the abnormal distribution of granulocytes in the circulation, when a significant number of granulocytes shift from the bloodstream onto the wall of the blood vessels. The use of hydrocortisone causes a shift of granulocytes back from the vessel wall to the circulation, resulting in the WBCs count returning to normal values (Table I).

The number of WBCs needed to influence HGB concentration remains poorly understood. Some authors have suggested that leukocytosis of 250 G/L can interfere with HGB concentration, while others suggest that values of 100 G/L or even 50 G/L can falsely increase the true HGB value [20, 21]. In patients with hematological disorders, especially in the case of leukopenia or leukocytosis, a flagged CBC is reported. In such cases, a differential WBCs count should be performed on a peripheral blood smear assessed under a microscope.

It is essential to prepare and stain the smear properly: inadequate dye proportions and incorrect staining times can result in the granules in the cells being too dark or even completely obscured. Such improper staining of the blood smear can result in interpretation errors e.g. blast cells may be taken for lymphocytes or *vice versa*.

Several factors can influence smear quality. For example, if the blood drop is too small or if the smearing is too slow, or if the smearing slide is applied to the blood drop at the wrong angle, the procedure can result in a thin smear, distorted erythrocytes or white blood cells being displaced onto the side edges and feathered edge (tail) of the slide. A similar effect can be observed when the HCT value is low, e.g. in anemia. In contrast, blood with high HCT values (e.g. in patients with polycythemia) may result in thick smears, making it difficult to evaluate erythrocyte morphology.

The presence of FRBCs in a peripheral blood smear indicates pathology, and usually requires urgent medical attention. In newborns however, both schistocytes and erythroblasts can be present in the peripheral blood in physiological conditions [26–30]. State-of-the-art analyzers are able to estimate the number of FRBCs by giving both their percentage and absolute value. The presence of schistocytes may result in anisopoikilocytosis [29, 31, 32]. Studies have noted examples where automatically-counted FRCs have been overestimated after PLTs transfusion [26, 27–32].

The distribution of WBCs may be presented as an absolute value or as a percentage. However, it should be underscored that the percentage of the CBC values has no clinical significance, and should not be taken into account at all as long as we have the absolute values, which are the parameters directly counted by the analyzers. An increase of the percentage of the population of WBCs does not necessarily lead to an increase in total WBCs number. Changes in WBCs percentage distribution, e.g. high lymphocyte counts in adults with normal or only slightly increased total WBCs counts, may indicate a viral infection but also some hematological disorders [e.g. monoclonal B-cell lymphocytosis (MBCL) or small lymphocytic lymphoma (SLL)]; this can also be noted in children [33, 34]. In such cases, it is recommended to use absolute values instead of percentages because they provide more accurate diagnostic information.

If the blood sample is mixed too vigorously, this can result in a significant increase in PLTs count due to the disruption of RBCs; these fragments are counted as PLTs by the analyzer. In contrast, insufficient mixing results in clot formation and thus false underestimation of WBCs, PLTs, RBCs count, HCT and HGB concentration [35].

Quality evaluation of hematological tests

In hematology, normal peripheral blood can be used as a control to calibrate hematological analyzers.

Quality evaluation of hematological tests requires the use of 3-level quality controls which enable the laboratory to minimize the risk of analytical errors and assess the linearity of the determinations. While day-to-day intra-laboratory control is the responsibility of laboratory staff, further calibration is usually performed by an external technical service responsible for the device. The results of the daily intra-laboratory control can be presented graphically using Levey-Jennings charts. The Westgard rules should be used to interpret the results of control material [36, 37].

Conclusions

Thanks to recent technical progress, modern analyzers are capable of fully-automated digital assessment of blood cell counts and blood smear staining. However, despite this

high degree of automation in medical laboratories, the results of laboratory tests can be influenced by a number of factors which may be sources of error. A thorough knowledge of preanalytical phase variables and their impact on the results of hematological tests and/or analytical phase pitfalls is necessary to obtain accurate results which reflect the patient's true condition and to minimize the need to repeat analyses of potentially pathological samples to avoid unnecessary treatment and ensure proper medical care.

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Authors' contributions

AK — writing the article, literature analysis and interpretationfinal, approval of article; EZ — literature analysis and interpretation, writing the article; AKW — writing the article, critical revision of the article, final approval of article.

Conflict of interest

The authors have no conflict of interest to declare.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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