Hematology: A Review of the Main Methodologies of Clinical Analyses

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Abstract Human blood is composed of liquid and solid elements, blood cells being extremely important for the assessment of the patient's health status. Blood consists of plasma and cellular elements, which include blood cells and cellular fragments. Erythrocytes are blood cells containing a large amount of hemoglobin which is the pigment responsible for the transport of oxygen, being numerous and the most found in our blood. Leukocytes are colorless cells whose main function is to defend our organism, still emphasizing that there is not only one type of leukocyte, but it is possible to identify five distinct types. In this scenario, the exam that analyzes blood cells is called a blood count, and for this reason, it is the most requested laboratory exam in the medical routine. Based on this, explaining the methodologies of this exam as well as performing its interpretation is essential to support scientific research and provide a background for health professionals in academic training.

Keywords Blood cells · Hematology · Blood count · Hematological devices · Cellular biology

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1 Introduction

The blood count is a laboratory test that is highly requested in the medical routine, as it directly provides the diagnosis of pathologies or is used as an indicator for the detection of various diseases. This exam is formed by the erythrogram, leukogram and platelet, which evaluate the quantity and morphology of erythrocytes, leukocytes, and platelets, respectively [1].

The erythrogram is the part of the blood count responsible for evaluating the total number of erythrocytes (RBC), hemoglobin (Hb), hematocrit (Htc), and hematimetric indices: Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Hemoglobin Concentration Mean Corpuscular (CHCM). The total erythrocyte count is used to detect hematological disorders. Reductions in the number of red blood cells are called erythropenia, which is a strong indication of the presence of anemia or blood loss. On the other hand, erythrocytosis is characterized by an increase in the production of erythrocytes, either by genetic or external factors, is responsible for the appearance of polycythemias [1, 2].

Anemias can be classified either of genetic origin (falciform anemia, thalassemia, spherocytosis anemia, among others) or by food deficit (iron deficiency anemia, megaloblastic anemia, among others). Both types inspire care and medical follow-up, as food anemias cause indisposition, decrease in work performance, pallor and vertigo. In turn, genetic anemias need lifelong care, as they are responsible for pain crises, hospitalizations and the need for periodic blood transfusions. Some types of polycythemia are of genetic origin and, like genetic anemias, need continuous care, as it is characterized by the exacerbated production of erythrocytes, causing damage to the organism such as infarction and strokes [3–5].

Hemoglobin is a protein present inside erythrocytes, being responsible for the reddish pigmentation of cells and for the transport of O_2 and CO_2 to tissues, through the association of these gases with iron molecules. The hematocrit, in turn, expresses in percentage the amount of blood cells present in the plasma [6].

Hematimetric indices are dependent on the values of hemoglobin, hematocrit and RBC, with the function of assessing the color and size of erythrocytes. The size of the erythrocytes is evaluated through the MCV, which classifies these cells into normocytic, microcytic and macrocytic. Normocytic red blood cells are those that are normal in size, while microcytes are smaller in size and macrocytes are larger in volume than those expressed by reference values. Both HCM and CHCM are responsible for evaluating the erythrocyte staining according to the parameters: normal coloration, insufficient staining or exacerbated staining, being classified into normochromic, hypochromic and hyperchromic, respectively [7].

It is important to emphasize that the decrease of just one parameter is not enough to conclude a diagnosis of anemia or leukemia. The confirmation of these pathologies depends on biochemical or cytochemical tests for the adequate conclusion of the hematological disorder subtype found. However, the suspicion of these pathologies is often detected through the blood count, which usually presents changes in more than one of the analyzed parameters [8]. Healthy patients have an RBC count

within the reference values, and the erythrocytes are classified as normocytic and normochromic. However, patients with iron deficiency anemia, for example, have a reduction in the total RBC count associated with microcytic and hypochromic red blood cells [9].

The leukogram is the part of the blood count with the function of evaluating the granulocyte and agranulocyte leukocytes through the quantitative evaluation of the total count and by the differential count of the leukocytes. The total WBC count indicates or rules out the presence of inflammations and/or infections from different sources. The decrease in the total leukocyte count is called leukopenia and occurs in cases of patients undergoing cancer treatment or in cases of a sedentary lifestyle. In turn, leukocytosis is represented by the increase in the amount of leukocytes, due to the presence of allergies; parasitic, viral or bacterial infections, or leukemias [10–12].

The differential leukocyte count, evaluates the amount of each leukocyte: neutrophils, eosinophils, basophils, monocytes and lymphocytes. Neutrophils are the most abundant blood cells, representing about 70% of the total circulating blood cells. The quantitative increase in neutrophils is called neutrophilia, indicating the presence of acute bacterial infections. However, neutrophilia is also present in exams of patients with burns, myocardial infarction, postoperative period, stress, use of glucocorticoids and intense physical exercises [11, 12].

Neutrophilia is divided into two types: neutrophilia with a left shift and neutrophilia with a right shift. The left shift is a condition present in cases of acute infections, when there is the release of a large amount of neutrophil rods into the bloodstream. In turn, the deviation to the right is characterized by the presence of segmented or hypersegmented neutrophils (more than 5 lobes), being indicative of chronic infections. The decrease in the amount of neutrophils is called neutropenia and may indicate, spinal infiltration, immunosuppression by the human immunodeficiency virus (HIV), alcoholism, food deficit, radiation exposure or use of cytotoxic drugs [13].

Eosinophils are cells that act mainly in the intestines, epithelium and the respiratory system. Thus, eosinophilia is characterized by the numerical increase of these cells, which is indicative of intestinal infections by helminths (schistosomiasis, hookworm, ascariasis, among others), dermatitis, dermatoses, acute myeloid leukemia, chronic myeloid leukemia, pernicious anemia or allergic processes respiratory. However, the decrease (eosinophilia) or the absence of eosinophils in the bloodstream is normal. Thus, eosinopenia is usually due to the administration of adrenocortical or corticoid hormones [13, 14].

Basophils are cells little present in the bloodstream; however, they play an important role during allergic reactions through the release of IgE and histamines. Basophilia is a process where there is a greater number of circulating basophils, being indicative of allergic reactions or the presence of leukemias. Basophenia, in turn, is not classified as a pathological process [3, 14].

Monocytes, on the other hand, are largely related to phagocytosis processes. Thus, the increase in the production and release of these cells is called monocytosis. This increase is suggestive of tissue repairs or indicates the presence of chronic infections. However, monocytopenia is indicative of decreased monocyte production and release

and is often associated with the lifestyle of the patient who is exposed to stress. All leukocytes present variations in their amounts according to the patient's age [14, 15].

Lymphocytes are blood cells active in the processes of viral infections. The increase in the number of these cells in the bloodstream (lymphocytosis) is directly related to the presence of viral pathologies such as hepatitis, HIV, rubella, among others [11–14]. The decrease in the amount of lymphocytes (lymphopenia) can be interpreted such as chronic HIV, acute stress or the presence of liver cirrhosis. It is important to note that lymphocytosis is a normal condition during childhood. Like lymphocytes, other blood cells undergo changes in their amounts according to the stage of life [16].

Thrombocytopenia occurs when the patient has a reasonable drop in the normal number of platelets. This analysis can be done after counting platelets in a blood sample. When a patient has thrombocytopenia, bleeding occurs more easily, as well as the appearance of purple spots on the body. The patient may also have blood in the stool, bloody vomiting, pain in the joints and muscles and have a weakness.

Thrombocytosis occurs when a patient has a high number of platelets in the blood, the number is greater than 1,000,000 platelets per mm^3. Thrombocytosis can be further classified into primary and secondary. The primary is related to myeloproliferative diseases (related to the abnormal production of blood cells), and the secondary is triggered by some underlying disease, such as infections and anemias [6].

However, in order to arrive at such quantifications of red blood cells and platelets, specific methodologies for each cell type are employed. Thus, the present study aims to present in a clear and brief way the main methodologies for total and differential blood cell counts.

2 Methodology

Considering the need to gather fundamental information regarding basic concepts of hematology, this study brought together the main concepts present in the literature regarding hematology, explaining from blood cells to the comparison between the methodologies employed.

3 Results and Discussion

Regardless of the methodology used to perform the complete blood count, this test must be performed by collecting 4 ml of venous blood collected in a tube containing EDTA anticoagulant ("Ethylenediaminetetraacetic acid") or heparin, which can be collected with or without fasting of the patient. The samples must avoid the presence of hemolysis (ruptured erythrocytes causing a lower count in the total amount of red blood cells), lipemia (presence of fat in the form of triglycerides which makes it

difficult to read results by colorimetric methods), clots (resulting in lower counts of platelets), use of antibiotics, anti-inflammatory and antiallergic agents hours before the exam (decreases the amount of leukocytes, being able to mask infectious or allergic conditions) and avoid smoking 2 h before the exam (change in hemoglobin rates, hematocrit and indexes hematimetric) [15].

Currently, the blood count can be performed using two methodologies: manual and automated, which are used according to the needs of each clinical analysis laboratory. Both the manual methodology and the automated methodology must be carried out with the help of a health professional such as doctors, pharmacists, biologists, biomedical and hematologists [1, 5].

The manual methodology is more dependent on the health professional to be performed, so the time taken to perform the exam is greater. It is also important to consider that mainly in underdeveloped and developing countries, health professionals are often subjected to long working hours. This reality directly impacts the accuracy of the results released. This is because in the manual methodology it is necessary to count blood cells one by one under optical microscopy. The biggest advantage of the manual methodology is the cost. This technique depends on low-cost reagents and instruments, thus being more present in small laboratories and/or in countries with low per capita income [1, 5].

The automated methodology has a higher cost, especially when applied to low-income populations, since the cost of acquisition, maintenance and reagents are passed on to the patient at the time of payment for the exam. However, the investment is worthwhile, as there is the less direct interaction between the health professional and the patient's blood. Thus, there is less chance of accidents involving blood samples. When such an accident occurs, the professional is exposed to a high risk of infection with Hepatitis types B, C, D, HIV, Syphilis, Malaria, Chagas disease, among others. Other benefits are the speed and accuracy of the results obtained, after all, the equipment is not subject to stress, fatigue and personal problems like a human [1, 5, 10].

3.1 Manual Erythrogram

The total erythrocyte count in a Neubauer chamber is performed by means of a 1:200 dilution, where 20 μl of whole blood is deposited in a test tube in 4 ml Hayen's liquid (sodium citrate diluted in physiological solution) with subsequent homogenization. Subsequently, part of this solution is transferred to the Neubauer chamber, which is covered by a glass coverslip and analyzed under optical microscopy, where erythrocytes will be counted under the objective lens at 400× magnification [10, 16].

It is also necessary to make a blood smear, by sliding a few microliters of blood on a glass slide and later using dyes, which allow the visualization of cellular structures. These dyes have the function of staining the nuclear and cytoplasmic structures of blood cells. There is a wide variety of dyes; however, the most used for blood smears are Leishman and panoptic. Only the final portion of the slide is used for counting,

as the anterior portions have clustered and/or overlapping cells, preventing reliable counting. This blood smear is used to analyze the red cell morphology and staining [16].

Quantitative and morphological analyzes of blood cells are fundamental parameters for the detection of anemia, since the vast majority of them result in anisocytosis (variation in shape), such as sickle cell anemia (sickle-shaped red blood cells), spherocytosis anemia (red blood cells with a circle format), hereditary ellipsis (elongated red blood cells), among others. Through the blood smear, it is also possible to identify blood parasites in the acute phase, such as leishmaniasis, nasturtium and malaria, among others. It is important to emphasize that it is only necessary to make a single blood smear during the entire blood count analysis process, with the same slide used for the analysis of red blood cells, leukocytes and platelets [17, 18].

The erythrocytes are responsible for the transport of oxygen, since inside these cells there is a protein called hemoglobin, which in addition to giving the blood a red color it binds to oxygen, which allows red blood cells to transport and distribute oxygen gas from the lungs to inside the cells of the body. The importance of analyzing these cells goes from both ends, related to the increase in the number of erythrocytes, is considered polyglobulia (known as polycythemia), is normally not clinically relevant; however, its reduction in relation to the level of red blood cells is considered hypoglobulia, which is a sign of anemia [18, 19].

Although in addition to assessing erythrocytes and hemoglobin, the eritrogram also performs a hematocrit check, which it is related to the volume of erythrocytes found in the centrifuged blood, since through that there is a possibility in determining the ratio between red blood cells and plasma, relative to the liquid part of the blood [15].

The determination of hematocrit consists of filling 2/3 of a glass capillary with whole blood. Subsequently, one end of the capillary must be sealed by means of heat sources or the use of mass, in order to prevent the escape of blood. This capillary must be placed in a microhematocrit centrifuge at a rotation of 1500 rpm (rotations per minute) for 5 min. After centrifugation, there is the separation of whole blood in 2 phases: a lighter one, formed by plasma, and another more dense, formed by the sedimentation of blood cells. To read the result, it is necessary to measure in millimeters the size of the sediment formed at the bottom of the capillary [6, 19].

Hemoglobin, in turn, can be determined using equipment called a spectrophotometer. Several methodologies are available in the market for the quantification of hemoglobin, with variations both in the time spent to perform the exam and in the amount of sample needed and reagents used during the process. The most used methodology for the measurement of hemoglobin is the cyanomethoglobin method, which uses Drabkin's liquid. This liquid can be composed of potassium ferrocyanide (K_3FeCN_6) potassium cyanide (KCN) or anhydrous potassium phosphate (KH_2PO_4) [5, 20, 21].

Upon contact with Drabkin's liquid, red blood cells are hemolyzed, with the release of free Hb, which in turn is converted to methemoglobin (iron hemoglobin in the iron form). Due to the action of potassium cyanide, methemoglobin is transformed into cyanomethemoglobin, a stable compound that absorbs light at 540 nm. The

hemoglobin concentration of the sample is calculated from a commercial hemoglobin standard, pre-determined by the manufacturer [5, 20, 21].

The hematimetric indices, in turn, are determined by means of mathematical formulas depending on the values obtained for total RBC, hemoglobin and hematocrit [5, 22]. Equation 1 is used to determine the MCV:

$$\mathrm{MCV} = \mathrm{Htc} \times 10\mathrm{RBC} \tag{1}$$

where Htc represents the hematocrit value obtained by centrifuging whole blood, and RBC indicates the total red blood cell count obtained by automated equipment or by manual counting in a Neubauer chamber. The result is expressed between 80–90 fl, which indicates the absence of pathologies [5, 22]. In Eq. 2, the HMC determination is presented:

$$\mathrm{MCH} = \mathrm{Hb} \times 10\mathrm{RBC} \tag{2}$$

where Hb indicates hemoglobin concentration and RBC is the total red blood cell count. The reference value is given between 26 and 34 picograms. CMCH, through Eq. 3:

$$\mathrm{CMCH} = \mathrm{Hb} \times 100\mathrm{Htc} \tag{3}$$

where Hb represents hemoglobin and Htc the hematocrit [5, 22]. The reference value is expressed as

$$31.5 - \frac{36\mathrm{g}}{\mathrm{d}L} \tag{4}$$

3.2 Manual Leukogram

The leukogram assesses white blood cells (WBC), called leukocytes, the defense cells responsible for fighting invading agents. Leukocytes are actually a group of different cells, with different functions in the immune system. Some leukocytes directly attack the invader, others produce antibodies, others only make the identification, and so on; this test helps in the diagnosis of viral, bacterial or parasitic infections, spinal dysplasias, leukemias and lymphomas [4].

The counting of WBC is performed through total counting and differential counting, which are counts with different methodologies, but complementary. It is important to note that the counts are expressed in absolute and relative values [23].

The total count of WBC is performed in the Neubauer chamber, through a 1:20 dilution, where 0.4 ml of Turk's liquid and 20 µl of whole blood are deposited in a test tube. Turk's liquid can consist of 2% glacial acetic acid or 1% hydrochloric acid,

which can be stained with methylene blue or gentian violet in order to differentiate it from the Hayer liquid [23].

Differential leukocyte counting is performed using a blood smear previously prepared for the evaluation of erythrocytes. In this process, 100 leukocytes present in the final portion of the blood smear slide should be counted. It is necessary to note manually, how many leukocyte subtypes were counted per field. Upon reaching the total of 100 cells counted, the professional must finish the identification and count of the leukocytes and perform the calculations of relative WBC and absolute WBC (value expressed as a percentage) [4, 23].

3.3 Manual Platelet Count

To manual platelet count is need still taking into account that to make thin smears, dry and stain using the usual methods. Examine under the microscope with an immersion objective. After count 1000 erythrocytes in different fields, noting the number of platelets found [8]. Apply the values in the formula:

$$\text{Number of platelets} \times \frac{\text{Number of erythrocytes per mm}^2}{1000} \tag{5}$$

3.4 Automated Counting

The automation of the blood count implies greater agility in the performance of the exams and in the release of the reports; however, they are a more expensive methodology when compared to the manual methodology. This methodology is able to count the three cellular types simultaneously. In the 1950s, Coulter Eletronic, Inc introduced the impedance principle for cell counts [1, 15].

The impedance principle is based on the fact that cells, bad conductors of electricity are diluted in an electrically conductive solution. This cell suspension is weighed through an orifice with a diameter around 100 μm, where an electric current passes. This electric current originates from two electrodes: one located on the inside of the orifice and positively charged, and the other located on the outside of the orifice, negatively charged. In this way, each time the cell passes through the hole, it interrupts the electrical current and there is a change in conductance; therefore, each interruption is counted as a particle [1].

The impedance principle, over the years, has been enabled with counters capable of measuring cell volume. Such evolution was the result of the color-relation of the proportionality of the magnitude of the interruption of the electric current (pulses) with the cell volume. Thus, it was observed that small pulses corresponded to small volumes, while large pulses were the result of larger volumes [15].

From this correlation between the magnitude of the electric current and the cell volume, a new concept called the threshold concept was created. The threshold concept is responsible for classifying cells according to their volume, thus allowing the detection of globular volume. The globular volume corresponds to the hematocrit performed on the manual blood count; however, it receives this name because it is performed without the need for microcentrifugation. Both the impedance principle and the threshold concept are responsible for the introduction of multiparameter devices on the market. These devices are capable of performing simultaneous cell counts using separate channels for counting [1, 15].

In the 1960s, the conductivity technique was developed, based on the high-frequency electromagnetic current, which is responsible for providing information regarding the cell volume, the size of the nucleus and the content of the cytoplasmic granulations. Subsequently, in 1970, the techniques of laser beam dispersion (laser light scatter) and hydrodynamic fluid were introduced. Both techniques preserve the leukocyte nuclei and granulations, retracting only the cytoplasmic membrane. These techniques are based on the principles of diffraction, refraction and reflection of the emitted light [15].

However, in these techniques, red blood cells are undetectable. To solve this problem, erythrocytes started to be quantified by means of flow cytometry and hydrodynamic focus, where these cells are counted one by one, through an extremely fine capillary. The red blood cells are then subjected to a laser beam, where the dispersion of the light is analyzed at different angles of deviation. In this context, the cell size is indicated at zero degrees, at 10° the internal structure is indicated and at 90° the leukocytes are identified and their lobularity characteristics and their granulation content [1, 15].

Currently, there are a large number of multiparameter devices, which use impedance, conductivity and scattered light techniques. These technologies can also be associated with the cytochemical characteristics of cells (such as myeloperoxidase) and the use of reagents that perform the analysis of certain cell types. However, before purchasing a hematological device, it is necessary to take into account the following parameters: Automation device versus type of patient attended; number of hemograms per day × samples/time spent by the device; cost of each hemogram; quality control; technical assistance; interfacing, training of employees [1, 15].

However, even with the acquisition of hematological equipment, manual hemogram is not a dispensed practice, being recommended for the confirmation of hematological reports of pediatric patients, patients over 75 years of age, cancer patients, patients with suspected leukemia or polycythemia, patients with leukocytosis and patients in severe condition (mainly in a state of hospitalization in the Intensive Care Unit—ICU) [1, 15].

4 Conclusions

The manual blood count is an examination that is totally dependent on human performance, which is used for the use of non-automated equipment. It is a cheaper exam, but more time-consuming and less reliable, as it depends on counts and calculations performed by health professionals. It is considered a good alternative for small laboratories, where the demand for tests is small and the cost of acquiring hematological equipment and reagents does not match the cost-benefit of the process.

Over time, the study of blood cells is no longer restricted to just observing the morphology of cells and has started to develop more specific tests, but that does not dispense with the use of conventional microscopy. This test responsible for the analysis of blood cells is called a complete blood count. Thus, review studies are extremely important to fundament and inspire new research, whether they refer to studies of cell biology, molecular biology, histology, cytology, hematology, or even for the creation of medical devices.

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