LEARNING GUIDE

RBC INDICES AND EXTENDED RBC PARAMETERS

CORELABORATORY.ABBOTT/HEMATOLOGY

Abbott

CONTENTS

SECTION 1

RED BLOOD CELLS AND ERYTHROPOIESIS

LEARNING OBJECTIVES

AFTER COMPLETING THIS SECTION, YOU WILL BE ABLE TO:

1. Describe the morphology and life cycle of RBCs
2. Explain the structure and function of hemoglobin
3. List the maturation stages in RBC development
4. Discuss the use of the traditional RBC indices

RED BLOOD CELLS AND ERYTHROPOIESIS

When a complete blood count (CBC) is requested by a physician, the red blood cell (RBC) count and the red blood cell indices are essential components in the report. Mature RBCs or erythrocytes circulate through the blood stream and are responsible for oxygen (O2) delivery to tissues throughout the body, and removal of carbon dioxide (CO2) from the tissues. They are anucleate biconcave disks, measuring 6-8 μm in diameter, and circulate in the blood for approximately 120 days. The main component of the RBC is hemoglobin (HGB), an oxygen transporting molecule. Hemoglobin is a large molecule that consists of a heme molecule and a globin protein. Heme consists of 4 pyrrole rings with ferrous iron (Fe2+) ion located in the center of the 4 rings. The globin protein is made up of 4 folded chains, 2 α-chains and 2 β-chains (**Figure 1**). Each iron ion in heme binds to one oxygen molecule, therefore each HGB molecule can transport 4 O2 molecules.[1]

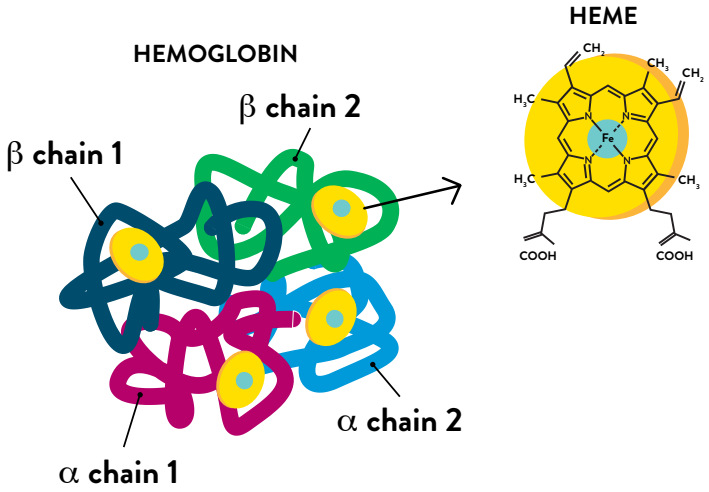

Figure 1. Structure of the hemoglobin molecule

RBCs are produced in the bone marrow through a process called erythropoiesis. Erythropoiesis is regulated by a hormone called erythropoietin (EPO) which is produced by peritubular cells of the kidney in response to changes in oxygen tension in the blood vessels. When the oxygen tension is low, EPO is released and travels to the bone marrow to stimulate the production of more erythrocytes. Approximately 2 million RBCs enter the bloodstream from the bone marrow daily in a typical healthy adult and under normal conditions, erythrocyte production is equal to erythrocyte destruction.

During erythropoiesis, committed erythroid progenitor cells differentiate into the earliest recognizable erythroid precursor, morphologically known as the pronormoblast. The pronormoblast in the bone marrow differentiates into basophilic normoblast, polychromatophilic normoblast and orthochromic normoblast (**Figure 2**). During maturation and differentiation hemoglobin content increases, the cell decreases in size and the nucleus becomes smaller and pyknotic. At the end of the othrochromic normoblast stage the nucleus is expelled from the cytoplasm. The cell remains in the bone marrow for about a day as a polychromatophilic erythrocyte before being released into the circulation as an immature erythrocyte (reticulocyte).

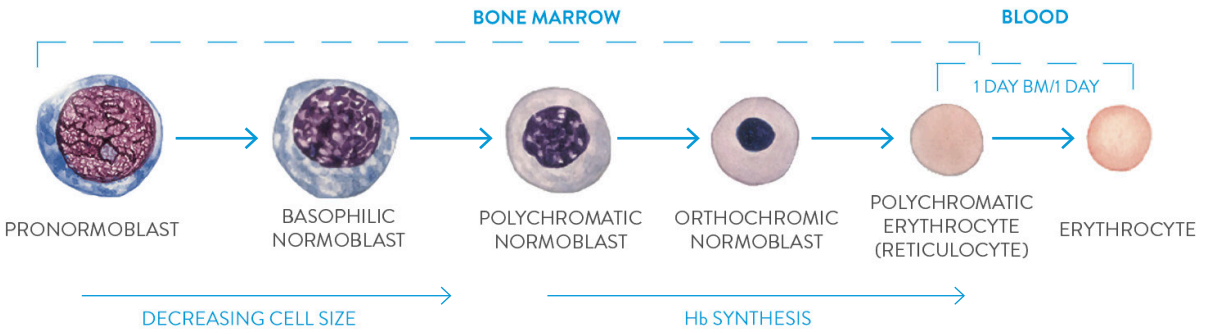

Figure 2. Hierarchy of erythrocyte maturation

QUIZ QUESTIONS

1. What molecule is responsible for binding to oxygen?

   A. Ferrous iron

   B. Magnesium

   C. Cobalt

   D. Amino acids

2. Under which condition is erythropoietin released for RBC production?

   A. High oxygen tension

   B. Low oxygen tension

   C. During homeostasis

   D. In acute renal failure

3. During which stage of erythroid development is the nucleus extruded from the developing RBC?

   A. Pronormoblast

   B. Basophilic normoblast

   C. Polychromatic normoblast

   D. Orthochromic normoblast

SECTION 2

TRADITIONAL RBC INDICES

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. List the traditional RBC indices
2. Explain how MCH and MCHC are calculated
3. Compare RDW-CV and RDW-SD

TRADITIONAL RBC INDICES

RBC indices as a part of the complete blood count (CBC) were initially introduced by Maxwell Wintrobe in the early 1930's, when the CBC was determined by a manual procedure. 3 WBC and RBC were counted using a hemocytometer and microscope. Hemoglobin (HGB) was determined spectrophotometrically, and the hematocrit (HCT) was determined by centrifugation as packed cell volume. This method is still the reference method for HCT.4 Wintrobe described three erythrocyte indices: the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) (**Table 1**). These were calculated based on the RBC count, the HGB concentration, and the packed cell volume. Today, most hematology analyzers directly measure the MCV and calculate HCT, MCH and MCHC from the automated CBC.

RBC indices are important screening and confirmatory tools in anemias and are helpful in the classification and in the assessment of the severity of anemias.

Table 1. The Wintrobe RBC indices[5]

RBC indices name	RBC indices abbreviation	Formula	Traditional units
Mean cell volume	MCV	MCV = HCT/RBC	80-100 fL
MCH mean cell hemoglobin	MCH	MCH = HGB/RBC	26 - 34 pg
Mean cell hemoglobin concentration	MCHC	MCHC = HGB/HCT	32 - 36 g/dL

MCV is the average volume of the RBCs in a sample. It may serve as a useful parameter to classify anemias based on RBC size.[6] Based on MCV, anemias can be categorized as macrocytic, normocytic and microcytic (with MCV above, within or below the reference range). The reference interval for the MCV is generally reported as 80 – 100 fL, but this varies by age, gender, technology and reference population. The MCV is determined automatically when RBCs are being counted on the hematology analyzer. It can also be calculated manually if the RBC count and the HCT are known (MCV = (HCT x 10) / RBC count). Since the MCV is a measure of the average volume of RBCs, it does not reflect the size variation of RBCs in the sample. A sample with a normal MCV may have groups of smaller-sized and/or larger-sized RBCs within the total population being counted. The excessive variation in RBC size is known as anisocytosis. It is assessed by laboratorians by reviewing a peripheral blood smear. This estimation, however, is based on diameter, rather than volume.[7]

The **MCH** is a measure of the average HGB content or mass of hemoglobin per RBC.[6] The reference interval for the MCH is generally reported as 26 – 34 pg, with some variation according to age and gender. The MCH is calculated using the hemoglobin and RBC values (MCH = HGB / RBC). The MCH primarily depends on the size of the RBC; when the MCV decreases, usually the MCH also decreases. Therefore, a low MCH is typically seen in microcytic anemias. The MCH is a sensitive parameter in anemias caused by impaired hemoglobin synthesis, such as thalassemias,[8] but usually is not considered in anemia classification.

The **MCHC** is the average concentration of hemoglobin in the RBCs[6]. The reference range is about 32-36 g/dL. It is a very stable measurand, with low within-person variation under normal circumstances.[6,7] When the MCHC is below or above the reference range, the RBCs are classified as hypochromic or hyperchromic, respectively. Morphologically, on peripheral blood smears, normal RBCs have an area of central pallor that is about 1/3 of the diameter of the RBC; these cells are referred to as normochromic. Hypochromic RBCs have increased size of central pallor, and are seen in many anemias, including iron deficiency. On the other hand, the MCHC only rarely exceeds the upper limit of the reference range. Hyperchromic RBCs have reduced or missing central pallor on peripheral blood smear. The presence of hyperchromic RBCs (high MCHC) is diagnostic of RBC cytoskeletal/ membrane disorders (hereditary spherocytosis or xerocytosis).[3]

The **RDW** (red cell distribution width) was not part of the classical Wintrobe indices but was introduced in the 1980's as an additional RBC parameter. RDW reflects the heterogeneity of the RBC volume in the sample. A normal RDW value implies a homogenous size distribution of RBCs, while an elevated RDW suggests a large variety in RBC size. RDW correlates with the degree of anisocytosis that can be observed on the peripheral blood smear.

The RDW is derived from the RBC volume distribution histograms and may be reported as a coefficient of variation (%CV) or size distribution (SD) (**Figure 3**). When expressed as %CV, it is calculated mathematically using the following formula: RDW-CV = (1 standard deviation of RBC volume / MCV) x 100. As a result of the mathematical formula, RDW-CV is affected by the average RBC size (MCV). This contributes to the decreased sensitivity of RDW-CV for detecting anisocytosis in macrocytic anemias.[9]

The RDW-SD (expressed in fL) is the width of the RBC volume distribution histogram, determined at the 20% frequency level. The RDW-SD is not influenced by the MCV and has shown better sensitivity for detecting anisocytosis in the normocytic and macrocytic range than RDW-CV.[9]

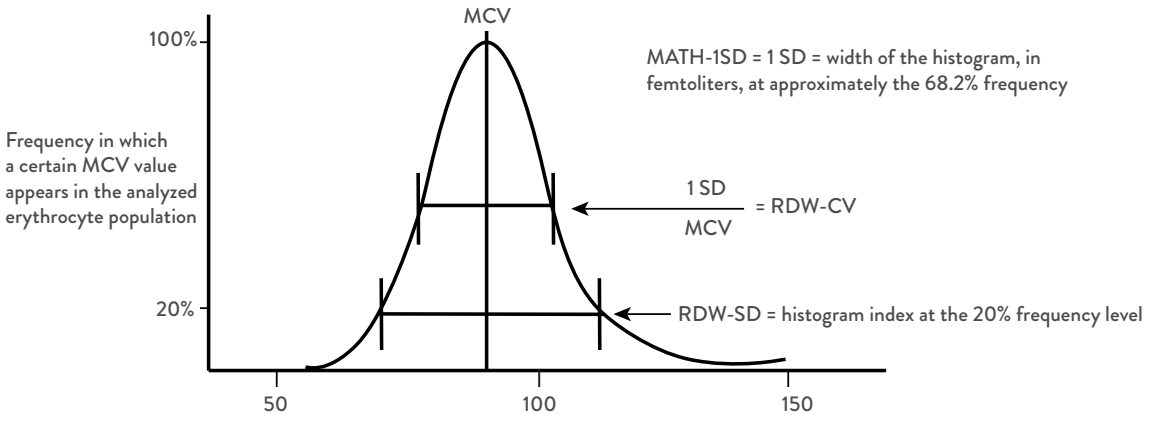

Figure 3. RDW-CV and RDW-SD from erythrocyte volume distribution histogram[9]

The reference range for the RDW-CV is approximately 11.5 – 14.5%.[10] This value, however, is somewhat technology dependent, as the measurements are not standardized. In addition to the technology used to measure MCV, the lack of harmonization between manufacturers in volume distribution curve measurement methodologies, particularly related to truncation of extreme values of the distribution, results in well-documented differences between platforms.[3,11]

The RDW is useful in the classification of anemias. It may become abnormal earlier in nutritional deficiencies compared to MCV and other RBC indices, as early changes in subpopulations of cells may not be readily detectable by changes in the mean volume. In a developing iron deficiency anemia, for example, the first sign of the increasing population of microcytic cells may be an increased RDW. The RDW has been proposed to be useful in differentiating between iron deficiency anemia and thalassemia.[12,13] Thalassemia is characterized by the presence of uniformly small RBCs, associated with a small RDW, while in iron deficiency anemia a mixed population of normocytic/normochromic and microcytic/hypochromic cells are usually present, associated with an above normal RDW.

QUIZ QUESTIONS

1. Which RBC index is defined as the average weight of hemoglobin per RBC?

   A. MCV

   B. MCH

   C. MCHM

   D. RDW

2. Under which condition is erythropoietin released for RBC production?

   A. MCV

   B. MCH

   C. MCHM

   D. RDW

3. During which stage of erythroid development is the nucleus extruded from the developing RBC?

   A. Are larger than the average RBC

   B. Have increased central pallor

   C. Are characteristic for hereditary spherocytosis

   D. Are seen in iron deficiency anemia

SECTION 3

ADVANCED RBC INDICES AND PARAMETERS

LEARNING OBJECTIVES

AFTER COMPLETING THIS SECTION, YOU WILL BE ABLE TO:

1. List advanced RBC parameters
2. Describe the clinical utility of advanced RBC parameters
3. Compare spectrophotometrically measured and calculated

With advances in technology over the last 10-15 years, additional RBC parameters have been introduced on automated hematology analyzers. Although many of these advanced indices are currently available on hematology analyzers as Research Use Only (RUO) parameters, there is increasing evidence of their clinical importance in the diagnosis and in the assessment of the severity of anemia, as well as in monitoring treatment response. These advanced RBC parameters are listed in **Table 2**; their names may vary depending on the manufacturer.

Table 2. The advanced parameters comparison by manufacturers[17]

Advanced RBC parameters	Abbott	Siemens	Sysmex	Beckman Coulter
Cellular hemoglobin concentration mean	CHCM	CHCM	NA	NA
Calculated cellular hemoglobin	cHGB	Calculated HGB	NA	NA
Hemoglobin distribution width	HDW	HDW	NA	NA
Percent microcytosis	%MIC	% MICRO	%Micro-R	NA
Percent macrocytosis	%MAC	%MACRO	%Macro-R	NA
Percent hypochromia	%HPO	%HYPO	%Hypo-He	LHD%
Percent hyperchromia	%HPR	%HYPER	%Hyper-He	NA

CHCM – corpuscular hemoglobin concentration mean
cHGB – calculated hemoglobin
HDW – hemoglobin distribution width
%MAC – percent macrocytic cells
%Hyper – percent hyperchromic cells
%Hyper-He – percent hyperchromic cells
%HYPO – percent hypochromic cells
%Hypo-He – percent hypochromic cells
LHD% – low hemoglobin density percent
%MIC – percent microcytic cells

The percent microcytic RBCs (%MIC) is a measure of the number of microcytic RBCs in a sample, reported as a percentage. The frequency of microcytic RBCs can also be assessed by visual estimation from a stained peripheral blood smear. The %MIC from automated hematology analyzers, however, offers a more precise quantitation of microcytic RBCs. The %MIC is derived from the RBC volume distribution histogram, and usually includes RBCs with a volume of < 60 fL.[18] (**Figure 4**)[19]

%MIC can be used in combination with other RBC parameters to differentiate between iron deficiency anemia (IDA) from thalassemia.[20]

The percent macrocytic RBC (%MAC) is a measure of macrocytic RBCs in a sample, reported as a percentage. Similar to %MIC, the %MAC can be assessed by visual estimation from a stained peripheral blood smear, but the automated analysis, based on large number of events, is more accurate. The %MAC is derived from the RBC volume distribution histogram, and usually includes RBCs with a volume of > 120 fL.[18] (**Figure 4**)[19]

Increased %MAC may be a sign of B12 or folate deficiency or can be an early indicator of myelodysplastic syndrome.[19]

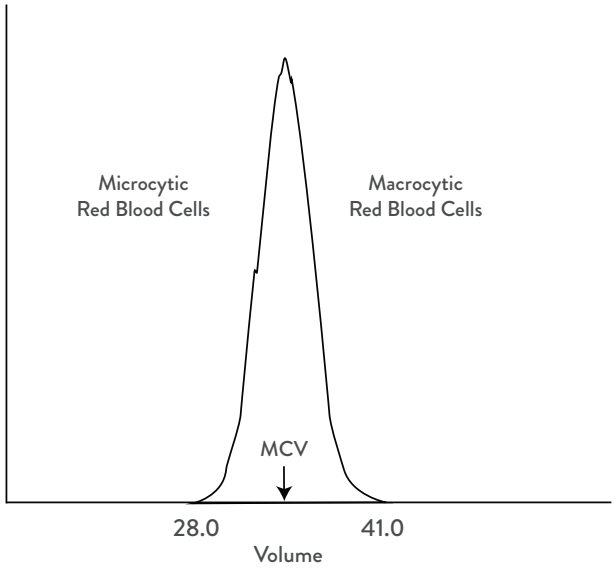

Figure 4. RBC distribution histogram depicting %MIC and %MAC

Automated hematology analyzers that use optical technology for measuring RBCs are capable of the simultaneous measurement of the volume and HGB concentration of individual RBCs. Based on the cell-by-cell HGB concentration, a hemoglobin concentration distribution curve (histogram) can be created (Figure 5), which is used to derive the cellular hemoglobin concentration mean (**CHCM**). CHCM can be used to calculate the HGB concentration in the blood sample using the MCV and the RBC concentration by the following equation: cHGB = CHCM x [(RBC x MCV) / 1000].[21] The CHCM and MCHC are equivalent parameters; however, the CHCM is directly measured whereas the MCHC is calculated based on the routine spectrophotometric analysis of the total HGB concentration.[22,23]

The spectrophotometric HGB method is considered to be sensitive to interfering substances causing plasma turbidity, such as lipemia,[22] while the calculated HGB value (**cHGB**) is not impacted by these conditions. The cHGB concentration, although not a reportable parameter on current hematology analyzers, can be used as an important quality check when compared to the traditional HGB concentration result. When discordance is are observed between the measured and the calculated HGB, it can be used to generate an alert, notifying the user about the potential presence of interfering substances, impacting the measured HGB value.[22]

The percentage of hypochromic RBC (%HPO) can be derived from the hemoglobin concentration distribution curve and is usually defined as the percentage of RBCs with a cellular hemoglobin content (CHC) of < 28.0 g/dL. %HPO is a very sensitive parameter for detecting iron deficiency and functional iron deficiency, for example in patients with chronic renal failure, who receive erythropoietin stimulating agent (ESA) therapy.[18] ESA therapy causes increased demand for the iron stores, potentially leading to transient deficiency. In iron supplementation during ESA treatment. A level of >10% is considered indicative for the need for iron supplementation.[24] Because of the long circulating life span of mature erythrocytes, %HPO values are related to iron status over the last 2-3 months.[25]

The percentage of hyperchromic RBC (**%HPR**) can also be derived from the hemoglobin concentration distribution curve, and is usually defined as the percentage of RBCs with a CHC of > 41.0 g/dL.[25] Hyperchromia of RBCs is rare. It is commonly seen in hereditary spherocytosis and may serve as in indicator of severity of disease.[25] It can also be present in hemolytic anemias and thermal injuries (burns).

Another parameter that can be derived from the hemoglobin concentration distribution curve is the hemoglobin distribution width (**HDW**). It is a measure of the heterogeneity of the RBC hemoglobin concentration.[26] HDW is reported as the %CV of the cellular hemoglobin concentration distribution.[26,27] A high HDW is a sign of increased variation in the cellular hemoglobin concentration. Increased HDW values have been reported in cord blood samples, sickle cell anemia, iron deficiency, β-thalassemia and spherocytosis.

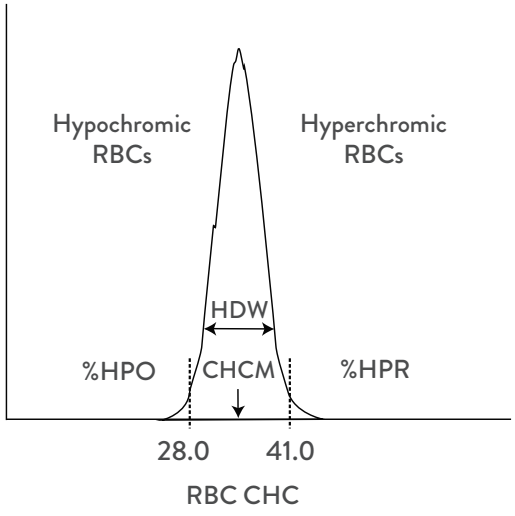

Figure 5. CHC (RBC) distribution histogram depicting the advanced hemoglobin parameters.

QUIZ QUESTIONS

1. Which RBC index is an equivalent of the MCHC and derived from cell-by-cell HGB concentration measurement?

   A %MAC

   B cHGB

   C CHCM

   D HDW

2. Which of the following indices is a very sensitive parameter for detecting functional iron deficiency?

   A %HPO

   B %MIC

   C %HPR

   D HDW

3. Which indice can be used as a quality check for hemoglobin

   A CHCM

   B cHGB

   C %MAC

   D %HPR

4. Which of the following indices has a potential use in diagnosing hereditary spherocytosis?

   A cHGB

   B %HPR

   C %MAC

   D HDW

5. Which of the following indices is derived from the RBC size distribution histogram?

   A %MAC

   B %HPO

   C CHCM

   D %HPO

SECTION 4

RBC COUNTING TECHNOLOGIES

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Describe the impedance technology for red blood cell analysis
2. Understand the limiting factors of the impedance technology
3. List the correction strategies to overcome challenges of impedance technology
4. Explain the optical light scatter technology and how it is used for red blood cell analysis
5. Describe the importance of isovolumetric sphering in optical red blood cell analysis

ELECTRICAL IMPEDANCE BASED RED BLOOD CELL COUNTING

This first semi-automated cell counting technology invented in the 1950's by Wallace H Coulter (also is known as Coulter Principle or electrical impedance.[28,29] It remained the only counting technology for almost two decades. This technology is based on suspending cells in an electrolyte solution (such as isotonic saline) that serves as a conductor of constant electrical current between two electrodes. As each cell passes through a small aperture, the non-conductive cells produce a momentary increase in resistance, resulting in an electrical pulse. The number of pulses indicates the cell count, and the amplitude of the pulse is proportional to cell volume[6] as shown in **Figure 6.**

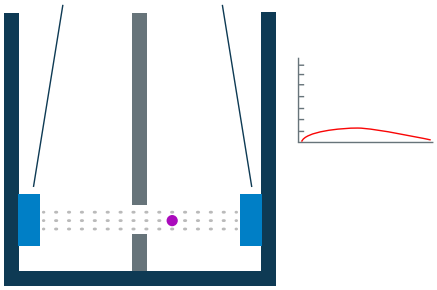

Figure 6. Impedance Technology; Cells pass through the detection zone of an aperture

Ideally, cells pass through the sensing zone one by one. However, simultaneous occupancy of the sensing zone by more than one particle may occur, especially when the concentration of the cells is high. This phenomenon is called "coincidence," and the resulting count variation is known as the coincidence error. Coincident passage of more than one cell at a time causes falsely decreased cell counts and creates artificially large pulses resulting in falsely increased cell volumes. Coincidence is predictable as it has a direct relationship with the cell concentration and the size of the aperture. A coincidence correction is typically applied by the manufacturer to correct for this phenomenon.

Another source of potentially aberrant pulses is cells passing through the aperture near its edge or at an angle, rather than at the center. This issue can be managed by pulse editing.

The deformability of the RBC may also contribute to altered pulse height. During measurement, the disc-shaped RBCs become elongated into a cigar shape as they pass through the aperture. The deformation of RBC is dependent on the hemoglobin concentration inside each cell (i.e. MCHC). Elongation is more pronounced when the MCHC is low, and deformability is decreased when the MCHC is high. This phenomenon may lead to overestimation of the MCV in hyperchromic RBC (such as in spherocytosis), and underestimation of the MCV in hypochromic RBCs, leading to spuriously low MCHC is the first case, and spuriously high MCHC in the latter.[30-32]

Finally, recirculation of the cells into the sensing zone after passing through the aperture can lead to double counting and falsely elevated cell counts. To prevent recirculation of cells into the sensing zone, a backwash or sweep-flow mechanism is typically added.

The use of hydrodynamic focusing, introduced in the 1970's addresses many of these potential problems inherent in an impedance-based system. In hydrodynamic focusing, the sample stream is surrounded by a sheath fluid as it passes through the aperture, creating a laminar flow (**Figure 7**). This creates a narrow single flow of cells, thereby reducing deformation and coincidence, and preventing pulse height irregularity.

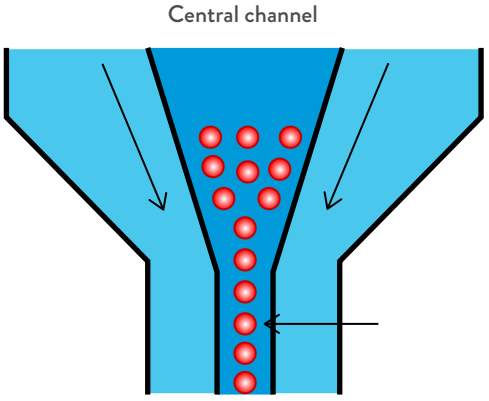

Figure 7. Hydrodynamic focusing

Despite the addition of hydrodynamic focusing, the main limitation of the impedance technology is the fact that it uses only size to discriminate between cell populations. For RBC measurements, the most important factor is to differentiate between RBC and PLT, as these cells are counted from the same sample dilution in the same channel. In certain pathological conditions, the size difference between RBCs and PLTs is diminished and volume events may overlap. For example, fragmented or microcytic RBCs could be counted as PLTs[22,33] leading to overestimation of PLT count, underestimation of RBC count, and inaccurate MCV and MPV measurements. Conversely, PLT clumps and giant PLTs can fall in the size range of RBCs and can be counted as RBCs, resulting in under-reporting of the PLT count.

There are other sources of potentially spurious results, apart from RBC/PLT discrimination. Autoagglutination of RBC, due to cold agglutinins, can lead to spuriously low RBC counts and to abnormally high MCV, as each small RBC clump is considered as one single particle. In addition, RBC and MCV results can potentially be incorrect in samples with extremely high WBC counts, especially when the RBC count is low, as small WBCs may be included in the RBC count.[22]

OPTICAL RED BLOOD CELL COUNTING

Optical RBC counting, utilizing the principles of optical flow cytometry, was developed in 1970s.[34] In this technique, hydrodynamically focused cells are illuminated by a laser light source as they pass through the flow cell one by one. The light is scattered and captured by photodetectors positioned at various angles.[35,36]

Optical RBC analysis requires three preconditions to produce reproducible scatter signals: monochromatic (laser) light source; efficient isolation of cells by hydrodynamic focusing; and isovolumetric sphering.[36]

Isovolumetric sphering is the process of changing the shape of the RBCs into a spherical shape without changing their volume. It is a critical in optical RBC analysis because the characteristic biconcave shape of the RBCs and their ability to deform while passing through the flow cell can result in orientation/shape dependent variation in light scatter. (Kim et al., 2003). In contrast, isovolumetrically sphered red blood cells behave as homogenous dielectric spheres and produce identical signals. This has been shown to achieve improved precision of volume measurements of RBCs by optical technology.

According to the Mie theory of light scatter for homogeneous spheres, isovolumetrically sphered RBCs produce light scatter signals that are proportional to the cell size (low forward angle light scatter) and refractive index (high forward angle light scatter). The latter is proportional to the hemoglobin concentration of the cell, therefore allowing hemoglobin concentration to be measured in individual RBCs.[37,38]

Optical technologies, based on independent and simultaneous measurement of volume and HGB concentration of individual cells, allow the visualization the RBC population according these two characteristics in a two-dimensional matrix (**Figure 7**). The resulting scatterplot provides important visual clues for assessing the distribution of the cells related to size (microcytosis and macrocytosis), as well as HGB concentration (hypochromia and hyperchromia). Visual inspection of this scatterplot can be used to trigger or supplement smear reviews.

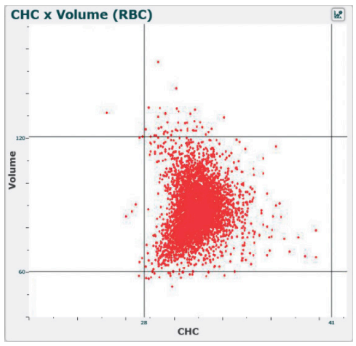

Figure 8. CHC x Volume (RBC): RBC hemoglobin concentration vs. RBC volume scatterplot by Alinity hq
The entire population of RBC is shown, and deviation from normal in terms of microcytosis, macrocytosis, hypochromia, and hyperchromia is visualized by using four fixed thresholds that define hypochromic (<28 g/dL), hyperchromic (> 41 g/dL), microcytic (< 60 fL) and macrocytic (>120 fL) RBC.
CHC: Cellular Hemoglobin Concentration

The above described two-dimensional analysis does not provide information on RBC morphological abnormalities.[37] A multi-dimensional approach, however, utilizing one or more additional intermediate angle light scatters, is capable of providing information on RBC morphology.[37]

In the CELL-DYN Ruby analyzer (Abbott), RBC analysis utilizes three angles of light scatter: 0°, 10°, and 90°, as part of Abbott patented MAPSS™ technology (Multi Angle Polarized Scatter Separation).[39] The Alinity h-series analyzers (Abbott) employ an advanced version of the MAPSS™ technology, utilizing six light scatter signals for counting and discriminating RBC and PLT. (**Table 3**). In this multi-dimensional approach, capturing the scattered light from multiple angles creates unique optical signal signatures for each cell, facilitating the differentiation of RBC, PLT and other cellular events or particles (microcytes, spherocytes, RBC fragments, giant PLT, debris, etc.)

Table 3. The six light scatter signals used by with the advanced MAPSS™ technology on the Alinity h-series analyzers for RBC and PLT measurements

Detector	Angle	Attribute applicable to RBC/PLT measurements
ALL	0 °	Cell size
IAS	3.2 – 7.6°	Cell contents
IAS1	2.4 – 4.5°	Hemoglobin
IAS2	4.7 – 5.7°	Cell volume
IAS3	5.9 – 7.6°	Cell complexity
PSS	90° (pol)	Intracellular structure/granularity

ALL: Axial Light Loss, IAS: Intermediate Angle Scatter, PSS: Polarized Side Scatter

BENEFITS OF OPTICAL LIGHT SCATTERING TECHNOLOGY

Compared to impedance measurements, optical technology allows for better separation and the differentiation of RBC and PLT, even when they are of similar size, because it collects additional signals that reflect the internal structure and complexity of the cells.[40] Multi-dimensional analysis produces additional optical signals that further improve the differentiation of populations in cases where dual angle light scatter technology may demonstrate signal overlap.[41]

The CHC x Volume (RBC) scatterplot produced by optical technologies (Figure 8) can provide important visual clues for differentiating between thalassaemia and iron deficiency anemia. In iron deficiency, RBCs tend to be more hypochromic than microcytic, while in thalassemia, RBCs are usually more microcytic than hypochromic, and are usually uniformly sized.[40] Multiple RBC populations can also easily be recognized on the CHC x Volume (RBC) scatterplot assisting in the identification of myelodysplastic syndrome, a response to treatment for iron deficiency, presence of cold agglutinins, or recent RBC transfusion.[40]

Cell-by-cell volume and hemoglobin concentration measurements make it possible to derive extended RBC parameters, including: %HPO, %HYP, CHCM and HDW[42-44] and extended reticulocyte parameters, such as reticulocyte volume (MCVr) and reticulocyte HGB content (MCHr).

Although some of these parameters are offered as Research Use Only parameters by hematology analyzers, their clinical utility is well documented. Please refer to Section 2 of this learning guide for more information on these parameters.

QUIZ QUESTIONS

1. The principle of impedance technology is:
   - A Size based discrimination of particles
   - B Refractive index-based discrimination particles
   - C Shape based discrimination of particles
   - D All the above

2. Correction strategies to overcome challenges of impedance technology include: (Choose all that apply)
   - A Coincidence Correction
   - B Using nonconductive medium for passage of cells
   - C Using alkaline solution as a medium for the passage of cells
   - D Introduction of hydrodynamic focusing

3. Hydrodynamic focusing directs the particles:
   - A In the periphery of the sensing zone aperture
   - B In the center of the sensing zone aperture
   - C Outside of the sensing zone
   - D None of the above

4. Measurements by scattered laser light during optical RBC analysis is performed on:
   - A Red Cells transformed into elongated cigar shape
   - B Red Cells under an electromagnetic current
   - C Red Cells transformed into spherical form
   - D Red Cells under a high frequency current

5. Benefits of the optical light technology are most prominent when:
   - A Red cells and white blood cells are of same size
   - B Red cells and platelets are of similar size
   - C Red cells under an electromagnetic current
   - D Red cells under a high frequency current

6. Advanced MAPSS™ Technology for RBC analysis uses
   - A Two angles of light scatter
   - B Impedance technology
   - C Six angles of light scatter
   - D Two optic benches

SECTION 5

CLINICAL UTILITY OF RBC INDICES AND PARAMETERS

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Classify anemias based on MCV and related parameters
2. Describe the utility of RDW in differentiating between iron deficiency anemia and thalassemia
3. Identify additional laboratory tests that aid the diagnosis of specific anemias

ANEMIA

Anemia is a common condition that affects an estimated 1.93 billion people worldwide.[45] It is defined by low hemoglobin concentration, usually accompanied by decreased RBC count. According to World Health Organization (WHO), hemoglobin levels < 13 g/dL in men and <12 g/dL in women suggest anemia.[46] Anemias can be classified based on etiopathogenesis or RBC morphology.[47] The pathogenic approach is based on mechanisms that lead to inadequate RBC production or to increased loss or destruction of RBC.

Factors resulting in decreased RBC production include disturbances in DNA synthesis (eg. B12 deficiency) or hemoglobin synthesis (eg. iron deficiency, thalassemia), and bone marrow failure (eg. aplastic anemia, chemotherapy induced bone marrow suppression). Factors leading to shortened RBC life span include intrinsic abnormalities such as enzyme defects, globin abnormalities, membrane defects (eg. sickle cell disease, hereditary spherocytosis), and extrinsic abnormalities, such as chemicals, drugs, antibodies, infectious agents (eg. autoimmune hemolytic anemia).

The morphologic approach characterizes anemias based primarily on cell size, and classifies anemias as microcytic, normocytic, and macrocytic. These can be further divided based on RBC parameters.

Table 4. Morphologic classification of anemias (based on the MCV).[48]

MCV	Classification	Types of Anemias
Decreased (<80 fL)	Microcytic	• Iron deficiency anemia • Thalassemia • Anemia of inflammation (chronic disease) • Sideroblastic anemia
Normal (80 – 100 fL)	Normocytic	• Anemia of inflammation chronic disease) • Aplastic anemia • Some hemolytic anemias • Anemia of renal disease
Increased (>100 fL)	Macrocytic	• Megaloblastic anemias • Anemias of myelodysplasia • Vitamin B12/Folate Deficiency • Chronic liver disease

When anemia is suspected in a patient, a CBC is ordered by the physician. The information derived from the HGB, HCT, MCV, RDW, and RBC count is used in making the initial diagnosis of anemia and allow for initial morphological classification. Identifying the cause and etiopathogenesis of anemia, however, require additional (biochemical, cytological, genetic, etc.) laboratory testing.

MICROCYTIC ANEMIAS

Microcytic anemias are characterized by an MCV less than 80 fL. The presence of small RBCs (smaller than the nucleus on a normal small lymphocyte) can be observed on the peripheral blood smear, sometimes associated with significant variation in size (anisocytosis). These small cells often have an increased central pallor compared to normal RBCs, suggesting hypochromia. Hypochromia is characterized by MCHC of less than 32 g/dL. Anisocytosis is usually associated with increased RDW.

The two most common causes of microcytic anemia are iron deficiency and α- or β-thalassemia.[49] If the decreased MCV is associated with an increased RDW, it usually suggests iron deficiency as the cause of the anemia. Whereas in cases of thalassemia, the MCV is decreased and the RDW is typically normal or low-normal. RDW values may overlap among conditions, therefore this categorization is not always absolute.[50]

Confirmatory diagnosis of iron deficiency requires measuring serum ferritin, serum iron, total iron-binding capacity, and transferrin saturation. In cases of iron deficiency, serum ferritin, serum iron and serum transferrin saturation are decreased, and total iron-binding capacity is increased.[51] Thalassemias are caused by mutations that either prevent or reduce the synthesis of one or more of the globin chains in the hemoglobin tetramer.[52] In thalassemia trait, MCV is often less than 70 fL, but the RBC count is usually above 5.5 x 1012/L.[53] The severity of the microcytosis and hypochromia will depend on the type of thalassemia. The RDW is typically normal because the RBCs are uniformly microcytic. When confirmatory iron studies are performed in thalassemia, serum iron, serum ferritin, and total iron-binding capacity are normal. Hemoglobin electrophoresis is used for the confirmation of the disease.

NORMOCYTIC ANEMIAS

Anemia of inflammation, also known as anemia of chronic disease (ACD) is the second most common cause of anemia after iron deficiency.[49] The pathophysiology involves dysregulation of iron metabolism due to chronic inflammation.[49] Most patients with ACD present with normocytic and normochromic or mildly hypochrocromic RBCs. The MCV is usually in the range of 75 -82 fL, and the HGB is rarely <9.0 g/dL.[53] The MCH, MCHC, and RDW are within normal limits. Additionally, serum iron, transferrin saturation and total iron-binding capacity may be decreased, but serum ferritin is normal or elevated.[51]

Functional iron deficiency (FID) is a condition that occurs when iron stores are transiently unable to meet the demands of increased erythropoiesis, usually during treatment with erythropoietin stimulating agents (ESA).[44]

It is commonly seen in patients with chronic kidney disease. FID is different from ACD in that there is no sequestration of the available iron. In FID, iron stores are low or borderline, and are not sufficient to satisfy the increased demand during ESA administration, resulting in inadequate response to ESA therapy. In FID the MCV, MCH, and MCHC may be within normal limits. %HPO have been shown to be useful in identifying patients with FID who might benefit from iron supplementation during ESA therapy.[54]

MACROCYTIC ANEMIAS

Macrocytic anemias are characterized by an MCV of greater than 98 fL, often as high as 120-140 fL.[55] Macrocytic anemias are classified as megaloblastic when they result from a vitamin deficiency such as vitamin B12 and/or folate, or non-megaloblastic when they result from other causes. The severity of the macrocytosis is often key to the differential diagnosis. An MCV greater than 110 fL is characteristic of vitamin deficiency, myelodysplasia, or some medications. In contrast, a mild macrocytosis where the MCV less than 110 fL is characteristic of alcohol abuse, liver disease, marked reticulocytosis, and hypothyroidism.[49] The MCH is also elevated in macrocytic anemias resulting from the increased volume of the RBCs, however the MCHC is usually within the reference interval, because hemoglobin production is not affected.[56]

Confirmatory tests for the differentiation and diagnosis of macrocytic anemias include serum levels of vitamin B12 and folate, red cell folate, methylmalonic acid and homocysteine.[55] Serum lactate dehydrogenase, and serum total and indirect bilirubin are usually elevated in both vitamin B12 and folate deficiency.[56]

QUIZ QUESTIONS

1. The combination of a decreased MCV and increased RDW is characteristic of which disorder below?
   - A Thalassemia
   - B Iron deficiency anemia
   - C Pernicious anemia
   - D Alcoholism

2. An MCV greater than 110 fL is consistent with which of the following disorders?
   - A Iron deficiency anemia
   - B Folate deficiency
   - C Anemia of inflammation
   - D Sickle cell anemia

3. In macrocytic anemias due to folate deficiency which of the following is correct?
   - A Vitamin B12 levels are normal
   - B The MCV is decreased
   - C RBC folate levels are in the normal range
   - D Absolute reticulocyte count is elevated

4. What level of hemoglobin in males satisfies the WHO definition of anemia?
   - A <10 g/dL
   - B < 11 g/dL
   - C <12 g/dL
   - D <13 g/dL

5. Which of the following conditions might result in a normocytic anemia?
   - A Severe B12 deficiency
   - B Severe iron deficiency
   - C Anemia of chronic disease
   - D Thalassemia major

SECTION 6

PREANALYTICAL VARIABLES AND INTERFERING SUBSTANCES AFFECTING RBC PARAMETERS

LEARNING OBJECTIVES

After completing this section, you will be able to:

1. Identify factors that lead to a false increase or decrease in the RBC indices
2. Recognize when an interfering factor or substance is present in a sample

PREANALYTICAL FACTORS

Preanalytical hemolysis of blood samples is a common problem in medical practice. Factors contributing to this issue include the use of intravenous catheters and the vacuum sampling technique, as well as inappropriate puncture sites, complicated blood sampling, prolonged tourniquet application, underfilling of tubes and excessive shaking of specimens.[57]

Overfilling or insufficient mixing of sample right after phlebotomy may lead to (partial) clotting, resulting in an aspiration error or incomplete aspiration, causing erroneous results not just for RBC count, but also for other cell types.[22]

When an EDTA-anticoagulated blood sample is stored at room temperature, MCV tends to increase, especially beyond 24 hours, also affecting associated RBC indices.[22]

INTERFERING SUBSTANCES OR CONDITIONS

A very high WBC count from an infection, leukemoid reaction or leukemia may cause a false elevation in the RBC count on some impedance-based analyzers, if the WBCs are small and are incompletely separated from RBC. As a result, MCV will be falsely increased, and related indices are also affected.[22]

Lipemia or the presence of an abnormally high concentration of emulsified fat, is well characterized as an interfering substance affecting spectrophotometric HGB measurements, as it increases the turbidity of the sample. Since the MCH and MCHC are calculated based on the HGB value, they would be falsely elevated as well.

High concentration of Immunoglobulins (as in monoclonal gammopathies or in cryoglobulinemia) may have the same effect.[22]

In situations associated with major intravascular hemolysis, caused by chemicals, mechanical hemolysis associated with heart valves, or hemolytic transfusion reactions, free plasma HGB may be elevated enough to affect total HGB measurement, and as a result, MCH and MCHC.[22]

Plasma glucose and sodium concentration may affect MCV results. MCV can be falsely elevated in the presence of high glucose in the sample, either related to severe hyperglycemia in the patient or to drawing blood near an intravenous glucose infusion.

As the glucose concentration within the cell is in balance with the plasma glucose concentration, dilution of such sample in the diluent used by an analyzer may result in RBC swelling.[22,58]

RBC autoagglutination due to cold agglutinins leads to spuriously low RBC counts and abnormally high MCV (as each small RBC clump is considered as one single particle). HCT will be spuriously low, and MCHC is usually >36 g/dl. This combination of results is highly characteristic to cold agglutinins. The values can be normalized if the sample is warmed to 37oC and then run immediately. However, re-warming the sample may results in significant in vitro hemolysis; therefore, obtaining a new sample and keeping it at 37oC all the time is recommended.[22,59]

Table 5. Frequent interfering substances affect the RBC parameters.

Interferent	MCV	MCH	MCHC
Elevated WBC	Elevated	Elevated	Elevated
Lipemia	NA	Elevated	Elevated
Hemolysis	NA	Elevated	Elevated
Intravenous glucose contamination	Elevated	N/A	Decreased
Cold agglutinins	Elevated	N/A	Elevated

QUIZ QUESTIONS

1. Which of the following RBC parameters is affected by the presence of a cold agglutinin antibody in a patient sample? (select all that apply)

   A. RBC count

   B. MCV

   C. HCT

   D. MCHC

2. Severe hypernatremia causes RBCs to swell during analysis

   A. True

   B. False

3. Intravenous glucose contamination in a sample may lead to which of the following? (select all that apply)?

   A. Falsely elevated MCV

   B. Falsely decreased MCV

   C. Falsely elevated MCHC

   D. Falsely decreased MCH

4. Which of the following parameters would potentially be affected by an extremely elevated WBC count? (Select all that apply)

   A. RBC count

   B. HGB

   C. MCV

   D. Platelet count

GLOSSARY, APPENDIX, AND REFERENCES

GLOSSARY

Anemia of chronic disease (ACD): anemia that results from dysregulation of iron metabolism

Calculated hemoglobin (cHGB): hemoglobin value calculated based on cell-by-cell measured hemoglobin

Cellular hemoglobin concentration mean (CHCM): hemoglobin measurement derived from a cell-by-cell measurement on some hematology analyzers

Erythropoietin stimulating agents (ESA): pharmaceutical agents used to stimulate erythropoiesis

Functional iron deficiency (FID): a state in which there is insufficient iron incorporation into erythroid precursors despite adequate body iron stores

Hematocrit (HCT): ratio of the volume of red blood cells to the total volume of blood

Hemoglobin (HGB): protein found in RBCs that transports oxygen and carbon dioxide

Hemoglobin distribution width (HDW): a measure of the heterogeneity of the red blood cell hemoglobin concentration

%Hyperchromia: percent of RBCs containing greater than 41.0 g/dL of hemoglobin

%Hypochromia: percent of RBCs containing less than 28.0 g/dL of hemoglobin

%Macrocytosis: percent of RBCs with an MCV greater than 120 fL

Mean cell hemoglobin (MCH): measure of the average HGB content or weight of hemoglobin per RBC

Mean cell hemoglobin concentration (MCHC): measure of the average concentration of HGN in each individual RBC

Mean cell volume (MCV): measure of the average volume of RBCs

%Microcytosis: percent of RBCs with an MCV less than 60 fL

Red cell distribution width (RDW): measure of the heterogeneity of RBC size

Red cell distribution width coefficient of variation (RDW-CV): measure of the heterogeneity of RBCs expressed as a coefficient of variation

Red cell distribution width size distribution (RDW-SD): measure of the heterogeneity of RBCs expressed as size (width) distribution

APPENDIX: QUIZ ANSWERS

SECTION 1 RED BLOOD CELLS AND ERYTHROPOIESIS

1. A
2. B
3. D

SECTION 2 TRADITIONAL RBC INDICES

1. B
2. D
3. B, D

SECTION 3 ADVANCED RBC INDICES AND PARAMETERS

1. C
2. A
3. B
4. B
5. A

SECTION 4 RBC COUNTING TECHNOLOGIES

1. A
2. A
3. B
4. C
5. B
6. C

SECTION 5 CLINICAL UTILITY OF RBC INDICES AND PARAMETERS

1. B
2. B
3. A
4. D
5. C

SECTION 6 PREANALYTICAL VARIABLES AND INTERFERING SUBSTANCES AFFECTING RBC PARAMETERS

1. A, B, C, D
2. A
3. A
4. A, C

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