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From Wintrobe to the XXI century. A Story of Inspiration and Effort

Review Article

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Abstract

In 2014 we are celebrating the 80th anniversary of the Maxwell Myer Wintrobe´s pioneer works, one of the most important contributions in Clinical Laboratory and Medicine. Red cell indices continue to provide an essential support to the diagnosis and classification of anemia.

The erythrocyte indices mean cell volume, mean cell hemoglobin concentration, mean cell hemoglobin are called the Wintrobe indices. Hematology automation has progressed steadily since Wallace Coulter first applied electrical impedance technology to counting red cells and white cells.

Technological advances being incorporated into hematology analyzers since then are now allowing the access to more cellular information than was ever available before through a "simple routine CBC".

Current research is beginning to demonstrate that this information also has great potential to identify cellular changes that typically occur in several important medical conditions—bringing us all one step closer to using hematology analyzers as more than simple cell counters, but instead as powerful tools for the management of any medical condition that impacts the biology of blood cells.

There are increasing amounts of data provided, which require specialist knowledge to interpret as well as understand the limitations in the measurement of the parameter. Both laboratory scientists and clinicians need to keep up to date with new parameters and methods in hematology, implying a stronger collaboration between them to improve clinical decision making.

Keywords: Anemia; Erythrocyte Indices; Red Cells; Hematology Analyzers.

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Remembering The History

MMWintrobe the pioneer

In 2014 we are celebrating the 80th anniversary of Maxwell Myer Wintrobe´s pioneer publications, one of the most important contributions in Clinical and Laboratory Medicine. Red cell indices, widely known as Wintrobe´s indices, continue to provide an essential support to the diagnosis and classification of anemia.

From the measurements of the average volume and Hemoglobin (Hb) concentration of erythrocytes, the underlying etiology of anemia was brilliantly unveiled [1-4].

Anemia is a disease itself, but it is often symptomatic of other illness; every clinician deals with anemia in daily practice, taking into account that concomitant anemia makes the prognosis of the underlying disease worse for those patients.

His textbook *Clinical Hematology*, first published in 1942 and now in its 13th edition [5], remains a prototype of excellence and for many years stood alone as the premier text in the field. He had written and edited the first six editions by himself, though always depending on the critical peer review of his talented colleagues at Utah University, with his wife Becky, as he said his severest and most helpful critic [6].

Hematology, the Blossoming of a Science: A Story of Inspiration and Effort was published on 1985, shortly before his demise [7]. Writing this book partially as his memoirs, partially as history, Wintrobe realized that he could never cover the lives of all who had contributed to what he called "the Golden Age of Hematology".

Wintrobe's methods were based on manual measurements of hemoglobin, hematocrit and erythrocyte count. Reading his articles published the 30's [1-4], his concern with the accuracy of the methods and the reliability of the measurements become evident. First, he proposed a classification of anemia, based on the new

indices. Then he brilliantly postulated the link with red cell morphology to deduce the real etiology of each type of anemia.

The erythrocyte indices mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) were derived from precisely measured values of Hb, hematocrit and red cell count are called the Wintrobe indices, in honor of Wintrobe's invention [8].

The complete blood count (CBC; also called full blood count or hemogram, depending on geographical region) is one of the most frequently requested tests by clinicians and the indices are still applied for classifying anemia. The analysis nowadays is totally automated and correct interpretation of the results requires reuniting knowledge about the characteristics of the measurement equipment and the clinical meaning of the results [9].

The birth of automation

The first attempt in automation of blood cell counts was the introduction of the Coulter Principle. While under contract to the United States Navy, Wallace H. Coulter developed a technology for counting and sizing particles using impedance measurements in the late 1940s, though a patent was not awarded until October 20, 1953. The technology was principally developed for counting blood cells quickly by measuring the changes in electrical conductance when cells suspended in a conductive fluid pass through a small orifice. Presently, the automated cell counters incorporate this technology, which is referred to as the Coulter Principle.

Using electronic count and pulse height analyzer circuits, the number of particles and volume of each particle passing through the aperture can be measured. When the volume of liquid passing through the aperture can be precisely controlled and measured, the cell concentration in the blood sample can also be determined A typical measurement using Coulter type instruments takes less than a minute, as counting and sizing rates of up to 10,000 particles per second are well possible. The precision of the size measurements is generally better than 1% [10].

Hematology automation has progressed steadily since Wallace Coulter first impedance his technology to blood. By the 1980s, most hematology laboratories were reporting out a 7-parameter CBC and three-part white blood cell (WBC) differential, all obtained from a single aspiration of a small volume of blood in a stand-alone, bench-top instrument. Eventually, this process was upgraded even further when it became possible to obtain these results without uncapping the sample.

An often overlooked fact is that individual red cell volume determination is dependent on the technology used by a specific hematology analyzers and that hematocrit value is calculated from the measured MCV. The only red cell index that is consistent throughout all hematology analyzers is MCH, which is derived from precisely measured values of Hb and red cell count [8].

In the 90's the analyzers became capable of quantifying the heterogeneity in red cell volume, which resulted in the red cell distribution width (RDW), which is adding remarkably useful information to MCV. MCV is the mean of the volumes of all erythrocytes, whereas RDW refers to the dispersion of cells with different volumes present in the whole population, so the contribution of marginally sized subsets to the calculated mean value can be assessed [11].

Although RDW is generally expressed as a coefficient of variation of the distributions of erythrocyte volume, some hematology analyzers calculate RDW from the direct measurement of the width of the distribution [12].

Technological Progress

Erythrocytes

Optical technology combined with flow cytometry for measuring physical and chemical characteristics of cells constituted another breakthrough in automated hematology analyzers.

The basic purpose of the optics bench in a flow cytometer is to detect light scattered by cells as they pass through a flow cell illuminated by a light beam, most often a laser beam. The scattered light harbors information on the cell size, its surface and internal structures. When scattered light is measured under different angles, these data can be combined in multiple dimensions, allowing discrimination of different cell types [13].

Optical flow cytometric technology for RBC measurement was first made available by the then Technicon Company in their H* series of instruments, later followed by the Advia® hematology analyzers (Bayer Diagnostics, presently Siemens Healthcare Diagnostics, Deerfield, IL, USA). This optical technology enables measuring, simultaneous and independently, the cellular Hb concentration of individual erythrocytes as well as their volume, derived from light scatter by isovolumetrically sphered RBC according to the Mie theory [14,15]. Mie theory describes the mathematics of light diffraction by spherical objects, in this case the red cells, which are transformed into isovolumetric spheres. When using monochromatic laser light, diffraction is only a function of the size and the refractive index of the object, which in erythrocytes is directly related to the cell's Hb concentration.

In the red cell/platelet channel, red cells are converted to spheres. Using Mie theory, low-angle (2°-3°) and high-angle (5°-15°) light scatter is measured and mathematical models in the software use these scatter signals for calculating the size and cellular hemoglobin concentration (CHC) of individual red cells.

Both measurements can be provided as a 2-dimensional scattergram. In this graph, the volume of individual red cells is plotted on the y-axis, and their intracellular hemoglobin concentration on the x-axis. In addition to a graphical representation, also quantitative data can be derived from these measurements: hypochromic RBC with CHC < 280 g/L, hyperchromic RBC with CHC > 410 g/L, microcytic with volume < 60 fL and macrocytic with volume > 120 fL

Technicon Instruments was the first manufacturer to offer a complete set of extended RBC parameters in their H*3 hematology analyzer [16] and this explains why the majority of literature on these parameters was produced using this analyzer and its successors of the Advia series (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

More recently, Abbott (Abbott Diagnostics, Santa ClaraCA, USA) introduced extended RBC parameters on the CELL-DYN Sapphire analyzer. These are calculated from three-dimensional laser light scatter, applying the principles of the Mie theory, which is basically the same as in the Advia analyzers [17,18].

Over the past years, other manufacturers have started offering extended RBC parameters, although differences in the technology used make it impossible to directly compare the numerical values of RBC parameters derived from different instruments.

Sysmex analyzers XE-5000 and XN-series (Sysmex Corporation, Kobe, Japan) report some RBC extended parameters, the percentages of erythrocyte subsets. In the reticulocyte channel hypochromic and hyperchromic erythrocytes are quantified, but it is important to highlight that is Hb content, not cellular Hb concentration, what is reported. Hypochromic red cells by Sysmex are those with an Hb content less than 17 pg, while hyperchromic are those with Hbover 49pg [19].

Because of the relatively long life span of mature erythrocytes, the classical Wintrobe indices reflect the overall erythrocyte production and iron incorporation into Hb over the last 2-3 months.

As previously mentioned in the 80s the analyzers were capable to quantify the heterogeneity in distribution of both mean cell volume (RDW) adding a remarkable useful information to MVC. This is not the case for MHC. Calculated from red blood cell count and Hb, MCH represents the average.

But different set of numbers can render nearly the same mean. In order to describe mathematically a set two types of measures are necessary centralization measures (more abundant individuals) and of dispersion, giving insight on the heterogeneity of the population.

The percentage of subsets can give complementary information of the contribution of cell with extreme values (hypochromic and hyperchromic cells) to the mean values, MCH. The percentages reflects *how* Hb is distributed in the individual cells, in a uniform way or not; if polychromasia is present, the fluctuations of iron availability to the erythron in the previous weeks can be highlighted [11,20].

Similar application of those technologies is being introduced by other companies (Mindray Biomedical Electronics, Nanchang, Shenzhen, China) on their analyzers [21].

Reticulocytes

Reticulocytes are immature red blood cells with a life span of only 1 to 2 days. The reticulocyte count represents an important test in the study of marrow erythropoietic activity. In clinical practice it can be used for diagnosis (distinguishing hemolytic or post-hemorrhagic anemias from hypoplasticanemias), in monitoring therapy (megaloblastic anemia, iron deficiency), or as a way of checking early regeneration after marrow or stem cell transplant.

The presence of cytoplasmatic RNA is the basic difference with mature red cells which is employed for detecting this minor fraction from the RBC whole population.

The reticulocytes are classically identified by optical microscopy, using supravital stain (brillantcresyl blue, new methylene blue) that binds to ribosomal RNA. The manual/visual method of counting reticulocytes evaluates the number of reticulocytes in a total of 1000 RBCs [22].

The additional technological progress in the last 10–15 years has resulted in automated measurements of the number of reticulocytes and cellular reticulocyte parameters. Starting in the mid-1990s [23], different stains binding RNA are used to distinguish reticulocytes, some fluorescence (cyanine dye, Abbott; Thiazole orange, Horiba; polymethine, Sysmex) or by absorbance (Oxazine, Siemens; new mehtylene blue, Beckman-Coulter) [8].

During the last years, flow cytometry has become the reference technique for measuring absolute reticulocyte counts and parameters of reticulocyte maturation [24,25].

Automated techniques for reticulocyte enabled the integration of reticulocyte analysis with the complete blood count in automated hematology analyzers. The advantages derived from automation are high throughput, improve precision and reliability of reticulocyte counts compared to microscope review (essentially owing to the high number of cells counted) [23],[26-28].

In summary, technological advances being incorporated into hematology analyzers since Wintrobe´s days are now allowing the access to more cellular information than was ever available before through a "simple routine CBC " [29,30].

The added information contained in the "new extended hemogram" has proven its usefulness in certain clinical conditions: anemia of chronic disease, functional iron deficiency and monitoring the availability of iron during treatment with erythropoietin [11,17],[31-40] thalassemia trait screening [41-43] spherocytosis [44,45] iron deficiency and latent iron deficiency [46-58].

In all these clinical conditions, common in our daily practice, the added information in the contemporary hemogram aids the clinicians to make a rapid and reliable diagnosis, without additional costs. Enabling the clinician to get a prompt accurate diagnosis reduces unnecessary testing and avoids inappropriate treatment, which results in an efficient use of the resources of the Health Systems.

The Promising XXITH Century

Current research is beginning to demonstrate that this information also has great potential to identify cellular changes that typically occur in several important medical conditions—bringing us all one step closer to using hematology analyzers as more than simple cell counters, but instead as powerful tools for the management of any medical condition that impacts the biology of blood cells.

In physiological conditions, bone marrow produces $200*10^9$ red blood cells per day; the body normally loses the same number through senescence, maintaining an overall steady state but little is known about the life cycle of 120 days of RBC.

Novel and promising research [59] has shed new light on the relationship between Hb content, Hb concentration and red cell volume. Using a systems- biology approach the dynamics of circulating red cells can be analyzed, linking the changes of volume and Hb concentration to red cell lifespan and removal.

The mechanisms that regulate the number, size, and hemoglobin concentration of normal red cells in circulation are not well understood. It has been established, however, that after their release from the bone marrow red cells undergo a reduction in volume and total hemoglobin content. Higgins and Mahadevan [59] used

theory from statistical physics together with standard red cell indexes derived from electronic cell counters and the information generated by flow cytometry, to develop a master equation for the maturation and clearance of red cells. Their mathematical model implies that the total number of red cells added to the circulation equals the number removed and suggests that there is a threshold value for the MCHC below which red cells are cleared from the circulation [60]. The model distinguishes the dynamics of red cell population in healthy subjects from those of patients suffering from iron deficiency, thalassemia or anemia of chronic disease [59].

As has been exposed, manufacturers have developed fine hematology analyzers that achieve high levels of precision and accuracy in cell counting through the examination and identification of many thousands, not hundreds, of cells in each sample analyzed. In this way there are increasing amounts of data provided, which require specialist knowledge to interpret as well as to understand the limitations in the measurement of the parameter. Both laboratory scientists and clinicians need to keep up to date with new parameters and methods in hematology, implying a stronger collaboration between them. Good laboratory practice ensures that reliable results of laboratory tests are reported to the clinician [61].

The modern clinical hematology laboratory is subject to strong pressure to provide clinically relevant information that can help clinicians to make a diagnosis in a fast, cheap and useful manner, but we must not forget the words by Max Wintrobe:

"*And yet, we must not stop exploring and measuring, for there is always more to learn*"

Happy anniversary!

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