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Review

Automated reticulocyte counting: state of the art and clinical applications in the evaluation of erythropoiesis

Elisa Piva[1,*], Carlo Brugnara[2], Lino Chiandetti[3] and Mario Plebani[1,4]

[1] Department of Laboratory Medicine, University-Hospital of Padova, Padova, Italy
[2] Department of Laboratory Medicine, Children's Hospital, Boston, MA, USA
[3] Department of Pediatrics, University of Padova, Padova, Italy
[4] Leonardo Foundation, Abano Terme General Hospital, Italy

Abstract

The reticulocyte count reflects the erythropoietic activity of the bone marrow and is thus useful in both the diagnosis of anemias and in monitoring bone marrow response to therapy. Starting in the mid-1990s, automated flow-cytometric analysis has replaced traditional microscopic quantitation of reticulocytes. Reticulocyte analysis now includes measurements mRNA content and the maturity of reticulocytes, cell volume, hemoglobin concentration and content. The immature reticulocyte fraction is a reliable early predictor of hematopoietic engraftment following allogeneic stem cell transplantation, while the reticulocyte hemoglobin content provides an indirect measure of the functional iron available for new red blood cell production over the previous 3–4 days. Especially in anemic newborns, reticulocyte analysis is useful to help clinicians follow erythropoietic changes, to monitor response to recombinant human erythropoietin therapy, to gauge transfusion needs, and to evaluate jaundice. Despite improved accuracy and precision, significant problems still persist in maintaining adequate levels of precision and comparability across different laboratories. In the absence of better laboratory standardization, having a single reference range for the parameters provided by flow-cytometric studies of reticulocytes remains problematic.
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Keywords: automated reticulocyte counting; erythropoiesis evaluation; prematurity.

*Corresponding author: Elisa Piva, MD, Dipartimento Medicina di Laboratorio, Azienda Ospedaliera-Università di Padova, Via Nicolò Giustiniani 2, Padova 35128, Italy
Phone: +39 049 8211917, Fax: +39-049-8211915,
E-mail: elisa.piva@sanita.padova.it
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Introduction

Described as transitional forms of red blood cells by Wilhelm H. Erb in 1865, reticulocytes are the developing cells between erythroblasts and mature erythrocytes (1). Approximately 3 days before the cell leaves the bone marrow, the nucleus of the erythroblast is expelled and the cell becomes a reticulocyte. On the fourth and final day of maturation, the reticulocyte enters the bloodstream and becomes a mature red blood cell one day later (2). In vitro, the time required for nascent murine reticulocytes to mature into erythrocytes, as defined by loss of reticulin staining, is about 3 days. The development of a biconcave shape requires an additional day or two after the reticulin is completely lost (3). Mammalian erythroblasts proliferate and differentiate in bone marrow microenvironmental compartments called erythroblastic islands. These islands consist of a central macrophage, which extends cytoplasmic protrusions to a ring of surrounding erythroblasts. The interaction of cells within the erythroblastic island is essential for both the early and late stages of erythroid maturation. It has been proposed that early in erythroid maturation the macrophages provide nutrients, proliferative and survival signals to the erythroblasts, and phagocytose extruded erythroblast nuclei at the conclusion of erythroid maturation. There is also accumulating evidence for the role of macrophages in promoting enucleation itself (4). Complex, regulatory feedback pathways within erythroid niches generate 2×10^6 reticulocytes/s during steady state, with an ability to increase production by 15–20-fold under stress conditions (5). The maturation of reticulocytes into erythrocytes involves morphological, biochemical and functional changes (6). The molecular mechanisms underlying the removal of organelles during terminal differentiation of erythroid cells remain undefined, although for erythroid maturation, autophagy has been suggested to mediate the elimination of organelles (7, 8). Because of the dynamic, short-lasting nature of reticulocyte maturation, reticulocyte analysis is an important tool in assessing erythropoietic activity. The advent of automated analysis has renewed the interest in the clinical and biological relevance of reticulocyte counting.

Definition, properties and methodologies for recognition of reticulocytes

The reticulocyte derives its name from the reticulum of ribonucleic acid and protein precipitated by fixation and staining that appears microscopically after treatment with supravital

dyes (9). In 1976, Gilmer and Koepke defined the criteria for morphological identification of reticulocytes (10). This definition has been revised a number of times. The term ''proerythrocyte'' was also proposed, but that term currently defines the reticulocyte in a morphological rather than biochemical sense (11). In 1997, the H44-A document from the National Committee for Clinical Laboratory Standards (now called Clinical and Laboratory Standards Institute) and the International Council for Standardization in Hematology (NCCLS-ICSH) defined reticulocytes as cells presenting at least two blue-staining granules, visible without fine microscope adjustment and away from the cell margin (12). Compared with mature erythrocytes, reticulocytes are larger and less dense. They are irregularly shaped, and their volume is about 25% greater than that of mature cells although the surface/volume ratio seems to remain constant for both cell types (13). Early reticulocytes are biochemically more active than mature cells while in the bone marrow, and continue to synthesize hemoglobin (Hb) and are capable of absorbing iron (14–16). The Hb content of circulating reticulocytes is similar to, or slightly higher than, that of erythrocytes, while the Hb concentration is lower (17, 18).

The identification of reticulocytes is based on the recognition of stainable nucleic acids, i.e., cellular RNA, by new methylene blue (NMB) or other cationic dyes (19). The term ''supravital'' technique is used because the dye is added before fixation of fresh blood; the dye crosses the erythrocyte membranes and interacts with RNA. Criteria that include the affinity of reticulocyte RNA for either chemical or fluorescent staining have been adopted for automated methods: among the former, the most commonly used are brilliant cresyl blue, new methylene blue and Azur B. A wide variety of fluorochromes have been used for reticulocyte enumeration; the first being auramine O, while acridine orange has become popular in fluorescence microscopy. Fluorochromes, such as propidium iodide, ethidium bromide, thioflavin T, thiazole orange or polymethine cross the cell membrane and bind to nucleic acids

Microscopic reticulocyte counting

Microscopic reticulocyte counting is based on supravital staining of cytoplasmic ribosomal RNA, and the morphologic differentiation of reticulocytes from erythrocytes is performed by a qualified laboratory examiner. This traditional technique, which has been the standard method since the late 1940s, is rather imprecise and is affected by manual steps. Sources of imprecision are due to the techniques used for preparation, such as different procedures, staining variation, distributional variability for quality of blood film and intra- and inter-observer variations. Variability among observers in morphological identification of reticulocytes and in the size of the total number of cells counted represent other limitations of the method. It is critical to evaluate a sufficient number of red cells to ensure that the measurement is adequately precise. The minimum sample size for erythrocytes, within which reticulocytes must be enumerated, is 1000, as defined by Poisson statistics. This is because reticulocyte counting follows the statistical law of binomial distributions (20). The ICSH reference measurement procedure specifies the number of cells to be counted at different coefficient of variation (CV) values (Table 1) (12). For routine purposes, a CV of 10% is acceptable. Manually counting of such a large number of cells is obviously labor intensive. To improve the overall precision of manual reticulocyte analysis, the use of Miller ocular micrometer disc (also called disks) has been proposed. The Miller disc is a counting aid that provides a standardized area in which to count erythrocytes. The disc incorporates a large square counting area within which there is a smaller square that is one-ninth the size. This is a labor-saving device that eliminates the need to manually count all of the erythrocytes in the total sample, and facilitates the counting of the reticulocyte proportion (12).

Data derived from laboratory proficiency testing (PT) programs shows that manual reticulocyte counts have shown unacceptably poor performance. Intra-laboratory reproducibility is poor and CVs range from 20% to 50% on samples with normal to low reticulocytes counts (21, 22). Surveys carried out by the College of American Pathologists (CAP) in 1983 and 1984 on samples with a mean of 1.4% and 6.65% reticulocytes showed a coefficient of variation of 17%–54.1% (23). In 2008, the External Quality Assessment Scheme (EQAS) for reticulocytes promoted by the Italian Center of Biomedical Research (CRB), Castelfranco Veneto, found that for a sample with a median reticulocyte count of 1.35%, the CV for a small number of participants (n = 4) was 34.04%. In the US, in the CAP 2009 proficiency testing survey for reticulocyte counting, the CV among 281 participants was 48.6% for a sample with a median reticulocyte count of 0.8%. Using the Miller disk as recommended, the CV among 261 participants was higher (50.5%). Standardized NMB staining procedures have been recommended to improve the comparability of manual reticulocyte counting. Unfortunately, the NMB procedure requires counting of at least 2000 red cells or up to the required number as noted in Table 1, which does not represent the current standard of practice in clinical laboratories. Measurement procedures and the NMB method described in NCCLS-ICSH H44-A2 guide-

Table 1 NCCLS-ICSH proposed number of erythrocytes to be counted in order to achieve acceptable imprecision at various reticulocyte percentages (H44-A2, 2004).

Reticulocyte percentage (unit fraction)	Number of RBC to be counted for CV of 5%	Number of RBC to be counted for CV of 10%
0.01	39,600	9900
0.02	19,600	4900
0.05	7600	1900
0.10	3600	900
0.20	1600	400
0.50	400	100

If a Miller disc is used, the actual number of erythrocytes counted would be 1/9 of the numbers listed above. The imprecision is inversely proportional to the number of erythrocytes counted.

lines have been recommended as the reference method when evaluating the performance of automated techniques (24).

Automated reticulocyte counting

Automated techniques for reticulocyte counting became available in the mid-1990s, enabling the integration of reticulocyte analysis with the complete blood count (CBC) in high throughput automated hematology analyzers. Fully automated methods offer the advantage of substantially increasing measurement precision by analyzing a much greater number of cells compared with manual methods (approximately 10 times more). These methods eliminate the variability due to pre-analytic staining, dilution, and incubation, and provide a much faster turn around time with the opportunity for reflex reticulocyte testing based on established algorithms. In addition, these methods also eliminate the variability due to the subjective elements associated with manual identification of reticulocytes. This increased precision has made it possible to provide reliable counts on a very low numbers of elements, which was not possible with the manual method. Reticulocyte analysis has also been extended from the simple enumeration of reticulocytes to reliable measurements of mRNA content and cellular indices, such as volume, Hb concentration, and content. Assessment of reticulocyte maturity is based on the fluorescence or absorbance intensity of reticulocytes due to the RNA content. Although automated methods for detection of RNA are based on principles of light scatter and absorption or fluorescence, there are many differences between the specific analytical techniques. Details about the most commonly used automated reticulocyte analyzers and the parameters they provide are shown in Table 2. The first comparative evaluations showed significant differences between methods. However, instruments were considered equivalent for daily routine enumeration of reticulocytes (25). When methods that use the fluorescent dye thiazole orange were compared with those using the new supravital dye methylene blue for reticulocyte staining, significant differences were shown (26). Following this, the performance of reticulocyte counting of five automated reticulocyte analyzers was evaluated in comparison to the reference method based on microscopic evaluation. Agreement was satisfactory for all automated methods that were evaluated, although a tendency for overestimation at low counts was noted (27). The evaluation performed by the Hematology study group (GdSE) of the Italian Society of Laboratory Medicine (SIMeL) compared five fully automated flow cytometers for enumeration of reticulocytes compared with the NMB method, in accordance with the NCCLS-ICSH H44-A document (24). For each method, imprecision was determined and the data is shown in Table 3. Imprecision was compared to that reported in previous studies. Analytical goals derived on the basis of biological variability described in the literature were also reported (28). In 1994, a multi-institutional inter-laboratory correlation study by Davis et al. assessed the CVs amongst all methods and institutions and found ranges from a mean value of 69% for samples with $<0.5\%$ reticulocytes, to 24.1% for those with $>2.5\%$ reticulocytes. The best performance was observed with the TOA R-1000, the first dedicated reticulocyte analyzer available on the market. Using this instrument, the CV was 18.4% for samples with $<0.5\%$ reticulocytes and 4.6% for samples with $>2.5\%$ reticulocytes (30). The 2009 EQAS program for automated reticulocyte counts by the Italian CRB involved eight method-groups with a total of 104 instruments. Amongst all instruments, global CVs ranged from 39.14% for samples with median reticulocyte count of $1.25\% \pm 0.49\%$, to 16.09% for samples with a median value of $4.06\% \pm 0.65\%$. For method-specific groups, the CVs were lower. For two optical light scatter detection methods, a sample with median of $1.25\% \pm 0.49\%$ showed CVs of 17.11% and 10.17%, respectively, while those for two fluorescence detection methods were 8.83% and 11.04%, respectively. On a sample with a median reticulocyte count of $4.06\% \pm 0.65\%$, the CVs were 16.74% and 4.12% for the optical methods and 9.25% and 6.81% for the fluorescent methods. The same inter-method variability was shown for the absolute reticulocyte count. Although substantial improvements were seen among the different methodologies, some variability, which should be lower, still remains. Critical aspects are due to lack of an acceptable automated reference method, lack of reference material for instrument calibration and harmonization among the different technologies.

Due to these differences, method-specific reference intervals have been established which are not interchangeable, and thus do not allow for easy monitoring of patients tested at different laboratories. Until this problem is solved, every laboratory should determine reference values which are instrument- and method-specific to their own instrument and method. Reference reticulocyte values obtained using fluorescence are reported in the literature to be higher than those obtained utilizing automated methods with supravital dyes (24). Whether the reticulocyte count is reported as a percentage (%) or as a number per volume of blood (10^9/L), the distribution of values from the general population is skewed, and the reference range should therefore be calculated as the central 95% percentile of results (31). A comparison of reference ranges reported by different authors is shown in Table 4 (13, 27, 29, 32–37). Among the different methods, the differences are evident. Standardization and harmonization of automated reticulocyte analysis should be a major focus of work for the next few years.

Variation on reticulocyte measurements: biological and pre-analytical components

Biological and pre-analytical variation, together with analytical and post-analytical variation, represents important sources of variation for laboratory results. Numerous studies on biological variation have been published over the years, but few were concerned with issues related to hematological quantities, and to reticulocytes in particular. The components of biological variation are used to set analytical quality

Table 2 State of art of reticulocyte analytical capabilities for the majority of hematology analyzers. Principles of methodology, techniques and parameters are provided.

Company	Instrument	Method	Dye	Parameters provided by the reticulocyte measurements	Notes
Abbott	CELL DYN Sapphire	Fluorescence detection	Cyanine dye (Sybr II)	IRF (immature reticulocyte fraction)	Fully automated
	Cell Dyn 4000	Fluorescence detection	CD4K530	IRF	Production has been discontinued
	Cell Dyn 3700	Absorbance	New methylene blue	IRF	Manual operation required
	Cell Dyn Ruby, Cell Dyn 3200*	Absorbance	New methylene blue	ND	Manual operation required *Production of the Cell Dyn 3200 has been discontinued
Beckman Coulter Inc.	UniCel DxH 800	Flow cytometric digital analysis using different angles of light scattering detection, impedance, radiofrequency	New methylene blue	IRF, MRV (mean reticulocyte volume), HLR (high light scatter reticulocytes), RDWR-CV (reticulocyte distribution width, coefficient of variation), RDWR-SD (reticulocyte distribution width, standard deviation), MSCV (mean sphered red cell volume), LHD (low hemoglobin density), RSF (red cell size factor), MAF (microcytic anemia factor)	Fully automated
	Coulter LH 700 series (780-785-750)	Light scattering, impedance and conductivity (VCS technology)	New methylene blue	IRF, MRV, RDWR-CV, RDWR-SD, HLR, MSCV, RSF, MAF	Fully automated
	Coulter LH 500	Light scattering, impedance and conductivity (VCS technology)	New methylene blue	IRF, MRV, MSCV	Fully automated
	Coulter HmX	Light scattering, impedance and conductivity (VCS technology)	New methylene blue	IRF, MRV	Manual operation required
	Coulter Gen*S	Light scattering, impedance and conductivity (VCS technology)	New methylene blue	IRF, MRV, HLR, MSCV	Fully automated Production has been discontinued
	Coulter STKS, MAXM	Light scattering, impedance and conductivity (VCS technology)	New methylene blue	IRF, MRV	Manual operation required Production has been discontinued
Horiba	ABX Pentra DX 120	Impedancemetry technology and fluorescence detection	Thiazole orange	IRF, MRV, RETH% (high fluorescent reticulocytes), RETM% (medium fluorescent reticulocytes), RETL%	Fully automated

(Table 2 continued)

Company	Instrument	Method	Dye	Parameters provided by the reticulocyte measurements	Notes
				(low fluorescent reticulocytes), IMM% (immature reticulocytes), MFI% (mean fluorescent index), CRC% (corrected reticulocyte count)	
Siemens	ADVIA 2120	Absorbance and optical light scatter detection	Oxazine 750	IRF, MCVr, CHr, CHCMr, RDWr, HDWr, CHDWr, H-RET% (high adsorbance reticulocytes), M-RET% (medium adsorbance reticulocytes), L-RET% (low adsorbance reticulocytes), in development IRF	Fully automated
	ADVIA 120	Absorbance and optical light scatter detection	Oxazine 750	MCVr, CHr, CHCMr, RDWr, HDWr, CHDWr, H-RET% (high adsorbance reticulocytes), M-RET% (medium adsorbance reticulocytes), L-RET% (low adsorbance reticulocytes)	Fully automated Production has been discontinued
	H3	Absorbance and optical light scatter detection	Oxazine 750	MCVr, CHr, CHCMr, RDWr, HDWr, CHDWr, H-RET% (high adsorbance reticulocytes), M-RET% (medium adsorbance reticulocytes), L-RET% (low adsorbance reticulocytes)	Manual operation required Production has been discontinued
Sysmex	XE 5000, XT 4000i	Fluorescence and light scattering	Polymethine	IRF, Ret-He, (reticulocyte hemoglobin equivalent, formerly Ret-y), RBC-He (red blood cell hemoglobin equivalent), MicroR (microcytic red blood cell ratio), MacroR (macrocytic red blood cell ratio), LScRBC (low scattered red blood cell ratio), HScRBC (high scattered red blood cell ratio), DELTA-He (delta hemoglobin equivalent), RPI (reticulocyte production index)	Fully automated
	XE 2100, XT 2000i	Fluorescence and light scattering	Polymethine	IRF, Ret-He (formerly Ret-y), RPI	Fully automated
	SE 9500, R Series (1000-2000-3000-3500)	Fluorescence	Auramine 0	IRF, LFR (low fluorescence ratio), MFR (medium fluorescence ratio), HFR (high fluorescence ratio)	Fully automated Production has been discontinued Instruments of the R-series are dedicated cytometers

Table 3 Analytical imprecision of the most common high throughput hematology analyzers, as reported in two different studies. Imprecision has been expressed as the coefficient of variation (CV), calculated in duplicate measurements of reticulocyte (10^9/L) and IRF, for values within the method-specific reference range (n = 126 and n = 225, respectively).

Company	Instrument	Reticulocytes mean, 10^9/L	CV, %	IRF mean	CV, %
Abbott	CELL-DYN 4000[a]	69.9	5.1	0.26	7.5
	CELL-DYN 4000[b]	57.8	6.5	0.26	7.6
Beckman Coulter	LH 750[a]	51.6	11.4	0.30	10.2
	GEN*S[b]	45.4	10.3	0.29	10.8
Horiba	ABX PENTRA 120[a]	101.2	10.9	0.12	11
	VEGA RETIC[b]	64.9	11.7	0.16	20.5
Siemens	ADVIA 120[a]	62.2	6.4	0.12	14.6
	ADVIA 120[b]	60.1	10.9	0.11	19.9
Sysmex	XE 2100[a]	53.2	6.4	0.06	25.3
	SE 9500[b]	46.7	6.7	0.15	20.5

The desiderable performances for analytical imprecision range from 1.4% to 10%, the lower and higher value calculated utilizing data from published intra- and inter-individual biological variation studies. [a]Evaluation carried out by Hematology study group (GdSE) of the Italian Society of Laboratory Medicine (SIMeL) and [b]Buttarello et al. See references (27–29) in the text.

Table 4 Reference ranges for absolute reticulocyte count and reticulocyte parameters in adults, as reported by different authors.

Authors	Reticulocytes and parameters	Company/instrument				
		Abbott CELL-DYN 4000	Beckman Coulter LH 750	Horiba ABX PENTRA 120	Siemens ADVIA 120	Sysmex XE 2100
Garzia et al., 2007 (n = 55)	CHr, pg				29.2–33.6	
	Ret-He, pg					30.5–35.5
Banfi et al., 2006 (n = 73)	MRV, fL		93–117.8			
	IRF		0.19–0.42			
Noronha, 2005 (n = 50)	Reticulocytes, 10^9/L					20.5–122.8
	IRF					0.01–0.18
Bovy et al., 2005 (n = 57)	Reticulocytes, 10^9/L				38.3–65.1	
	MCVr, fL				103.3–109.9	
	CHr, pg				32.2–32.8	
Canals et al., 2005 (n = 196)	Ret-He, pg					30.2–36.7
GdS-Simel-Emat, 2003 (n = 127)	Reticulocytes[a], 10^9/L	29–146	18–117	30–148	30–111	18–104
(data not published)[a]	IRF	0.16–0.42	0.19–0.43	0.07–0.19	0.04–0.23	0.01–0.13
	MCVr, fL		91–111	98–120	100–114	
	CHr[a], pg				28.0–35.1	
Van den Bossche et al., 2002	Reticulocytes, 10^9/L		GEN*S			SE 9500
(n = F:175; M:142)		21–98 (F)	24–73 (F)	22–95 (F)	19–64 (F)	16–66 (F)
		30–110 (M)	30–90 (M)	31–130 (M)	29–69 (M)	16–70 (M)
	IRF	0.14–0.35				
Buttarello et al., 2002 (n = 225)	IRF		GEN*S	VEGA RETIC		SE 9500
		0.15–0.35	0.20–0.37	0.06–0.23	0.04–0.25	0.05–0.21
Buttarello et al., 2001 (n = 225)	Reticulocytes, 10^9/L	25–108	GEN*S	VEGA RETIC	27–125	SE 9500
			20–85	30–130		23–95
d'Onofrio et al., 1995 (n = 64)	MCVr, fL				H*3	
					103.2–126.3	
	CHr, pg				H*3	
					25.9–30.6	
Piva et al., 2007 (n = 126)	Reticulocytes, 10^9/L				22–85	27–90
(data not published)[a]	MCVr, fL				98–115	
	CHr, pg				30.2–35.9	
	Ret-He, pg					30.7–37.0

[a]Healthy adults for reference population were selected according to NCCLS-ICSH H44A2 document. Reference ranges (95% interval, non-parametric method) are calculated from the selected reference population in a parallel evaluation of the listed instruments. See text for the references.

specifications for bias and imprecision, evaluate serial changes in individual analytes, and assess the clinical utility of population-based reference intervals (38). Biological variation should be considered when counting reticulocytes (39). In an article from 1998 by Sandberg et al., reticulocyte measurements were performed for 7 weeks on 13 healthy subjects utilizing two different instruments (Sysmex R-1000 and Bayer H*3). The reticulocyte count showed a within-subject biological variation of 11% on both instruments. However, the between-subject variation was different for the two instruments; 33% for the fluorescent method and 26% for the adsorbance-optical method (40). Values seem to be similar to those for leukocytes, and according to Costongs et al. cells have within-subject variation that is inversely related to their life span (41). For both methods, the three fractions of maturation, low (LFR), middle (MFR) and high (HFR) showed biological variation that was similar to that of the reticulocyte count. However, variability of reticulocyte Hb content (CHr), reticulocyte mean cellular volume (MCVr) and reticulocyte Hb concentration (CHCMr) was smaller and comparable to those of the corresponding erythrocyte parameters, conforming with normal erythropoiesis. The index of individuality obtained for both methods was <0.5 for the reticulocyte parameters, whereas it was 0.6 for MCVr (40). In a subsequent study, the biological variability of reticulocytes was assessed over a period of 7 consecutive days in 20 healthy subjects. Within- and between-subject variation, expressed as %CV, was 5.8% and 32.4%, respectively. The CVs were calculated from the average of results from five different instruments (42). Within-day variation in reticulocyte percentage was described, although this data was not confirmed by others studies (43, 44). As far as for gender-based differences, studies differ significantly with respect to methods, number of subjects enrolled and age range (45–48). Significant changes have been reported with age (47, 49). In our experience, no significant gender differences have been found (data not shown), whereas differences have been seen in neonates. Menarche and menopause do not cause variation in reticulocyte counts (47). During pregnancy, reticulocytes decrease slightly during the 24th–28th week, and then increase (50). Variation caused by the application of a tourniquet during blood collection did not generate any meaningful differences in the reticulocyte count (51). Storage at room temperature causes significant changes in reticulocyte counts after 24 h, whereas at 2–6°C counts remain unchanged, although with certain limitations for parameters derived or calculated from cellular volumes (52, 53). The variation with storage seems to depend on the hematology analyzer that is used (54). However, measurement uncertainty for reticulocytes, estimated as several pre-analytical uncertainty components combined with data on analytical variation and literature reports on biological variation, was reported to be 41%. This was larger than that obtained for platelet and leukocyte counts, which were 24% and 31%, respectively. The analytical component of measurement uncertainty for reticulocytes was relatively high (12%) but was exceeded by sample-related uncertainty (13%), resulting with the largest combined sample- and analysis-related uncertainty of hematological measurements in this study (18%). Also, reticulocytes seem to be more sensitive than chemistry measurands with respect to the pre-analytical factors that were examined (55).

Reticulocyte maturation parameters and cellular indices

Attempts have been made to classify the maturity of reticulocytes based on the quantity of reticulum they contain. The first attempt was made by Heilmeyer, who divided cells into four groups (the fourth being the most mature). These groups were designated using Roman numerals and characterized by a progressive reduction in the amount and compactness of reticulum (56). Under normal conditions, in the peripheral blood more than 90% of the reticulocytes are at stage 3 and 4 of maturation (57). This evaluation of reticulocyte maturity was difficult and poorly reproducible due to the high degree of subjectivity. Automated methods classify the maturity of reticulocytes based on the amount of stained RNA content. The immature reticulocyte fraction (IRF) has been proposed as a means of assessing reticulocyte maturation. Originally called the ''reticulocyte maturity index'' (RMI), the ''immature reticulocyte fraction (IRF)'' has become the internationally accepted term to indicate the least mature fraction of reticulocytes (58). In an attempt to harmonize the results obtained by different automated methods, it has been proposed that instruments that identify three populations of differing maturity should define the IRF as the sum of the populations of high and medium immaturity (59). Few studies have compared the performance of automated methods in estimating the immature reticulocyte fraction. Results highlight the substantial differences in the reference intervals which are strictly method-dependent (29). Even though standardization and diagnostic efficiency still require substantial improvement, a level of inter-method agreement seems to have been achieved for some instruments (60, 61). In another study involving elite athletes, the values of IRF determined with two different technologies showed good correlation (62).

The most important application of IRF seems to be as early marker of engraftment in hematopoietic stem cell transplantation (63). Several studies have demonstrated that using IRF changes as an indicator of engraftment precedes other changes, such as those in white cell counts (63–68). Although the information conveyed by increasing IRF is important for patients and physicians, it still remains to be determined whether it can be translated into clinical decision-making, leading to benefits for both the patient and the health care system. Some instruments provide measurements of reticulocyte cellular parameters, i.e., mean reticulocyte volume, MCVr, reticulocyte distribution width (RDWr) and reticulocyte Hb content (CHr or Ret-He). CHr is measured during reticulocyte analysis with automated hematology analyzers manufactured by Siemens (Advia 2120 and 120). CHr is the product of the cellular volume and cellular Hb concentration. The volume and Hb concentration of individual

reticulocytes are determined independently from measurement of light scatter at two different angles following isovolumetric sphering of stained reticulocytes. Ret-He, (formerly RET-Y), is measured by automated hematology analyzers from Sysmex (XE 5000 and 2100), and is similar, but not identical, to CHr. Ret-He is a measure of the forward scatter of stained reticulocytes and has a curvilinear relationship with CHr (69). The MCVr multiplied by the number of reticulocytes gives the ''hematocrit'' (Ht) value of the reticulocytes. As shown in Table 4, MCVr shows differences between various instruments, and therefore requires the use of method-specific reference intervals and does not allow these to be interchangeable for monitoring of patients (70). If the clinical usefulness of these parameters are to be confirmed in future studies, there will be the need for quality control procedures and appropriate reference materials to calibrate the method (71). These parameters should present a challenge to EQAS providers. The quality control procedures and external programs should evaluate not only analytical processes, but also other steps, such as, for example, the calculation of reference intervals. Results obtained in EQA schemes could improve clinical laboratories by adopting more appropriate reference intervals.

Several studies have been published on the value of the reticulocyte Hb content (CHr or Ret-He) for assessing the availability of iron for erythropoiesis and the appropriateness of the bone marrow response to erythropoietic stimulating agents (ESA). The reticulocyte Hb content estimates the amount of Hb in the reticulocytes, which is a reasonably good reflection of how much iron was available for RBC production in a clinically relevant timeframe, i.e., a few days prior. Measurement of the reticulocyte Hb content provides a direct assessment of the incorporation of iron into erythrocyte Hb, reflecting recent functional availability of iron, and may assist in the assessment of iron status (72, 74).

Studies have shown that CHr is helpful in identifying iron deficient states prior to the development of anemia in young children, thus providing a possible early and cost-effective method of intervening to prevent the cognitive impairment associated with iron deficient states at these early ages (75–77). In adolescent females, the use of an algorithm, including CHr, to screen for iron deficiency anemia may increase the diagnostic accuracy, enabling early detection and treatment of iron deficiency without the need for additional costly iron studies. The algorithm is based on Hb/CHr and the micro/hypo ratio, which is used to identify subjects with thalassemic traits (78, 79). Treatment of anemia with ESAs has been shown to produce a transient iron deficient state, defined as iron-restricted erythropoiesis or functional iron deficiency, due to a discrepancy between the iron needs of the bone marrow and the availability of iron (80, 81). The KDOQI Clinical Practice Guidelines define CHr as an appropriate test to assess the adequacy of iron for erythropoiesis (82, 83). In patients on dialysis for chronic renal failure and treated for anemia with ESAs (recombinant erythropoietin), studies have shown that CHr may be valuable for identifying functional iron deficient states and in optimising IV iron therapy (84–86). CHr has some diagnostic limitations. Independent of iron status, CHr is often reduced in alpha and beta thalassemias and hemoglobinopathies that cause microcytic anemia. In the case of beta thalassemia, the reduction in CHr seems to follow ''pari passu'' the severity of impairment in beta chain synthesis (87). It can also be increased in iron-deficient patients with confounding megaloblastic anemia due to the high mean reticulocyte volume associated with megaloblastosis (13). Therefore, it is important that CHr values be interpreted in the context of the patient's overall erythrocyte physiology, including knowledge of recent blood transfusions, iron therapy, vitamin B12 or folate deficiency, and the results of Hb analysis (88). For individual patients, reticulocyte Hb values can be paired and data can be plotted using the ''Thomas'' plot. This plot describes the relationship between functional iron deficiency (reticulocyte Hb content) and iron supply to erythropoiesis [soluble transferrin receptor (sTfR)/ferritin ratio]. In normoproliferative and hypoproliferative erythropoiesis, this plot allows the differentiation of classic iron deficiency from anemia of chronic disease and the combined state of functional iron deficiency with anemia of chronic disease. The therapeutic implications of the plot are to differentiate patients into those who should be administered iron supplements, ESA, or a combination of ESA and iron. In patients receiving ESA therapy, the plot is an important tool for monitoring erythropoietic activity, functional iron deficiency, and adequate iron stores for new red cell production (89–91).

A recent study by Mäkelä et al. provided data for the natural changes in reticulocyte and iron parameters that occur following birth of preterm infants (92). CHr and IRF were highest at birth and declined thereafter. The reticulocyte count reached a nadir at 1 week and sTfr at 9 weeks. CHr values are also higher at birth in at term newborns (93).

We have performed a study of reticulocytes in premature newborns hospitalized in the Department of Pediatrics at Padova University Hospital. Preterm newborns were divided into three groups based on gestational age: a) <32 weeks (mean 28.5 ± 2.6); b) between 32 and 36 (mean 34 ± 1.6) weeks; c) >36 (mean 39 ± 1.49) weeks. During a 3-month period in 2008, all newborns were investigated using left-over samples collected with venipuncture for routine, clinically-indicated studies. Inclusion criteria were stable respiratory status, absence of prior transfusions, anemia, and/or infections. CBC and reticulocyte studies were performed with the XE 2100 instrument (Sysmex Corporation, Kobe, Japan). Results are shown in Table 5. No significant statistical differences were found among groups of neonates with respect to Hb, reticulocyte counts and IRF. The IRF values were found to be higher than those seen in adults. Thus, prematurity does not seem to appreciably alter reticulocyte and IRF values, suggesting that in the absence of anemia, erythropoiesis is unaffected by prematurity. A recent study by Kasper et al. provides an important assessment of the applicability of the Thomas plot in very low birth weight premature infants that receive iron and/or erythropoietin therapy. The data seem to indicate that the plot provides a useful assessment of iron status as it relates to erythropoiesis, and correctly identifies iron deficient and functional iron deficient states (94).

Table 5 Hematologic values for preterm infants.

Parameter	Gestational week		
	<32 n=11 mean±SD	Between 32 and 36 n=29 mean±SD	More than 36 n=41 mean±SD
Hemoglobin, g/L	152.4±22.07	157.90±25.84	148.04±22.38
Reticulocytes, 10^9/L	165.06±100.75	196.59±96.35	165.50±93.29
Reticulocytes, %	4.02±2.72	4.38±1.79	3.80±2.11
IRF, %	26.89±11.59	26.93±13.74	26.79±11.65

p-Value not statistically significant among groups (one-way analysis of variance; statistical analyses were performed using MedCalc for Windows, version 9.5.0.0, MedCalc Software, Mariakerke, Belgium).

Reticulocyte reporting

The percentage of reticulocytes is still the most commonly reported value. Using the microscopic method, if the red blood cell count is available, the absolute reticulocyte count can be calculated and reported. With the introduction of automated methods, it has become mandatory to report the absolute count which gives more accurate information on bone marrow output than the reticulocyte percentage (95). The peripheral reticulocyte count is, in fact, the result of two factors: (i) the rate of release of reticulocytes into the peripheral blood and (ii) the maturation time of reticulocytes in the peripheral blood. If the working method is the microscopic count, in patients with anemia or during erythropoietic stimulation the percentage of reticulocytes may be uncorrected. Thus, two corrections of the manual reticulocyte count have been proposed to reflect changes associated with anemia and stress erythopoiesis. An apparently normal reticulocyte count can be demonstrated to be low if an allowance is made for anemia. The ''hematocrit correction'' uses Ht rather the red cell count to correct the % reticulocyte value, and is similar in its intent to the absolute reticulocyte count (96). At present, absolute reticulocyte counts generated by automated instruments make it unnecessary to calculate. In the presence of intense erythroid stimulation, a ''stress macroreticulocytosis'' or shift in circulating reticulocyte maturity indicates premature release of reticulocyte from the marrow and more time spent in the circulation prior to maturation. The ''reticulocyte production index'' (RPI) corrects the reticulocyte count both for Ht and maturation time. Early release shortens the amount of time reticulocytes mature in the marrow, and proportionally prolongs their maturation time in the circulation. The result of early release is an increase in the total reticulocyte count. However, this increase reflects the premature shift to the circulation, not an increase in erythroid production. Normal maturation time in the circulation is 1 day. The expected maturation time, presuming a sufficient erythropoietin response to anemia, increases to 1.5 days at Hb values between 100 and 130 g/L, or when the Ht is 0.35±0.5, 2 days at Hb values between 70 and 100 g/L or with the Ht 0.25±0.5, and 2.5 days at Hb values between 30 and 70 g/L or when the Ht is 0.15±0.5 (71). Conversely, reticulocytes persist for a longer time in the peripheral blood when anemia is present. A RPI >3 is associated with hemolytic anemias (i.e., hereditary spherocytosis), recent hemorrhage and response to therapy. A RPI <2 is associated with hypoproliferative disorders (i.e., aplastic anemia) and ineffective erythropoiesis seen in megaloblastic anemias (97). With the development of automated reticulocyte analysis, reticulocyte maturation parameters, such as the IRF provides the same clinical significance as the RPI, making this calculation unnecessary.

What information should be reported to clinicians and how?

Information overload is a concern in the reporting of new hematological parameters, given the large number of parameters already reported in the routine CBC. We believe that laboratories should report the reticulocyte count as the absolute number of reticulocytes, accompanied by a properly determined normal range, with the percentage value being optional. However, the reticulocyte percentage is still crucial for monitoring the bone marrow response when plasma volume is fluctuating, as occurs in blood boosting in athletes or in kidney diseases. Since some reticulocyte cellular parameters, such as IRF and cell Hb content have proven to be clinically useful, we believe that they should be reported if available, with the method-specific reference ranges. MCVr, even though still being studied, could in some instances provide useful information. Obviously, if MCVr is reported in the laboratory report, it needs method-specific reference ranges. Physicians seem to be more interested in the reticulocyte percentage and absolute number, as shown by a study aimed at ascertaining which components of the reticulocyte count reports are perceived as being useful in clinical practice (98). A subset analysis according to specialty indicated that a small number of pediatric and adult hematologist–oncologists and nephrologists also wanted the IRF to be included in the report. In 2006, an internal audit was carried out at Padova University-Hospital to assess how reticulocyte reporting was used by clinicians. One hundred responses to a questionnaire were obtained, equally divided between the Departments of Surgery and Medicine. The audit did not include Pediatrics. In the Department of Medicine, 57% of interviewed physicians indicated that in their opinion, absolute counting was more useful than the percentage of reticulocytes. On the contrary, only 4% of surgeons found the absolute count to be more useful. Only 50% of physicians

and 33% of surgeons found it useful to include the IRF in the report. Fifty-seven percent of physicians and 40% of surgeons judged the CHr to be clinically useful. It may be useful for laboratories to consider providing an interpretation of the reticulocyte analysis when done with flow cytometric techniques in conjunction with the routine CBC. For example, if the absolute reticulocyte count and IRF are simultaneously increased, an interpretative comment could be added to emphasize the increase in erythropoietic activity. The comment could help physicians assess a case of suspected hemolytic anemia, or to monitor the treatment of anemia.

Conclusions

Automated methods for reticulocyte analysis provide acceptable precision and bias, improving the quantitative evaluation of erythropoiesis. However, efforts in standardization should be encouraged in order to avoid reference ranges that are specific to particular methods or instruments. Since a qualitative evaluation is performed with reticulocyte maturation parameters and cellular indices, EQA programs should be provided. Finally, interpretative reporting should be offered to clinicians.

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