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SEED HAEMATOLOGY

The importance of reticulocyte detection

Production of reticulocytes

All blood cells emanate from a stem cell. Under pronounced proliferation they differentiate into cells of the three blood cell lines (erythropoiesis, granulopoiesis and thrombopoiesis). Focusing on the red blood cell (RBC) line, we see that the life span of circulating erythrocytes is approx. 120 days. Nearly 1% of this total is lost daily and is replenished by new cells. Every second, about two millions of RBC are being produced. In the bone marrow the young erythroblasts eject their cell nucleus, becoming reticulocytes which then enter the peripheral bloodstream.

As a rule, the reticulocyte remains in the bone marrow for a further three days and for one day in the peripheral bloodstream. The name 'reticulocyte' originates from the web-like structure ('reticulum' in Latin), which becomes visible after staining with supravital stains, such as brilliant cresyl blue or new methylene blue (precipitation of ribonucleic acid fragments; Fig. 1). By removing the endoplasmic reticulum, the reticulocyte develops into a mature red blood cell within four days.

History

- 1865 First description of reticulocytes by *Erb*, who discovered an intracellular reticulum using picric acid.
- 1881 Using supravital staining, *Ehrlich* demonstrated an intracellular network that was described as *substantia reticulo-filamentosa*.
- 1891 *Smith* identified reticulocytes as immature red blood cells.
- 1932 Classification of maturation stages by *Heilmeyer* (see Table 1).
- 1953 *Seip* quantifies the maturation stages with reference ranges (see Table 1).
- 1960 Counting of reticulocytes with methods based on fluorescence (acridine orange), developed by *Kosenov & Mai*.
- 1983 *Tanke* automates the measurement of reticulocytes by using the fluorescence method and flow cytometry.

Table 1 *Maturation stages according to Heilmeyer and quantification according to Seip*

Maturation stages according to *Heilmeyer*	Morphological description	Quantification according to *Seip* (normal %)
Stage 0	Nucleus	
Stage I	Reticulum consists of dense clots	< 0.1
Stage II	Loosely arranged reticulum	7.0
Stage III	Diffusely arranged reticulum	32.0
Stage IV	Some scattered granulae	61.0

The maturation stages I and IV are often interpreted incorrectly: stage I is sometimes described as an erythroblast and stage IV as a mature RBC as its low content of RNA is not detected. The correct interpretation of stage IV is especially important, as this maturation stage is dominant in the peripheral bloodstream.

In 1986 the National Committee for Clinical Laboratory Standards (NCCLS) classified as a reticulocyte 'any non-nucleated red cell containing two or more particles of blue stained material corresponding to ribosomal RNA' [1]. The International Council for Standardization in Haematology (ICSH) also accepted this definition [2].

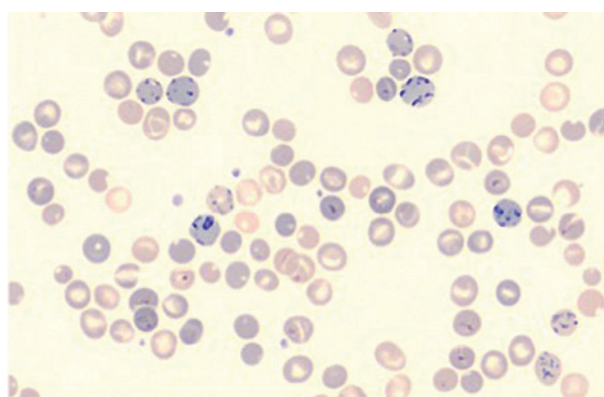

Fig. 1 *Supravital stain of a smear of human blood with different stages of reticulocytes*

The reticulocytes reflect the regeneration of erythropoiesis. In a balanced system, > 90 % of the very mature stages of reticulocytes (stages III and IV) are present in the peripheral bloodstream.

If erythropoiesis is stimulated, the earlier level of maturation shifts into the peripheral blood (similar to a 'left shift' in granulopoiesis).

Reticulocyte count

The normal fraction of reticulocytes in the blood depends on the clinical situation but is usually 0.5 % to 1.5 % in adults [3] and 2 % to 6 % in newborns [4]. The number of reticulocytes is a good indicator of bone marrow activity because it represents recent production and shows the erythropoietic status of the patient and if the production is healthy or not. Therefore, a reliable count of the reticulocytes is needed. A comparison of different reference ranges as reported by different authors can be found in a review article published by Piva *et al.* [5]

Indications for counting reticulocytes

- Basic diagnostic work-up in all types of anaemias
- Therapeutic monitoring during iron, vitamin B12 or folate replacement
- Therapeutic monitoring under erythropoietin treatment
- Monitoring during stem cell transplantation
- Newborns and paediatric patients

Determining reticulocytes from EDTA blood remains reliable for up to 72 hours after blood sampling. The storage temperature of +4 °C or 20 °C, respectively, has no significant effect on the measured result [6].

Manual count

(Materials: supravital stains, such as brilliant cresyl blue or new methylene blue, a prepared microscope slide and a microscope).

1. Whole blood is mixed with equal volumes of a supravital stain.
2. After incubation the sample is smeared on a microscope slide and air-dried.
3. The reticulocytes are counted under the microscope at oil immersion magnification (1,000-fold magnification).
4. 1,000 red blood cells are counted. At 1,000-fold magnification this corresponds to approx. five visual fields, each consisting of approx. 200 red cells.
5. Reticulocyte counts are given per mill [‰] or per cent [%].

Error rates for manual counting are quoted in the literature at 25–50% CV [7–8] and higher, depending on the number of reticulocytes. Counting 1,000 cells is recommended as standard. According to a recommendation by the ICSH (1998) [9] on the counting rate in the reference range of reticulocytes, at least 40,000 cells should be counted to avoid exceeding a statistical error of 5% (Table 2).

Table 2 *Effect of the number of counted RBC on the statistical error in reticulocyte counts. The numbers quoted refer to a CV of 5%.*

Reticulocyte count in blood (%)	Number of cells to be counted to achieve a CV of 5%
1	39,600
2	19,600
5	7,600
10	3,600
20	1,600
50	400

Automated counting

To accurately measure reticulocyte counts, automated counters use a combination of laser excitation, detectors and a fluorescence marker that labels RNA and DNA (such as thiazole orange or polymethines) [10].

To measure the reticulocytes, the sample is incubated with an RNA-binding fluorescence marker and counted by flow cytometry. Automated reticulocyte counters use objective thresholds for the classification of cells. This ensures a high level of reproducibility of the results. In automated counts the measurement signals of up to 30,000 red blood cells are evaluated. This results in both high count rates and a high degree of precision. Compared to manual reticulocyte counting, automated counting results (Fig. 2) are available much faster, actually in less than one minute.

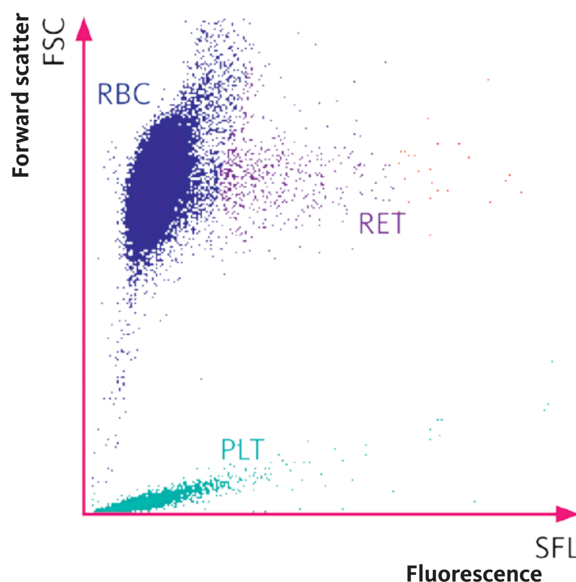

Fig. 2 *Scattergram of the reticulocyte channel (RBC: mature red blood cells; RET: reticulocytes; PLT: fluorescence-optical platelets)*

Reticulocyte count in the clinical diagnosis

The interpretation of the reticulocyte count is problematic in severe anaemias. A moderately increased relative reticulocyte count in severe anaemia does not indicate a sufficiently strong regeneration of erythropoiesis, but merely indicates a shortened life span of the red blood cells. It is preferable to report the absolute reticulocyte concentration as reticulocytes/µL, as this provides a direct measurement of erythropoietic performance.

For example, a value of 20‰ reticulocytes is considered increased. However, in severe anaemia with 2 million red blood cells, 20‰ reticulocytes merely represent 40,000 reticulocytes/µL, a value within the reference range.

The relative number of reticulocytes (‰ and %) gives some information about the life span of the red blood cells, whereas their cell concentration (reticulocytes/ µL) reflects the erythropoietic productivity of the bone marrow.

With a simple formula, relative counts (%) can be converted into cell concentrations (RET/µL):

$$\frac{\text{RET [\%]} \times \text{RBC } [10^6/\mu\text{L}]}{100} = \text{reticulocyte concentration } [10^6/\mu\text{L}]$$

The reference ranges – in percentage and absolute – of reticulocytes according to Cavill *et al.* [6] are the following:

Relative reticulocytes:	m/f 0.43 – 1.36 %
Reticulocyte count:	f 17.0 – 63.8 x 10^9/L m 23.0 – 70.1 x 10^9/L

It is necessary to remember that published reference ranges from other analysers may be used on newer analysers, but only after having validated them. Since there are also differences between haematological reference intervals from different populations, every lab will need to select which of the published reference ranges fits better their own population.

Reticulocyte-associated parameters

Reticulocyte index (RI)

The relative portion (‰ and %) of reticulocytes may increase, if either the reticulocytes are in fact increased or the red blood cells are decreased. Corrective measures can be carried out via the patient's haematocrit by reference to a normal haematocrit of 0.45 [L/L]. This type of correction is recommended with anaemias:

$$\text{RI} = \frac{\text{RET [\%]} \times \text{HCT [L/L]}}{\text{0.45 [L/L] (standard HCT)}}$$

Reticulocyte production index (RPI)

The RPI is an index, which helps to evaluate erythropoiesis efficiency and thus the productivity of the bone marrow. The physiological maturation of reticulocytes is divided into the bone marrow maturation (about 8 – 10 days since the first division of the proerythroblast) and about 1 – 2 days in the peripheral blood stream until they become mature red blood cells.

The idea of the RPI is to assess whether the bone marrow is producing an appropriate response to an anaemic state. After an acute haemorrhage, the reticulocyte production should increase within 2 – 3 days in response to the loss of RBC, and reach its peak in 6 – 10 days [11]. If that does not happen, it could point out a defect of the erythropoietic process in the bone marrow.

In case of a pronounced red blood cell production, the maturation of the reticulocytes shifts into the peripheral blood as the reticulocytes are passed into peripheral blood at an earlier stage (the altered dwell time in peripheral blood is called a 'shift'). This leads to a pronounced increase in circulating reticulocytes, but does not represent proof of erythropoietic performance. The maturation time of reticulocytes in bone marrow is proportional to the haematocrit, i.e. it decreases with the haematocrit, and the maturation time in peripheral blood increases. To give an indication of the efficiency of the bone marrow, the reticulocyte count is corrected by this haematocrit-dependent factor.

Table 3 *Link between haematocrit and maturation time of reticulocytes in peripheral blood*

Haematocrit	Reticulocyte survival in blood = maturation correction
36 – 45 %	1 day
26 – 35 %	1.5 days
16 – 25 %	2 days
15 % and below	2.5 days

The RPI should be between 0.5 % and 2.5 % for a healthy individual [11]. In case of anaemia, an RPI < 2 % indicates inadequate production of reticulocytes, while an RPI > 3 % indicates compensatory production of reticulocytes to replace the lost red blood cells [12].

$$\text{RPI} = \frac{\text{RET [\%]}}{\text{RET maturation time in blood in days}} \times \frac{\text{HCT [L/L] (patient)}}{\text{0.45 (standard HCT)}}$$

Example:
Patient values: HCT= 0.25 L/L, reticulocytes = 20

$$\text{RPI} = \frac{20\ [\%]}{2} \times \frac{0.25}{0.45} = 5.5$$

Immature Reticulocyte Fraction (IRF)

The IRF value is an early marker for evaluating the regeneration of erythropoiesis. Whereas the IRF percentage increases after only a few hours, the reticulocyte count increases after 2 – 3 days. If the IRF value does not increase during the treatment of deficiency anaemias with erythropoietin or vitamins, this indicates a lack of response to therapy. In addition, it helps to classify hypo-, normo- and hyper-regenerative anaemias.

Together, the IRF value and the reticulocyte count have proven themselves as monitoring parameters for bone marrow and stem cell transplants. With successful transplants, in 80 % of the cases the IRF value reaches its 5 % mark earlier than the granulocytes their classic threshold of 0.5 x 10^9 granulocytes/L [13].

An example of this utility can be seen in Fig. 3, where different pathologies are represented with different levels of immature and mature reticulocytes. As an example, in the case of aplasia, both immature (IRF) and total reticulocytes are low. This type of anaemia affects the precursors of red blood cells but not the ones of the white blood cell line. The bone marrow ceases to produce red blood cells, and that is why neither immature nor mature reticulocytes can be observed.

The reference range [14] of IRF is 1.6 – 10.5 %, for both men and women.

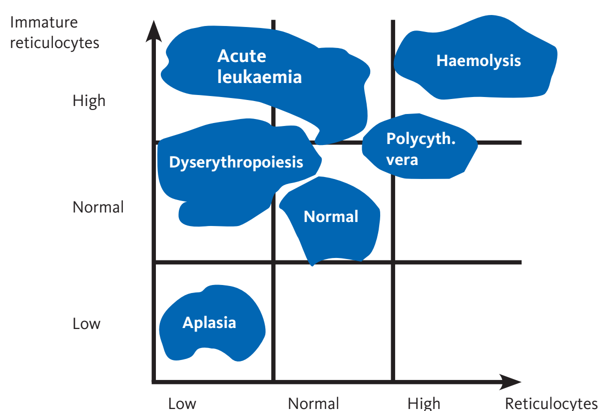

Fig. 3 *The importance of reticulocytes for clinical diagnosis*

Reticulocyte maturation

In addition to conventional reticulocyte measurement, the fluorescence flow cytometry method allows the classification of reticulocytes into three maturation stages. These maturation stages are defined by the RNA content of the reticulocyte, measured on the analyser as fluorescence intensity. The higher the RNA content, the less mature the reticulocytes are.

Reticulocytes are fractioned according to their fluorescence intensity into the following three categories representing different maturity stages (Table 4):

- LFR (low-fluorescence reticulocytes) – 'mature' reticulocytes
- MFR (medium-fluorescence reticulocytes) –'semi-mature' reticulocytes
- HFR (high-fluorescence reticulocytes) – 'immature' reticulocytes

IRF is the sum of MFR plus HFR, i.e. the immature reticulocytes, and is also referred to as the 'reticulocyte maturation index'.

IRF = MFR + HFR

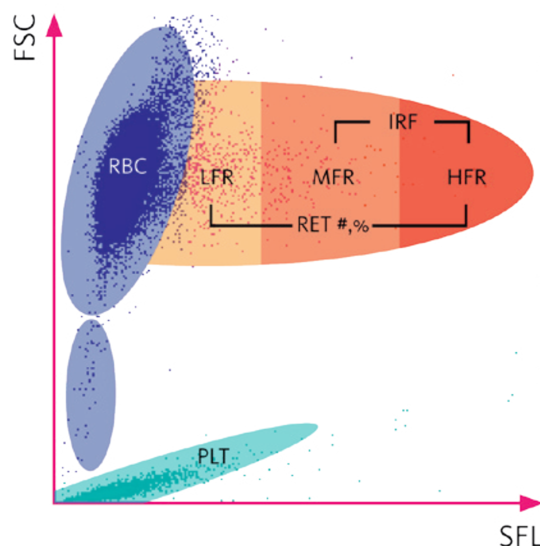

Fig. 4 *Scattergram of the reticulocyte channel*

Table 4 *Maturation stages of reticulocytes*

LFR	MFR	HFR
Low-Fluorescence Reticulocytes	Medium-Fluorescence Reticulocytes	High-Fluorescence Reticulocytes
Little content of RNA	Medium content of RNA	High content of RNA
Mature reticulocytes	Semi-mature reticulocytes	Immature reticulocytes
Reference range: 86.5 – 98.5 %	Reference range: 1.5 – 11.5 %	Reference range: 0 – 1.4 %

Reticulocyte Haemoglobin equivalent (RET-H_e)
In addition to the quantitative determination of the blood count parameters (RBC, HGB, RET#/%, IRF, MCV), RET-H_e offers a qualitative dimension. Red blood cells have a 120-day lifetime. Therefore, using them for detecting iron deficiencies and changes in the iron status of erythropoiesis is only possible at a relatively late point in time. In contrast to that, RET-H_e represents the HGB content of young RBC, the reticulocytes, and thus offers real-time information on iron supply to erythropoiesis.

Measuring the haemoglobin content of the reticulocytes means you can look at the current iron supply to erythropoiesis and judge the 'quality' of the newly produced cells. This lets you detect changes in iron status far earlier than through the haemoglobin content of mature red blood cells.

The determination of RET-H_e can be performed on the haematology analyser together with the routine parameters obtained during the whole blood test. A major advantage over the parameters ferritin or transferrin is that RET-H_e is not affected by the acute phase reaction. These conventional biochemical markers are drastically disturbed e.g. during inflammation, pregnancy, or in the presence of many other severe diseases, so that a clinical interpretation of the results would then be difficult or impossible. Measuring the haemoglobin content of the reticulocytes as a direct assessment of the iron actually used for the biosynthesis of haemoglobin can indicate even in these cases whether there is enough iron available for erythropoiesis.

The clinical usefulness of the RET-H_e parameter has been proven and it is now an established parameter in advanced haematological analysis.

Indication

- Classification of normochromic and hypochromic anaemias
- Monitoring the therapy of chronic infections or tumours
- Monitoring erythropoietin therapy and iron substitution
- Determining the trend of the current iron status
- Early marker for diseases. Together with RET#, it lets clinicians draw conclusions on both the quality and quantity of the young RBC fraction.

The reference ranges of RET-H_e [14] are (for both men and women) 1,996 – 2,407 fmol or 32.1 – 38.8 pg.

RET-H_e alone provides information on the current bioavailability of iron – a low value means there is a lack of iron or iron is not bioavailable for erythropoiesis. It is often used together with ferritin – a high or normal ferritin value together with a low RET-H_e value can suggest functional iron deficiency while low ferritin values together with a low RET-H_e suggest a classic iron deficiency. Since ferritin is falsely increased during the acute phase of diseases, presence of inflammation should be checked, e.g. by CRP.

RET-H_e is used for monitoring erythropoietin (EPO) and/or IV iron therapy. If the value increases it indicates the therapy is having a positive effect.

Thomas-Plot index

In 2006, Thomas *et al.* described a diagnostic plot model in order to differentiate iron-deficient states and predict those patients who will respond to erythropoietin therapy [15]. The balance of the iron in our body is regulated by the rate of erythropoiesis and the size of the iron stores. The relationship between erythropoietic haemoglobin incorporation (RET-H_e) and iron stores (ferritin) can be described in a diagnostic plot. This plot, called 'Thomas plot', allows the differentiation of classical iron deficiency from anaemia of chronic disease. The plot indicates the correlation between the ratio sTfR/log ferritin (ferritin index), a marker of iron supply for erythropoiesis, and the RET-H_e (Fig. 5) [16]. The Thomas plot can also be used to monitor the patients that are under treatment and see how they move from one quadrant to another.

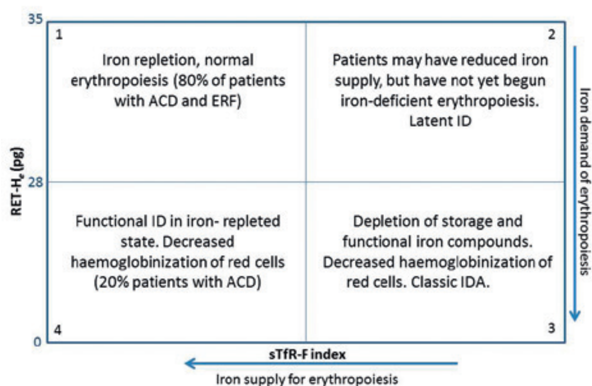

Fig. 5 *Thomas plot to identify different phases of advancing iron deficiency (ACD: anaemia of chronic disorders, ERF: end-stage renal failure, IDA: Iron deficiency anaemia, ID: classic iron deficiency)*

Conclusions

A reliable reticulocyte count including some more parameters associated with reticulocytes (IRF, RPI, etc.) are important to see if the bone marrow is working properly to develop new red blood cells, but the quantity aspect is not the only one that is important. We have seen that the reticulocyte haemoglobinisation, RET-H_e, is also of high importance in order to define the quality of the newly produced reticulocytes.

Reliable reticulocyte information, regarding quality and quantity, helps in the differential diagnosis of anaemias as well as in the monitoring of patients.

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