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Critically Appraised Topic

Verification and diagnostic value of the Advanced RBC Application on DI-60 (Sysmex)

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CLINICAL BOTTOM LINE

Background: Analysis of red blood cell (RBC) morphology is regarded as an essential part of the evaluation of hematological disorders. Manual microscopy is a sensitive, but labor intensive and subjective procedure. In the prospect of globalization and the ongoing merging of hospitals and laboratories to large, multi-center hospitals and laboratories, standardization becomes increasingly important. The use of the Advanced RBC Application on the DI-60 (Sysmex) could improve the level of reproducibility and thus lessen the variability in reporting results while reducing the hands-on time needed by our medical laboratory technologists (MLTs).

Methods: The relevant literature concerning the origin and clinical utility of abnormalities of erythrocyte morphology was reviewed and summarized. An online survey, investigating the clinical experience regarding RBC morphology, was sent to the internal medicine residents, the internal medicine specialists, gynecologists and pediatricians of the Jessa hospital in Hasselt. The performance characteristics of the Advanced RBC Application on DI-60 were verified by selecting 104 abnormal peripheral blood films and comparing the count obtained with the Advanced RBC Application with the manual microscopic count (both expressed in percentage of RBCs). Imprecision was assessed by measuring analytical within-run and between-run variability. We used Pearson's correlation coefficient and Passing-Bablok regression analysis to calculate new cut-off values for the Advanced RBC Application, starting from the cut-off values provided by the ICSH in 2015. If this method was not suitable, we used ROC curve analysis to estimate the 'ideal' cut-off for positivity.

Results: The reviewed literature demonstrates that abnormalities of red blood cell morphology can be helpful (and sometimes even essential) in the diagnostic work-up of a patient. However, our survey shows that only a minority of hospital physicians is aware of this clinical utility. We were able to calculate cut-off values for 1+, 2+ and 3+ grading of acanthocytes, echinocytes, elliptocytes, ovalocytes, polychromasia and target cells by Passing-Bablok regression analysis. For the other categories we needed to rely on ROC curve analysis to determine a cut-off value for positivity.

Conclusion: With the Advanced RBC Application we can obtain a sensitive and reproducible measurement for most RBC morphological abnormalities, provided that cut-off values are adjusted. However, specificity is low for the majority of categories and not all clinically relevant categories can be detected and classified by the application software. As a consequence, evaluation of the digital images by our MLTs remains necessary for most categories. By changing our grading levels to those recommended by the ICSH, we aim to report only the truly important abnormalities and convince our clinicians that the RBC morphology should not be overlooked. We still need to implement the Advanced RBC Application and the new grading levels, so at this point we do not know what the effect on workload, reproducibility and perceived clinical utility will be.

CLINICAL/DIAGNOSTIC SCENARIO

The examination of a peripheral blood film coupled with the complete blood count information is regarded as an essential part of the diagnostic work-up. Despite continuous improvements of laboratory technologies, advancements in hematology instruments and the publication of guidelines, the methods of reporting abnormal red blood cell morphology still vary among laboratories and hospitals throughout the world. This variability is due to the heterogeneity of grading systems and levels, differences in microscopic technique and inconsistencies in identification criteria. (1,3)

Different reference textbooks and guidelines use different grading systems (quantitative, semi-quantitative, descriptive or present/absent) and grading levels (cut-off percentage per level) to report abnormal morphologic findings. For example, when we look at the default values of the DI-60 (15), spherocytes are graded as 1+ (1-2%), 2+ (3-6%) and 3+ (>6%), while the UZ Leuven guidelines (17), based on 'Difboekje' NVKC, revised to own population) rate the same abnormality as rare (0,1-0,3%), small excess (0,4-0,9%), moderate excess (1-2%) and large excess (>2%). Yet another guideline reports the same red cell changes as slight/few (1-5%), moderate (6-20%) and marked (>20%).(3) Although there is no evidence that one grading system is superior to the others, the lack of uniformity can lead to inconsistent and confusing results as reflected in these examples.(1,3) Besides inconsistency of grading, the variability in reporting of red blood cell morphology may also depend on differences in the counting method (i.e. general overview or accurate enumeration), the area of the smear assessed, the number of RBCs counted, the use of counting aids such as an eyepiece grid or the Miller disk and the morphological criteria used for the classification of abnormal red blood cells.(2)

Recognizing this problem, the International Council for Standardization in Hematology (ICSH) published the 'ICSH recommendations for the standardization of nomenclature and grading of peripheral blood cell morphological features' in 2015. The guidelines were decided on by an international groups of morphology experts (from New Zealand, UK, USA, Italy and Australia) using consensus opinion. The recommendations emphasize that a red cell morphology report should provide the physician with comprehensible information that is useful in the differential diagnosis. In small numbers, most morphologic variations are ambiguous and of little diagnostic significance. They become significant only when they appear in considerable numbers. Therefore, instead of providing bits of data that are not clinically significant, the ICSH developed a two-tiered grading system for 2+ (moderate) and 3+ (many). Only for schistocytes, which can be clinically significant even in small numbers, the designation for 1+ (few/rare) should be used. See Table 1 for specific grading levels per morphological category.(1) Unfortunately, the ICSH provides only a brief description of red blood cell morphology, associated clinical conditions and microscopic counting technique, providing room for future variability and inconsistencies in reporting red blood cell morphology.

In our hospital (Jessa hospital Hasselt) we have been using the UZ Leuven guidelines for grading and classification of abnormalities in red blood cell morphology since 2009 (Table 1).(17) The introduction of the digital microscope in our laboratory in 2011 changed our way of evaluating peripheral blood films significantly. Instead of performing the classic manual microscopic count, our medical laboratory technologists (MLTs) screen the digital overview image (approximately 2500 erythrocytes) for abnormalities in red cell morphology. In March of 2018 the new DI-60 replaced the DM-96 as the digital microscope in our laboratory. With the DI-60 the 'Advanced RBC Application' was introduced in our laboratory. The Advanced RBC Application is an optional module for analysis of the red blood cell morphology. By using an artificial neural network that is trained by highly qualified field experts, a preliminary characterization and grouping of approximately 2500 red blood cells into 21 morphological categories is automatically performed. The categories included are: polychromatic cells, hypochromatic cells, anisocytosis, microcytes, macrocytes, poikilocytosis, target cells, schistocytes, helmet cells, sickle cells, spherocytes, elliptocytes, ovalocytes, teardrop cells, stomatocytes, acanthocytes, echinocytes, Howell-Jolly bodies, Pappenheimer bodies, basophilic stippling and parasites. The results are presented as a percentage value and a grading, which is based on a conversion table defined by the user in the settings file. The operator can verify and modify the classification of each red blood cell manually if necessary ('post-reclassification'), see Figures 1-2. The performance of digital microscope systems for the assessment of white blood cell morphology has been thoroughly evaluated and is widely accepted. This is in contrast to the digital morphological analysis of red blood cells, which has not been as extensively verified.(5-9)

Even with use of the ICSH recommendations, the classification of RBC morphologic abnormalities by MLTs remains subjective, influenced by skill and experience, labor intensive and subject to significant statistical variance.(5) As hospitals and laboratories are more and more joining with others, forming large multi-center hospitals and laboratories, and people are travelling (and developing disease) all over the world, this lack of standardization can become a major issue in the future. (3,1) The use of the Advanced RBC Application on the DI-60 could improve the level of reproducibility and thus lessen the variability in reporting results while reducing the hands-on time needed by our MLTs.

In the first part of this appraisal, we will summarize the relevant literature concerning the origin and clinical utility of abnormalities of erythrocyte morphology. In the second part, we will discuss the results of a survey,

investigating the clinical experience regarding RBC morphology in our hospital. In the third and last part we will present a verification study of the Advanced RBC Application on the DI-60.

QUESTION(S)

1) What is the diagnostic value of red blood cell morphology abnormalities?
2) How does the clinician use and interpret RBC morphology? Do we need to change our way of reporting RBC morphology in order to provide more comprehensible and clinically relevant information?
3) How can we make use of the Advanced RBC Application on DI-60 in our laboratory?

RELEVANT EVIDENCE/REFERENCES

1) Guidelines and Recommendations (most recent topics on top)

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12. Ford J., Milner R., et al. Red Blood Cell Morphology Reporting. Journal Of Pediatric Hematology/Oncology. 2011; 33(1), 10-14.

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16. Rodak B.F., Carr J.H.. Clinical hematology atlas, 5th edition. St. Louis, Missouri: Elsevier, 2017.

5) Posters, "grey literature", presentations

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1. Background and diagnostic value of red blood cell morphology

On a stained peripheral blood film, normal erythrocytes are round or slightly oval in outline and show only minor variations in shape and moderate variations in size. The average diameter is about 7.5 µm. In the area of a film where cells form a monolayer, a paler central area occupies approximately the middle third of the cell. (14,16,1). To review RBC morphology there are five important aspects: size, color, shape, inclusions and arrangement.(4). The categories between brackets will not be included in the verification of the Advanced RBC Application because of preferable use of analyzer parameters or because the category is not included in the application software. See Table 2 for an overview of common clinical conditions associated with abnormalities of red blood cell morphology and images per morphological category. For more extensive, detailed lists of associated pathologies we refer to Bain B.J. Blood Cells: A Practical Guide, 5th edition.

1.1 Size and colour

A. *(Microcytosis)*
Microcytes are small red blood cells with a diameter of less than 7-7.2 µm. If all or most of the erythrocytes are small there is a reduction in the MCV (< 80 fL), but a small population of microcytes can be present without the MCV falling below the reference range. It is important that red cell size is interpreted in the light of the age of the subject. In normal infants and children red blood cells are microcytic, while in healthy neonates red cell size is increased in comparison with that of infants, children and adults. (1,14) The ICSH recommends to use the MCV to gauge red cell size rather than grading by visual microscopic examination. Only in the case of normal MCV with abnormal RDW or red cell histogram visual microscopic examination and grading is recommended.

B. *(Macrocytosis)*
Macrocytes are enlarged red cells with a diameter greater than 8,5 µm. If this reflects a generalized change the MCV will be raised (> 100 fL). Fetal and neonatal erythrocytes are larger than those of adults. A slight degree of macrocytosis is also seen as a physiological feature of pregnancy and in older adults. Macrocytes may be round or oval in outline, the diagnostic significance being somewhat different.(14) The ICSH recommends to use the MCV to gauge red cell size rather than grading by visual microscopic examination. Only in the case of normal MCV with abnormal RDW or red cell histogram visual microscopic examination and grading is recommended.

C. *(Anisocytosis)*
Anisocytosis is a non-specific feature of almost any blood disorder. The term implies more variation in size than is normally present and is reflected by an increase in the red cell distribution width (RDW). The ICSH recommends to report the RDW as a measure of anisocytosis. (13,14,1)

D. *(Dimorphism)*
Dimorphism indicates the presence of two distinct populations of red blood cells which may be clearly seen on an analyzer red cell histogram with a corresponding increase in RDW. The ICSH recommends to report and describe the two populations. (1,14)

E. *(Hypochromia)*
Hypochromia is a reduction in RBC staining with an increase in central pallor to greater than one-third of the RBC diameter. The MCH will be decreased as will the MCHC in severe hypochromia. Red cells of healthy children are often hypochromic if assessed in relation to the red cells of adults, so it is important to take into account the age of the patient. The ICSH recommends to use the MCH to gauge hypochromia rather than grading by visual microscopic examination. Any of the conditions leading to microcytosis may also cause hypochromia, so they will often be associated. (1,16,14)

F. *(Hyperchromia)*
The term hyperchromia is rarely used in describing blood films. It can be applied when cells are more intensely stained than normal, but it is more useful to indicate why a cell is hyperchromic (e.g. presence of spherocytes or irregularly contracted cells).(14)

G. *Polychromasia*
Polychromasia describes red cells that are pinkish-blue as a consequence of uptake both of eosin (by hemoglobin) and of basic dyes (by residual RNA). They represent the most immature portion of reticulocytes, seen when there is either an intense erythropoietic drive or when there is extramedullary erythropoiesis. They are readily recognized in May–Grünwald–Giemsa (MGG)-stained films by their greater diameter, their lack of central pallor and their polychromatic qualities. On average, in patient samples, the reticulocyte count is about double the visual estimate of polychromatic cells. The ICSH recommendation is to grade polychromasia and perform a reticulocyte count if necessary. It should be noted that in certain circumstances the absence of polychromasia is

significant: in a patient with severe anemia it indicates that the bone marrow response is inadequate (e.g. in aplastic anemia and pure red cell aplasia).(16,13,14,1)

1.2 Shape

The normal shape and flexibility of a red cell are dependent on the integrity of the cytoskeleton to which the lipid membrane is bound. An abnormal shape can be caused by a primary defect of the cytoskeleton or membrane or be secondary to red cell fragmentation or to polymerization, crystallization or precipitation of hemoglobin.

A. *Acanthocytosis*

Acanthocytes are spheroidal, hyperchromic red blood cells bearing between 2 and 20 spicules that are of unequal length and distributed unevenly over the red cell surface. Some spicules have club-shaped rather than pointed ends. Acanthocyte formation probably results from a preferential expansion of the outer leaflet of the lipid bilayer that comprises the red cell membrane. They are more commonly observed in patients with hyposplenism, liver disease and a variety of dyslipidemias. Acanthocytosis as an inherited phenomenon is associated with a number of different syndromes and its presence may help in their diagnosis. Several rare degenerative neurological diseases associated with acanthocytosis have been collectively designated neuroacanthocytosis.(17,3,4,13,1,14)

B. *Echinocytosis*

Echinocytes are red cells that have lost their disc shape and are covered with 10-30 short blunt projections or spicules of fairly regular form. Echinocytosis, often referred to as 'crenation', may be seen in many blood films even in those from healthy subjects. The number one reason is excess EDTA or prolonged storage of EDTA-anticoagulated blood. The formation of echinocytes in vivo seems to be related to the exposure to fatty acids in plasma, ATP depletion and lysolecithin formation. Echinocytosis, other than as a storage artefact, is probably most common in critically ill patients with multiorgan failure including both hepatic and renal failure. It has been described in severely burned patients, heparinized patients on cardiopulmonary bypass, in patients with hypophosphatemia, patients with liver disease (however, acanthocytosis is more common) and as a physiological feature in pre-term neonates.(1,17,13,14)

C. *Elliptocytosis and ovalocytosis*

Elliptocytes are cells with an elliptical shape (the long axis is more than twice the short axis), while ovalocytes have an oval shape (the long axis is less than twice the short axis).(1) When elliptocytes or ovalocytes are numerous and are the dominant abnormality it is likely that the patient has an inherited abnormality affecting the red cell cytoskeleton, such as hereditary elliptocytosis. Smaller numbers of elliptocytes or ovalocytes are typical of iron deficiency, where the number of elliptocytes even seems to correlate with the severity of iron deficiency anaemia.(13) Elliptocytes and ovalocytes may also be seen in some patients with thalassemia, megaloblastic anemia, primary myelofibrosis, myelodysplastic syndromes (MDS) and occasionally in pyruvate kinase deficiency. In these conditions it is likely that elliptocytes reflect dyserythropoiesis. Macrocytic ovalocytes or oval macrocytes are characteristic of megaloblastic anemia and South-East Asian ovalocytosis.(1,17,
14)

D. *(Poikilocytosis)*

A poikilocyte is a red cell of abnormal shape. Poikilocytosis is a common, often non-specific abnormality in many blood disorders. However, the presence of poikilocytes of certain specific shapes, e.g. spherocytes or elliptocytes, may have a particular significance. Therefore the ICSH recommends to report the abnormal specific cell shape rather than use poikilocytosis.(1,17,14)

E. *Schistocytosis*

Schistocytes are fragments of red blood cells. In 2011 the ICSH published precise morphological criteria for the recognition and enumeration of schistocytes. According to the ICSH, schistocytes are always smaller than intact RBCs and should be defined as follows:

1. Small fragments of varying shape, sometimes with sharp angles or spines (triangles), with straight borders or sometimes with a round outline on one side (microcrescents), often distorted, usually staining darkly, occasionally pale as a result of loss of hemoglobin at the time of fragmentation.
2. Helmet cells, which are damaged erythrocytes with one single, rarely a double, amputated zone highlighted by a straight border, with sharp angulated edges.
3. Damaged cells larger than small fragments, which have a pair of spicules separated by a semicircular concave segment of membrane, sometimes even two or three pairs: they are usually named keratocytes.
4. Small-sized hyperdense RBCs with a round shape and increased staining: they are named microspherocytes. Microspherocytes are a secondary manifestation of fragmentation and

should be included within the schistocyte count only in the presence of the schistocyte shapes mentioned in points 1-3. They should not be confused with spherocytes of hereditary spherocytosis or immune hemolysis, which have decreased diameter but are not so small (although partial overlapping in both morphology and terminology does exist)

According to the ICSH Schistocyte Working Group irregularly contracted cells, blister cells and bite cells (that are features of oxidant damage) should not be included within the schistocyte count.

Schistocytes are formed in the following situations:(13,2)

1. In certain genetically determined disorders (e.g. thalassemias, glucose-6-phosphate dehydrogenase deficiency, …)
2. In acquired disorders of red cell formation when erythropoiesis is megaloblastic or dyserythropoietic (e.g. megaloblastic anemia, primary myelofibrosis)
3. As the result of direct thermal injury (as in severe burns) or mechanical trauma (as in march hemoglobinuria)
4. As the consequence of extrinsic mechanical damage to the membrane caused by filaments of fibrin in the microvessels, increased turbulence, shear stress and RBC adhesion to abnormal endothelium (e.g. in the thrombotic micro-angiopathies (TMA), malfunctioning cardiac valves and cardiac assist devices, HELLP syndrome, preeclampsia, DIC, metastatic malignancy, malignant hypertension, Kasabach Merritt syndrome, …)

In situations one to three the count of schistocytes rarely has a specific clinical diagnostic value. In situation four however (especially when there is a clinical suspicion of TMA), an accurate schistocyte count is of the utmost importance as early diagnosis and treatment can be life saving. The ICSH recommends to differentiate by taking into account the overall RBC morphology. In case one to three the schistocytes show highly variable shapes and are associated with marked anisopoikilocytosis and a wide range of additional RBC size and morphological changes, while in TMA and related disorders schistocytes are seen as the main morphological abnormality, sometimes in association with moderate signs of stimulated erythropoiesis, such as polychromasia, basophilic stippling, and circulating nucleated RBCs. The ICSH group recommends providing a qualitative report for those samples where schistocytes are observed in a context of multiple heterogeneous RBC shape aberrations. The quantitative assessment (expressed as a percentage, after counting at least 1000 RBCs) should be reported only when schistocytes are the dominant RBC abnormality on the smear.

Schistocyte normal ranges in adults are poorly defined and variably reported in different laboratories. The ICSH Schistocyte Working Group agreed that a schistocyte percentage above 1% in a peripheral blood smear in adults is a robust cytomorphological indication in favor of a diagnosis of TMA, when additional features suggesting an alternative diagnosis are absent. It has to be noted that schistocytes are frequent soon after birth, with values up to 1.4–1.9% in normal newborns and up to 4.9–5.5% in preterm newborns.(11,2,1,14)

F. *Sickle cells*

Sickle cells are red cells that become crescent or sickle-shaped with pointed ends as a result of polymerization of HbS. A sickle cell is a very specific type of cell that is confined to sickle cell anemia or any of the severe sickling syndromes (including $S\beta^0$, SD and SO-Arab). Sickle cells are almost always present in films of adults with homozygosity for hemoglobin S. However, sickle cells are usually absent in neonates and are rare in adult patients with a high hemoglobin F percentage.(4,1,13,14)

G. *Spherocytosis*

Spherocytes are cells that are more spheroidal (less disclike) than normal red cells but maintain a regular outline. They are cells that have lost membrane without equivalent loss of cytosol. As the diameter of a sphere is less than that of a disc-shaped object of the same volume, they appear smaller than normal erythrocytes, but can have either a normal or decreased MCV. In a stained blood film, spherocytes lack the normal central pallor. Some patients with hereditary spherocytosis (HS) will demonstrate occasional 'mushroom cell' or 'pincer cell' variants. Spherocytes most frequently result from genetic defects of the red cell membrane (as in HS), from the interaction between immunoglobulin- or complement-coated red cells and phagocytic cells (as in delayed transfusion reactions, ABO/Rh hemolytic disease of the newborn and autoimmune hemolytic anemia) and from the action of bacterial toxins (e.g. Clostridium perfringens lecithinase).(13) It should be noted that spherocytosis may also be seen in neonates with gram-negative sepsis, in patients with thermal burns, in other hemolytic anemias including G6PD deficiency and after prolonged storage of EDTA-anticoagulated blood.(4,14,17)

H. *Stomatocytosis*
Stomatocytes are cup-shaped red blood cells that appear on a stained blood film with a slit-like area of central pallor. Stomatocytosis results from a variety of membrane abnormalities but probably essentially from expansion of the inner leaflet of the lipid bilayer that comprises the red cell membrane. Stomatocytes have been associated with a great variety of clinical conditions but an etiological connection has not always been established. The commonest cause of stomatocytosis is alcohol excess and alcoholic liver disease, in these cases there is often associated macrocytosis. This combination of stomatocytosis with macrocytosis is also occasionally seen in MDS. Certain inherited erythrocyte membrane abnormalities are characterized by stomatocytes, either alone in hereditary stomatocytosis and hereditary xerocytosis or in association with other abnormalities in South-East Asian ovalocytosis. (13,1,14)

I. *Target cells*
Target cells have an area of increased staining, which appears in the middle of the area of central pallor. Target cells result from cells having a surface that is disproportionately large compared with their volume. They may also be thinner than normal cells. Target cells can be formed because of an excess of red cell membrane, as when there is excess membrane lipid. This is the mechanism of formation in obstructive jaundice, severe parenchymal liver disease and hereditary deficiency of lecithin-cholesterol acyl transferase (LCAT). An alternative mechanism of target cell formation is a reduction of cytoplasmic content without a proportionate reduction in the quantity of membrane. This is the mechanism of target cell formation in a group of conditions such as iron deficiency, thalassemias and certain hemoglobinopathies.(13,14,1,17)

J. *Teardrop cells*
Teardrop cells are erythrocytes that are pear or teardrop in shape. They are particularly characteristic of megaloblastic anemia, thalassemia major and myelofibrosis (either primary myelofibrosis or myelofibrosis secondary to bone marrow infiltration). In both thalassemia major and primary myelofibrosis, the proportion of teardrop cells decreases following splenectomy, suggesting either that they are the product of extramedullary hemopoiesis or that they are formed as the result of the action of splenic macrophages on abnormal erythrocytes.(14,1)

K. *(Bite cells, blister cells and irregularly contracted cells)*
These cells are formed when there is oxidant damage to erythrocytes or damage to red cell membranes by precipitation of unstable hemoglobin or free α or β chains. Bite cells have an irregular gap in their outline, probably caused by removal of Heinz bodies by the spleen. In blister cells (or hemighosts), the hemoglobin appears to have retracted to form a dense mass occupying half the cell while the rest of the cell appears empty. If hemolysis is very acute, a few complete 'ghost cells' may appear. Irregularly contracted cells lack central pallor and appear smaller and denser than normal erythrocytes without being as regular in shape as spherocytes.(14,16)

1.3 Inclusions

A. *Basophilic stippling*
Basophilic stippling describes the presence of fine, medium, or coarse basophilic inclusions due to abnormally aggregated ribosomes, uniformly dispersed through the erythrocyte cytoplasm. In contrast to Pappenheimer bodies (see below), they do not give a positive Perls' reaction for iron and are distributed regularly throughout the cell. Very occasional erythrocytes with basophilic stippling can be seen in normal subjects. Increased numbers are indicative of disturbed erythropoiesis. They are seen in the presence of thalassemia, megaloblastic anemia, unstable hemoglobins, hemolytic anemia, sideroblastic anemia, erythroleukemia, primary myelofibrosis, liver disease and poisoning by heavy metals. Basophilic stippling is a prominent feature of hereditary deficiency of pyrimidine 5'-nucleotidase, an enzyme that is required for RNA degradation.(13,14,1,17)

B. *Howell-Jolly bodies*
Howell-Jolly bodies are usually single, medium-sized (1 μm), dense, perfectly round cytoplasmic inclusions that have the same staining characteristics as a nucleus. They are fragments of nuclear material (DNA). They appear in the blood following splenectomy and are also present in other hyposplenic states, which makes searching for Howell-Jolly bodies a reliable technique for screening for significant hyposplenism. In megaloblastic anemias the rate of formation of Howell–Jolly bodies is increased, so they may be numerous in a patient with concomitant splenic atrophy. It should be noted that they can be a normal finding in neonates (in whom the spleen is functionally immature).(17,13,1,14)

C. *Pappenheimer bodies*
Pappenheimer bodies are small basophilic erythrocyte inclusions of variable size, shape and

distribution. They are clustered towards the periphery of the cell, usually in a limited area of the cytoplasm. They are composed of ferritin aggregates or iron-laden mitochondria and phagosomes, therefore they stain positively for iron (Perls' Prussian blue reaction). They are smaller than Howell–Jolly bodies. Usually only a small number of Pappenheimer bodies are present in a cell. Their presence is related to sideroblastic erythropoiesis, lead poisoning and hyposplenism. (13,14,1,17)

D. *Micro-organisms*
Micro-organisms may be seen free between or within RBC in patients with bacterial, fungal, protozoan or parasitic infections. The only micro-organisms that are observed fairly frequently are malaria parasites, but the fortuitous observation of other micro-organisms in a blood film can also be diagnostically useful.(14, 1)

E. *(Intracellular crystals)*
Crystalline aggregates of hemoglobin may be seen in hemoglobin C and sickle cell/hemoglobin C disease. These crystals stain densely, vary in size and have straight edges with pointed ends. They are usually contained in a cell that appears to be otherwise empty of hemoglobin. Slender purple-violet crystals, often radially arranged, have been observed in red cells in congenital erythropoietic porphyria. They are likely to represent crystallized porphyrins.(1,14)

1.4 Arrangement

A. *(Agglutination)*
Agglutination is the irregular clumping of red blood cells into grape-like clusters. A falsely increased MCV and falsely reduced RBC count will be obtained from the analyzer leading to an erroneous elevation in the MCH and MCHC. Reticulocytes may form agglutinates when their numbers are increased, this is a normal phenomenon. Mature erythrocytes agglutinate when they are antibody-coated. Small agglutinates may be seen in warm auto-immune hemolytic anemia. Agglutinates are more common in paroxysmal cold hemoglobinuria and in chronic cold hemagglutinin disease there may be massive agglutination. (1,14,17)

B. *(Rouleaux formation)*
Rouleaux are stacks of erythrocytes resembling a pile of coins. They occur to some extent in all films (thick part of blood smear), but increased rouleaux formation is significant. Rouleaux formation is increased when plasma protein concentrations are high. The most common causes are pregnancy (in which fibrinogen concentration is increased), inflammatory conditions (in which polyclonal immunoglobulins, α2-macroglobulin and fibrinogen are increased) and plasma cell neoplasms such as multiple myeloma (in which increased immunoglobulin concentration is caused by the presence of a monoclonal paraprotein). Rouleaux formation may be artifactually increased if a drop of blood is left standing for too long on a microscope slide before the blood film is spread.(13,14,1,17)

2. Survey: clinical utility regarding RBC morphology

An online survey was sent to the internal medicine residents, the internal medicine specialists (included subspecialties were hematology, nephrology, pulmonology, rheumatology and cardiology), gynecologists and pediatricians of the Jessa hospital in Hasselt. With this survey we wanted to evaluate the perceived clinical utility of the reporting of red blood cell morphology. The physicians were able to reply anonymously, but they were asked to list their specialty. The survey respondents consisted of 8 internal medicine residents and 12 specialists. The specialist group included 2 hematologists, 4 nephrologists, 2 cardiologists, 1 pediatrician, 1 gynecologist, 1 pulmonologist and 1 infectious disease specialist.

75% of respondents (15/20) reported they 'regularly' or 'always' check the MCV, MCH, MCHC or RDW on their patients' lab results, while only 20% (4/20) reported they 'regularly' or 'always' read the RBC morphologic changes if provided on the report. We asked the physicians to define the clinical utility of each RBC morphology term, the results are shown in Table 3. The presence of agglutination, schistocytes, Howell-Jolly bodies, hypochromasia, macrocytosis, microcytosis, poikilocytosis, rouleaux, spherocytes and target cells was considered clinically significant by ≥ 50% of the survey group. The RBC morphology terms with ≥ 50% of clinician respondents designating 'I do not know this term' were: bite cells, blister cells, dimorphism, echinocytes, elliptocytes, ghost cells, irregular contracted cells, ovalocytes, Pappenheimer bodies, stomatocytes and teardrop cells. We also asked the physicians which percentage of schistocytes would make the diagnosis of TTP (thrombotic thrombocytopenic purpura) more likely in a clinically suspect patient. Answers ranged between 1% and 30%, with a mean value of 9%. Six physicians (30%) answered 'I do not know'. Remarkably, only the answers of the two hematologists and two of the nephrologists were close to the 1% cut-off recommended by the ICSH.

In spite of the small size of this survey, it has shown us that the perceived clinical utility of RBC morphology among hospital physicians is low. Only a fraction of our physicians regularly reads the reported RBC morphologic changes. Even regarding schistocytes, which are generally considered as an important diagnostic element in TMA (thrombotic micro-angiopathy), the overall knowledge is poor. Our results are in line with two larger surveys concerning RBC morphology (one among pediatricians and one among veterinarians).(10, 12) Laboratory staff needs to be aware that a large proportion of hospital physicians do not know the clinical significance of some of the RBC morphology terms. We should consider educational initiatives to inform our clinicians and we should reconsider our way of reporting RBC abnormalities. A red cell morphology report should provide the physician with information that is useful in the differential diagnosis. As mentioned before, most morphologic variations are of little diagnostic significance in small numbers. They become significant only when they appear in considerable numbers. Therefore, we decided to change our grading system from the UZ Leuven cut-off values to the ICSH cut-offs (see Table 1) and no longer report minor morphological changes.

3. Evaluation of the Red Blood Cell Advanced Software Application on the DI-60

3.1 Materials and methods

A. Samples

From September 2018 until March 2019, 104 abnormal peripheral blood films were selected in the Jessa Hospital (Hasselt). These slides were selected by our MLTs during routine screening for patient RBC morphology on the DI-60. The blood samples were collected in 3 mL EDTA-K2-anticoagulated tubes (Vacuette – Greiner Bio-One). The blood smears were made and stained (May-Grünwald-Giemsa) by the SP-50 (Sysmex) slide maker/stainer. Enumeration of red blood cell morphology abnormalities was performed both by automated counting using the Advanced RBC Application on the DI-60 analyzer and by manual microscopic analysis. Each slide was selected for a specific red blood cell abnormality, but all morphological categories were evaluated so we could use each slide as a negative control.

B. Manual microscopic analysis

To evaluate the Advanced RBC Application we needed to develop a solid reference method that could be used as a 'golden standard' for accurate enumeration of abnormalities of RBC morphology. In 2011, the ICSH Schistocyte Group agreed that a count on 1000 RBCs offered a reasonable compromise between the precision required and the time needed for the count.(2) The manual microscopic technique that is widely used in Belgium consists of analyzing 10 microscopic fields of approximately 1000 RBC/field (i.e. microscopic field where the radius equals 18 erythrocytes) at a medium magnification. At the start of our verification study we compared the calculated mean value per 1000 RBC using the aforementioned method with a precise count of 1000 RBCs using an eyepiece grid. Both counts were performed on a Leica DMLB microscope (Leica Microsystem). We concluded that the first method underestimated the amount of red blood cell abnormalities, as the microscopic field selected by the MLTs (and considered to consist of 1000 RBC) actually enclosed significantly less erythrocytes. Therefore, we choose to use the precise count of 1000 RBCs with an eyepiece grid as our reference method.

As recommended by standard morphology textbooks we performed the count within a smear area of correct thickness, behind the tail, where RBCs are just beginning to separate from each other (the monolayer). The 'feather edge' at the tail of the smear was excluded to avoid a false impression of spherocytosis. Microscope evaluation was carried out at medium magnification with a 50 power objective, using immersion oil, in combination with 10x eyepieces. The 100 power objective was used only for looking at fine details such as basophilic stippling or malaria parasites.(2,13, 4) Each slide was presented in a blinded fashion and scored by two experienced morphologists (one MLT and one resident). In cases of observer disagreement, a third MLT served as a tie-breaker. Mean values of counts were used for statistical analysis.

C. Advanced RBC Application on the DI-60 digital microscope system

The Advanced RBC Application is an optional module for analysis of the red blood cell morphology. We used software version 6.0.3 for the present evaluation. As discussed in 'Clinical/Diagnostic scenario', a preliminary characterization and grouping of red blood cells into 21 morphological categories is automatically performed. The operator can subsequently verify and manually modify, if necessary, the proposed classification of each RBC class. As this is a tedious and time-consuming procedure, we will not use this 'post-reclassification' possibility. The results are presented as a percentage value and a grading, which is based on a conversion table defined by the user in the settings file. The default values are those by Gulati G. published in 2009.(15) The software makes the distinction between 'schistocytes' and 'helmet cells', with the possibility to group both categories under 'schistocytes'. For this evaluation, the grouped category was used.

D. Statistics

Statistical analysis was performed using Analyze-It (version 4.5.1) for Microsoft Excel (version 14.0). Within-run and between-run variabilities were calculated using the coefficient of variation (CV). Pearson's correlation coefficient was used to evaluate the degree of association between the Advanced RBC Application and manual microscopy. For evaluating the degree of agreement, Passing–Bablok regression analysis was used to determine proportional and constant bias via, respectively, slopes and intercepts of the regression lines of pairwise assay comparisons. Additionally, Bland-Altman analysis was used to assess the mean differences between the two methods and to reveal concentration-dependent differences between assays. Receiver operating characteristic (ROC) curves were drawn for determination of the optimal cut-off for positivity.

3.2 Evaluation of digital microscopy image quality

The quality and interpretability of the erythrocyte digital images were evaluated by all MLTs and Clinical Biology staff. Despite some slides having a macroscopic striped pattern, most digital images were of good quality and easy to interpret. During the verification process, we noticed that the Advanced RBC Application occasionally classifies normal erythrocytes as stomatocytes or as having pappenheimer bodies. When looking back at the digital images, we concluded that mainly overlapping erythrocytes were causing the erroneous classification. Finding a good 'monolayer' in these samples was difficult even with manual microscopy. However, there was no obvious relationship between the different samples so we could not define a clear reason for the DI-60 to select an inappropriate microscopic field.

3.3 Within-run variability

For assessment of the within-run variability three samples were used per morphological category: a normal sample, a sample with an intermediate and a sample with a high percentage of abnormal RBCs (if possible). One slide of each sample was analyzed in 10 consecutive runs on the DI-60. Quantitative expression (percentage of all analyzed RBCs) of the precharacterization results was used to calculate SD and coefficient of variation (CV). The results are presented in Table 4. Within-run variabilities demonstrated CVs ranging from 5% to 105% with the highest values measured for basophilic stippling (97%) and malaria parasites (105%). High CVs were due to low numbers (normal samples). The within-run CV of 27% in samples with low amounts of schistocytes should be taken into account, due to the small categories proposed by the ICSH. For example, a sample with 0.8% schistocytes (1+) can be reported as 1% (2+) and vice versa.

3.4 Between-run variability

Between-run variability was assessed by analyzing three samples per morphological category: a normal sample, a sample with an intermediate and a sample with a high percentage of abnormal RBCs (if possible). One slide of each sample was analyzed twice a day for five consecutive days. Quantitative expression (percentage of all analyzed RBCs) of the precharacterization results was used to calculate SD and coefficient of variation (CV). Results are shown in Table 4. Between-run variabilities demonstrated CVs ranging from 4% to 141%, with the highest values measured for acanthocytes and elliptocytes (both 141%). High CVs were due to low numbers (normal samples) and not clinically significant.

3.5 Method comparison

At first, our goal was to include at least 10 normal samples, 10 samples with low percentage (ICSH category 2+) and 10 samples with high percentage of abnormalities (ICSH category 3+) for every morphological category. Due to the low number of positive samples (especially samples with abnormalities graded 3+) we could not reach this goal. This means our results need to be interpreted with caution. The count obtained with the Advanced RBC Application was compared with the manual microscopic count (both expressed in percentage of RBCs). Results are shown in Figures 3-17 both in Bland–Altman and Passing–Bablok plots.

3.6 Reference ranges, sensitivity and specificity

As discussed previously, different reference textbooks and guidelines use different grading levels (cut-off percentage per level) to report abnormal morphologic findings. This means that for now there are no clearly defined and universally accepted reference ranges that can distinguish normal from abnormal samples. The department of laboratory hematology of the AZ St-Jan hospital in Bruges published two articles evaluating the performance of the Advanced RBC Application on the DM96 in 2015 and 2016. They calculated reference values from 40 (2015) and 38 (2016) adult healthy blood donors, using the precharacterization results. The calculated cut-off values for 'positive' samples are summarized in Table 5. With these cut-offs, the working group from Bruges could not reach an acceptable sensitivity and specificity so they turned to 'clinical' cut-offs taking into account the clinical significance of the RBC abnormality (8). In june 2018, we set up a small clinical study to define our own reference values. During this study we noticed the appearance of 'honeycomb' red blood cells in some samples, so the settings of the SP-50 needed to be changed. Therefore, we could not use all our data and will need to repeat our reference value study. In Table 5, we provide mean values and ranges for the 19 samples from healthy

volunteers that were usable.

We decided to use Pearson's correlation coefficient and Passing-Bablok regression analysis to calculate new cut-off values for the Advanced RBC Application, starting from the cut-off values provided by the ICSH in 2015. If this method was not suitable, we used ROC curve analysis to estimate the 'ideal' cut-off for positivity. A disadvantage of using the ROC curve analysis instead of Passing-Bablok regression is that only a cut-off value for 'present' can be determined and not for semi-quantitative grading. To determine the 'true' positivity or negativity of a certain slide, we applied the ICSH cut-off values to the results of manual microscopy count. For sickle cells a definite diagnosis of sickle cell anemia (by hemoglobin electrophoresis) was used as the criterion for positivity. Sensitivity and specificity of the Advanced RBC Application using the newly defined cut-offs were calculated by comparing precharacterization results to the golden standard manual microscopy. For Howell-Jolly bodies we also calculated sensitivity and specificity for detection of splenectomy.

3.7 Conclusion of the verification study

In Table 6 the cut-off values, corresponding sensitivity and specificity are summarized per morphological category. The primary goal during the verification of the Advanced RBC Application was to increase reproducibility and decrease workload, but we also wanted to maintain an excellent sensitivity for all categories. In comparison to Criel et al, we tend to use lower cut-off values which increases sensitivity, but decreases specificity. To reduce the amount of false positive samples, the grading of 9 of the 15 evaluated categories still needs to be reviewed by our MLTs if the sample reaches a certain cut-off value ('+++' in Table 6). By evaluating the individual cell images for false positives, the MLT can adjust the grading if necessary. As mentioned previously, we could not include enough true positive samples (ICSH category 2+ or 3+). Therefore, we need to keep in mind that our results may not reflect the true sensitivity/specificity for some categories and need to be completed with more positive samples.

Not all of the RBC morphology categories discussed in part 1 were verified in this study. The categories hypochromia, microcytosis, macrocytosis, anisochromasia and anisocytosis were not included because of preferable use of the analyzer parameters MCV, MCH, MCHC and RDW. Poikilocytosis was not included because this term is no longer recommended by the ICSH, we prefer to report the specific cell shape instead.(1) The detection and classification of rouleaux, agglutination, irregular contracted cells, bite cells, blister cells and intracellular hemoglobin crystals is not included in the application software. As these findings can be associated with serious clinical conditions, the ICSH recommends to report their presence (for rouleaux, agglutination and intracellular hemoglobin crystals) and provide a grading (for irregular contracted cells, bite cells and blister cells). This means our MLTs will still need to screen the digital overview image for the aforementioned abnormalities.

COMMENTS

The first part of this appraisal demonstrates that abnormalities of the red blood cell morphology can be helpful (and sometimes even essential) in the diagnostic work-up of a patient. In the differential diagnosis of anemia, hemolysis, thrombotic micro-angiopathies and several (rare) congenital diseases the RBC morphology report gives crucial information that can direct the physician to an accurate diagnosis. However, the second part of this appraisal shows that only a minority of hospital physicians is aware of this clinical utility. In the last part, we evaluated the performance characteristics of the Advanced RBC Application on DI-60 in our laboratory. Our verification study was more meticulously executed than studies that have been published before.(5-8) So far, studies have always compared the quantitative count of the Advanced RBC Application to the semi-quantitative estimation of manual microscopy. This is the first time the count of the Advanced RBC Application has been compared to an accurate manual microscopic enumeration (both expressed as percentage of RBC). With this approach we were able to calculate cut-off values for 1+, 2+ and 3+ grading of acanthocytes, echinocytes, elliptocytes, ovalocytes, polychromasia and target cells using Passing-Bablok regression analysis. For the other categories we needed to rely on ROC curve analysis to determine a cut-off value for positivity.

We conclude that with the Advanced RBC Application we can obtain a sensitive and reproducible measurement for most RBC morphological abnormalities, provided that cut-off values are adjusted. However, specificity is low for the majority of categories and not all clinically relevant categories can be detected and classified by the application software. As a consequence, evaluation of the digital images by our MLTs remains necessary for most categories. By changing our grading levels to those recommended by the ICSH, we aim to report only the truly important abnormalities and convince our clinicians that the RBC morphology should not be overlooked. We still need to implement the Advanced RBC Application and the new grading levels, so at this point we do not know what the effect on workload, reproducibility and perceived clinical utility will be.

TO DO/ACTIONS

1) More (high) positive samples need to be analyzed to further determine the utility of the Advanced RBC Application.
2) We need large multi-center studies to define grading levels for RBC morphology abnormalities that are not based on consensus (like those provided by the ICSH), but on clinical relevance (the condition of the patient: e.g. AIHA, TTP, thalassemia, …).
3) For schistocytes, it could be useful to combine the automated FRC count with the count of the Advanced RBC Application as a screening method, but this needs to be further evaluated.

ATTACHMENTS

Table 1: grading levels per morphological category

RBC morphology	DI-60 default values			ICSH guidelines			UZ Leuven guidelines				Constantino 2014		
	1 (Slight)	2 (Moderate)	3 (Marked)	1 (Few)	2 (Moderate)	3 (Many)	Rare	Small excess	Medium excess	Large excess	1 (Slight/Few)	2 (Moderate)	3 (Marked)
Polychromasia	1	5	10	N/A	5	>20		0,3	>1	>2	3	6	>20
Hypochromic cells	6	25	50	N/A	11**	>20		1	>4	>12,5	5	16	>40
Microcytosis	6	25	50	N/A	11**	>20		1	>4	>12,5			
Macrocytosis	6	25	50	N/A	11**	>20		1	>4	>12,5			
Poikilocytosis	10	25	50	Report the specific cell shape									
Anisocytosis	15*	20*	25*	N/A	11**	>20							
Target cells	5	10	30	N/A	5	>20		0,6	>2	>4	5	11	>25
Schistocytes	1	3	6	<1	1	>2		0,3	>1	>2	1	6	>15
Helmet cells	1	3	6	Included in schistocyte count			Included in schistocyte count						
Sickle cells	5	10	30	N/A	1	>2		0,1	>0,3	>0,7	Report if present		
Spherocytes	1	3	6	N/A	5	>20	0,1	0,3	>1	>2	1	6	>20
Elliptocytes	6	20	50	N/A	5	>20		0,3	>1	>2	6	21	>50
Ovalocytes	6	20	50	N/A	5	>20		0,6	>2	>4			
Teardrop cells	1	3	6	N/A	5	>20		0,3	>1	>2	If > 4%: report as present		
Stomatocytes	5	10	30	N/A	5	>20		0,3	>1	>2	If > 30%: report as present		
Acanthocytes	5	10	30	N/A	5	>20	0,1	0,3	>1	>2	1	11	>30
Echinocytes	10	25	50	N/A	5	>20		0,6	>2	>4	If > 30%: report as present		
Howell-Jolly bodies	1	3	6	N/A	2	>3		0,1	>0,3	>0,7	Report if present		
Pappenheimer bodies	1	3	6	N/A	2	>3		0,1	>0,3	>0,7	Report if present		
Basophilic stippling	1	3	6	N/A	5	>20		0,1	>0,3	>0,7			
Malaria parasites	1	3	6	Report if present			Report if present				Report if present		
Anulocytes	1	1	2				0,1	0,3	>1	>2			
Pencil cells	5	5	20				0,1	0,3	>1	>2			
Rouleaux	1	1	2	Report if present			Report if present					11	>50
Agglutination	0	0	0	Report if present			Report if present				Report if present		
Dimorphism	0	0	0	Report and describe if present							Report if present		
Anisochromasia	1	3	6										
Irregularly contracted cells	0	0	0	N/A	1	>2					If > 4%: report as present		
Bite cells	0	0	0	N/A	1	>2					If > 4%: report as present		
Blister cells	0	0	0	N/A	1	>2							
Oval macrocytes				N/A	2	>5					Report if present		
Megalocytes							0,1	0,3	>1	>2			
Cabot rings								0,1	>0,3	>0,7			

N/A: not applicable

* expressed as area distribution width %

** The ICSH recommends that the analyser generated MCH, MCV and RDW be used rather than grading by visual microscopic examination, unless abnormal RDW or red cell histogram suggests the presence of macrocytes/microcytes even though the MCV is normal.

RBC morphology included in verification of Advanced RBC Application.

Not included in guideline

Figure 1: Advanced RBC Application

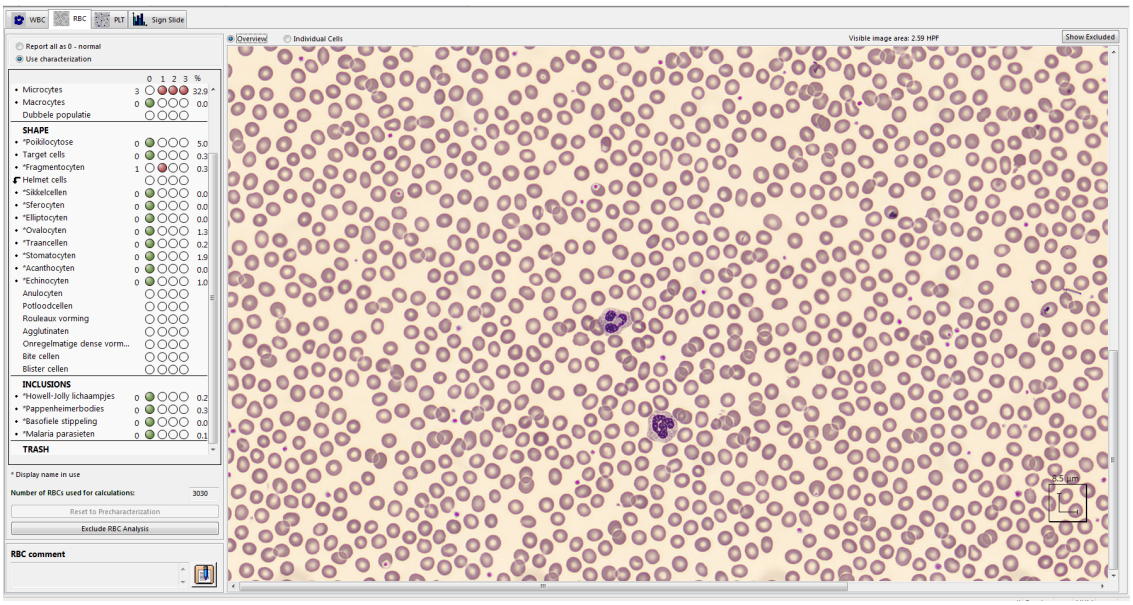


Figure 2: Advanced RBC application

Table 2: associated clinical conditions

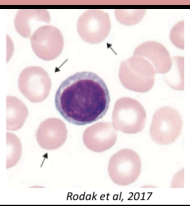
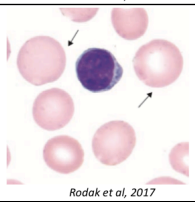
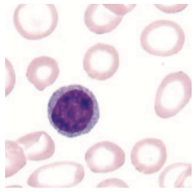
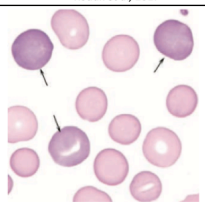
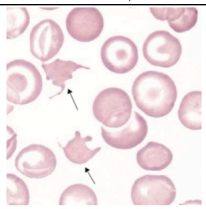
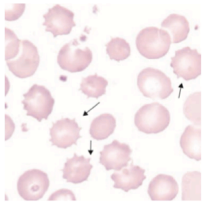
Recommended nomenclature	Morphology	Common clinical conditions associated with
Microcytosis	 *Rodak et al, 2017*	- iron deficiency anemia - thalassemias and some other hemoglobinopathies - chronic inflammation (some cases) - lead poisoning - sideroblastic anemia - slight degree can be normal for children
Macrocytosis	 *Rodak et al, 2017*	- liver disease - hypothyroidism - vitamin B12 deficiency - folate deficiency - medication-induced: methotrexate, hydroxyurea, doxorubicine, azthioprine, mercaptopurine, fluorouracil, hydroxycarbamide, … - reticulocytosis - MDS - aplastic anemia - slight degree can be normal for neonates, pregnant women and elderly
Anisocytosis		non-specific feature of almost any blood disorder
Dimorphism		- transfusion - myelodysplastic syndromes - sideroblastic anemia - double deficiency of iron and either vitamin B12 or folic acid - early in treatment process of vitamin B12, folate, or iron deficiency
Hypochromia	 *Rodak et al, 2017*	Any of the conditions leading to microcytosis may also cause hypochromia. Slight degree can be normal for children
Polychromasia	 *Rodak et al, 2017*	- hematopoietic stress: acute and chronic hemorrhage, hemolysis, … - effective treatment of iron, vitamin B12 or folic acid deficiency - extramedullary erythropoiesis: primary myelofibrosis, metastatic carcinoma of the bone marrow, … - normal in neonates
Acanthocytes	 *Rodak et al, 2017*	- advanced liver disease: alcoholic cirrhosis, severe viral hepatitis, hemochromatosis, ... - hyposplenism - anorexia nervosa, starvation and malabsorption of lipids - hypothyroidism - vit E deficiency - hereditary abetalipoproteinemia - associated with inherited degenerative neurological disease (neuroacanthocytosis): McLeod red cell phenotype, HARP syndrome, ...
Echinocytes	 *Rodak et al, 2017*	- storage artifact - liver and renal disease: described in critically ill patients with multiorgan failure, hemolytic uremic syndrome, ... - severe burn injuries - following cardiopulmonary bypass - phosphate deficiency - pyruvate kinase deficiency - premature neonates

Table 2 (continued)

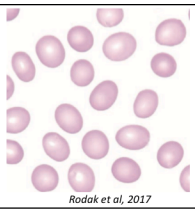
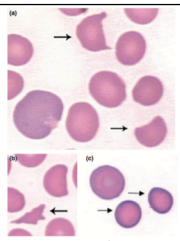
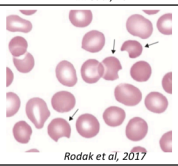
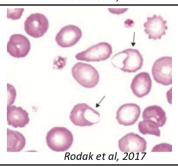
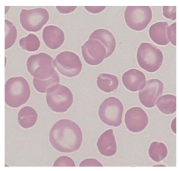
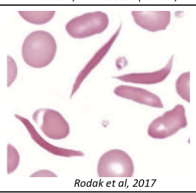
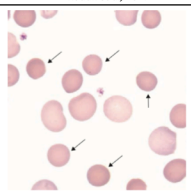
Ovalocytes	 *Rodak et al, 2017*	- Numerous and dominant abnormality: hereditary conditions affecting the red cell cytoskeleton e.g. hereditary elliptocytosis - Small number: iron deficiency, thalassemias, primary myelofibrosis, myelodysplastic syndromes, pyruvate kinase deficiency - Macrocytic ovalocytes or oval macrocytes are characteristic of megaloblastic anemia and South-East Asian ovalocytosis and are also seen in dyserythropoiesis, e.g. in primary myelofibrosis.
Elliptocytes	 *Rodak et al, 2017*	
Poikilocytosis		non-specific feature of many blood disorders
Schistocytes	 *Zini et al, 2011*	schistocytes are formed in the following situations - genetically determined disorders: thalassemias, glucose-6-phosphate dehydrogenase (G6PD) deficiency, ... - acquired disorders of red cell formation when erythropoiesis is megaloblastic or dyserythropoietic: megaloblastic anemia, primary myelofibrosis - direct thermal injury (as in severe burns) or mechanical trauma (as in march hemoglobinuria) - as the consequence of extrinsic mechanical damage to the membrane caused by filaments of fibrin in the microvessels, increased turbulence, shear stress and RBC adhesion to abnormal endothelium (e.g. in the thrombotic microangiopathies (TMA), malfunctioning cardiac valves and cardiac assist devices, HELLP syndrome, preeclampsia, DIC, metastatic malignancy, malignant hypertension, Kasabach Merritt syndrome, ...) ICSH: a schistocyte percentage > 1% in a peripheral blood smear in adults is a robust cytomorphological indication in favor of a diagnosis of TMA, when additional features suggesting an alternative diagnosis are absent.
Bite cells	 *Rodak et al, 2017*	- G6PD deficiency - hemoglobin C disease, hemoglobin C/β thalassemia, sickle cell/hemoglobin C disease, ... - unstable hemoglobins - severe oxidant stress (drugs or chemicals)
Blister cells	 *Rodak et al, 2017*	
Irregularly contracted cells	 *J. Burthem, M. Brereton (Palmer, 2015)*	
Sickle cells	 *Rodak et al, 2017*	sickle cell anemia and other forms of sickle cell disease
Spherocytes	 *Rodak et al, 2017*	- hereditary spherocytosis - auto-immune hemolytic anemia - ABO and Rh hemolytic disease of the fetus and newborn - drug-induced (oxidative/immune) hemolysis - delayed hemolytic transfusion reaction - severe burns

Table 2 (continued)

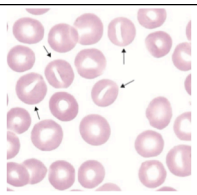
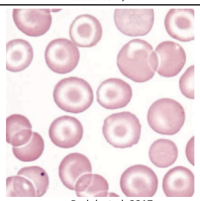
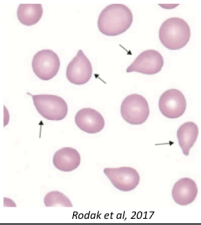
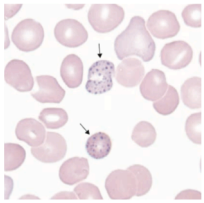
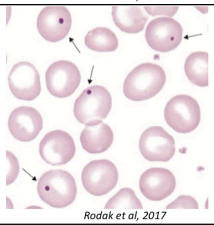
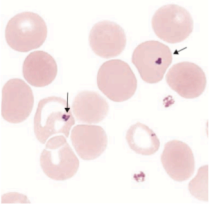
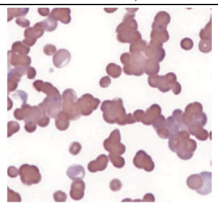
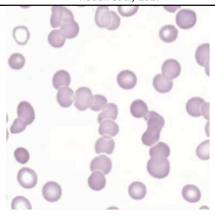
Stomatocytes	 *Rodak et al, 2017*	- alcoholism - liver disease - MDS } frequently associated macrocytosis - artifact - inherited erythrocyte membrane abnormalities: hereditary stomatocytosis, hereditary xerocytosis, South-East asian ovalocytosis (in association with ovalocytes/elliptocytes)
Target cells	 *Rodak et al, 2017*	- iron deficiency anemia - thalassemias and other hemoglobinopathies - hyposplenism - severe (obstructive) liver disease - hereditary LCAT deficiency - hereditary hypobetalipoproteinemia
Teardrop cells	 *Rodak et al, 2017*	- megaloblastic anemia - thalassemia major - myelofibrosis: primary or secondary to bone marrow infiltration
Basophilic stippling	 *Rodak et al, 2017*	- poisoning by lead and other heavy metals - thalassemias and other hemoglobinopathies - megaloblastic anemia - sideroblastic anemia - primary myelofibrosis - hereditary deficiency of pyrimidine 5'-nucleotidase
Howell-Jolly bodies	 *Rodak et al, 2017*	- postsplenectomy and hyposplenism - hemolytic anemia and megaloblastic anemia (especially if associated splenic atrophy) - normal in neonates
Pappenheimer bodies	 *Rodak et al, 2017*	- lead poisoning - sideroblastic anemia - postsplenectomy and hyposplenism - hemoglobinopathies
Agglutination	 *Rodak et al, 2017*	- small agglutinates: warm auto-immune hemolytic anemia - massive agglutination: paroxysmal cold hemoglobinuria and chronic cold hemagglutinin disease
Rouleaux	 *Rodak et al, 2017*	- pregnancy - acute and chronic inflammatory disorders - plasma cell neoplasms - artifact

Table 3: survey results

RBC morphology	**Never significant**	**Only significant if many (2+ of 3+)**	**Significant even in small numbers (1+)**	**I do not know this term**	**Number of responses**
Acanthocytes	11%	22%	22%	44%	18
Agglutination	17%	22%	28%	33%	18
Anisocytosis	12%	29%	12%	47%	17
Basophilic stippling	12%	24%	18%	47%	17
Bite cells	6%	6%	6%	83%	18
Blister cells	6%	6%	11%	78%	18
Dimorphism	6%	6%	22%	67%	18
Echinocytes	6%	11%	11%	72%	18
Elliptocyt	6%	22%	17%	56%	18
Schistocytes	17%	39%	28%	17%	18
Ghost cells	6%	11%	28%	56%	18
Howell-Jolly bodies	15%	20%	35%	30%	20
Hypochromasia	10%	45%	25%	20%	20
Irregular contracted cells	6%	6%	17%	72%	18
Macrocytosis	10%	40%	50%	0%	20
Microcytosis	10%	35%	55%	0%	20
Ovalocytes	6%	28%	17%	50%	18
Pappenheimer bodies	6%	6%	6%	83%	18
Poikilocytosis	16%	16%	37%	32%	19
Polychromasia	11%	21%	21%	47%	19
Rouleaux	11%	32%	42%	16%	19
Spherocytes	11%	42%	26%	21%	19
Stomatocytes	11%	17%	11%	61%	18
Target cells	11%	22%	33%	33%	18
Teardrop cells	22%	6%	22%	50%	18

Not all responders provided answers to every question, resulting in a variable number of responses for each question.

Abnormalities of RBC morphology considered as significant (in small or large numbers) by ≥ 50%of the survey group.

RBC morphology terms unknown by ≥ 50%of the survey group.

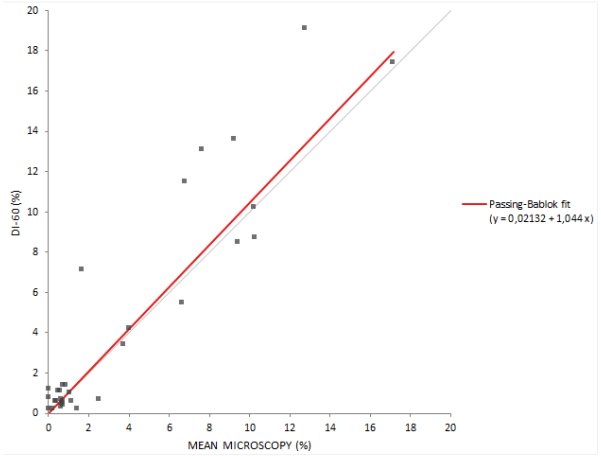
Table 4: evaluation of imprecision

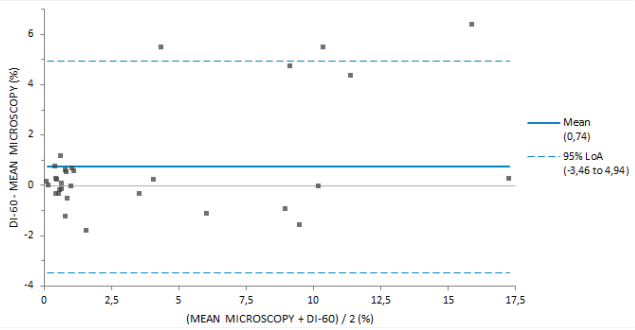
	Within-run						Between-run					
	Few / 1+		Moderate / 2+		Many / 3+		Few / 1+		Moderate / 2+		Many / 3+	
	Mean (± SD)	CV	Mean (± SD)	CV	Mean (± SD)	CV	Mean (± SD)	CV	Mean (± SD)	CV	Mean (± SD)	CV
Polychromasia	0,32(± 0,06)	20	6,89 (± 1,20)	17	-	-	0,25(± 0,10)	39	6,51 (± 1,51)	23	-	-
Target cells	0,36 (± 0,16)	46	12,79 (± 0,81)	6	22,13 (± 2,91)	13	0,32 (± 0,09)	29	12,82 (± 0,94)	7	21,35 (± 3,09)	14
Schistocytes	0,44 (± 0,12)	27	1,72 (± 0,35)	20	7,6 (± 0,46)	6	0,96 (± 0,25)	26	1,67 (± 0,54)	33	6,91 (± 0,34)	5
Sickle cells	0,72 (± 0,17)	23	-	-	-	-	0,92 (± 0,24)	27	-	-	-	-
Spherocytes	2,97 (± 0,81)	27	-	-	-	-	2,15 (± 0,76)	36	-	-	-	-
Elliptocytes	0,11 (± 0,06)	52	6,49 (± 0,59)	9	-	-	0,05 (± 0,07)	141	6,97 (± 0,55)	8	-	-
Ovalocytes	2,16 (± 0,70)	32	10,44 (± 1,18)	11	-	-	1,5 (± 0,29)	19	8,8 (± 0,75)	9	-	-
Tear drop cells	0,35 (± 0,14)	41	10,77 (± 0,51)	5	-	-	0,35 (± 0,14)	41	10,71 (± 0,40)	4	-	-
Stomatocytes	2,18 (± 0,65)	30	9,67 (± 1,07)	11	30,57 (± 9,52)	31	1,32 (± 0,34)	26	8,45 (± 0,96)	11	32,64 (± 1,70)	5
Acanthocytes	0,07 (± 0,05)	69	9,07 (± 1,25)	14	29,53 (± 4,56)	15	0,06 (± 0,08)	141	11 (± 1,56)	14	26,02 (± 2,40)	9
Echinocytes	1,34 (± 0,48)	36	12,88 (± 2,04)	16	56,41 (± 4,71)	8	0,97 (± 0,22)	22	14,19 (± 2,81)	20	58,95 (± 6,88)	12
Howell-Jolly bodies	0,59 (± 0,12)	20	-	-	-	-	0,59 (± 0,16)	27	-	-	-	-
Pappenheimer bodies	0,30 (± 0,10)	34	2,58 (± 0,57)	22	11,78 (± 3,61)	31	0,08 (± 0,06)	79	2,25 (± 0,28)	12	11,11 (± 2,47)	22
Basophilic stippling	0,25 (± 0,24)	97	10,44 (± 1,65)	16	-	-	0,25 (± 0,07)	28	8,61 (± 1,35)	16	-	-
Malaria parasites	0 (± 0,00)	-	0,05 (± 0,05)	105	0,84 (± 0,31)	36	0 (± 0,00)	-	0,08 (± 0,04)	53	0,76 (± 0,14)	19

Table 5: reference value study in healthy population

	St-Jan Hospital Bruges (Criel et al.)	Jessa Hospital Hasselt (limited data)
RBC morphology	*Cut-off value for positivity (%)*	*Range and mean value (%)*
Polychromasia	≥ 0,5	0 - 0,3 (0,07)
Schistocytes	≥ 2,6	0,1 - 1,1 (0,44)
Target cells	≥ 0,3	0 - 0,5 (0,15)
Sickle cells	≥ 0,1	0 - 0,1 (0,01)
Spherocytes	≥ 1,4	0 - 0,1 (0,01)
Elliptocytes	≥ 0,8	0 - 2 (0,17)
Ovalocytes	≥ 0,1	0,1 - 5,3 (2,47)
Tear drop cells	≥ 0,6	0 - 2 (0,46)
Stomatocytes	≥ 1,7	0,1 - 7,3 (2,97)
Acanthocytes	≥ 1,1	0 - 0,1 (0,03)
Echinocytes	≥ 2,1	0,4 - 5 (1,77)
Howell-Jolly bodies	≥ 0,2	0 - 0,1 (0,03)
Pappenheimer bodies	≥ 0,1	0 - 0,2 (0,06)
Basophilic stippling	≥ 0,1	0 - 0,6 (0,16)
Parasites	≥ 0,1	0 - 0,2 (0,03)

Figure 3: Acanthocytes



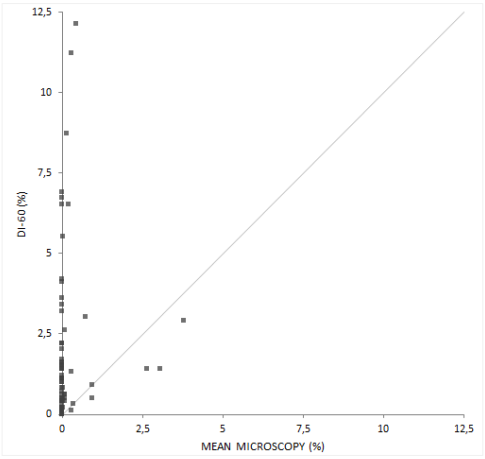


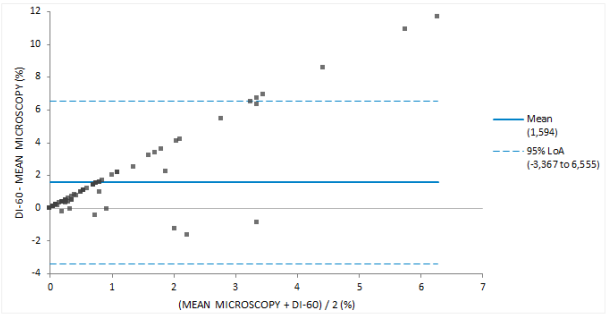
N	31

	Minimum	Maximum
MEAN MICROSCOPY	0,05	17,15
DI-60	0,20	19,10

Correlation - r	0,930

Figure 4: Basophilic stippling



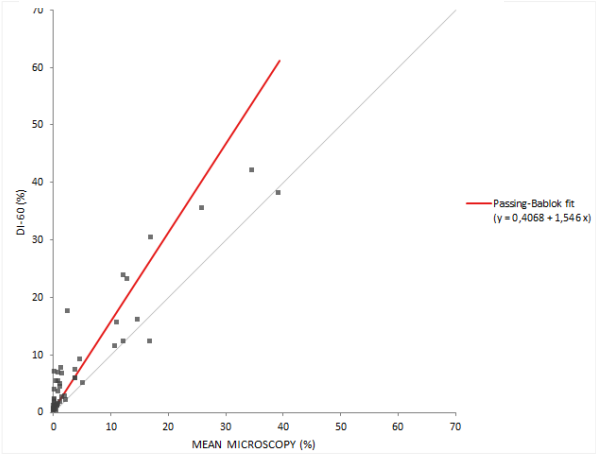


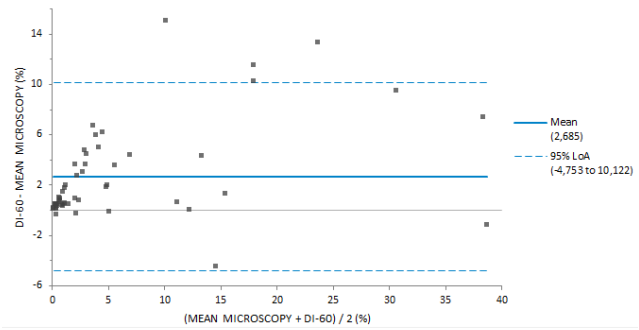
N	75

	Minimum	Maximum
MEAN MICROSCOPY	0,000	3,800
DI-60	0,000	12,100

Correlation - r	0,072

Figure 5: Echinocytes



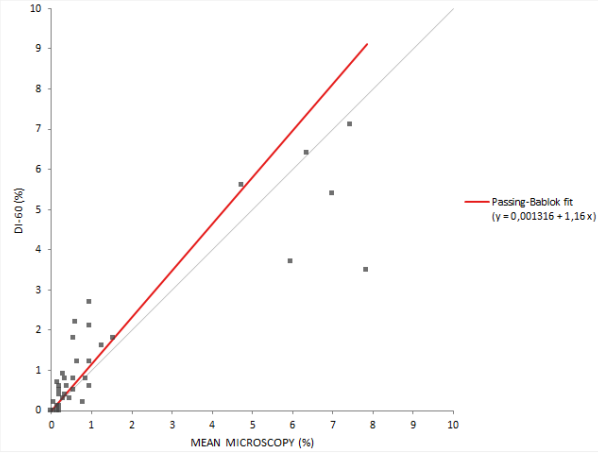


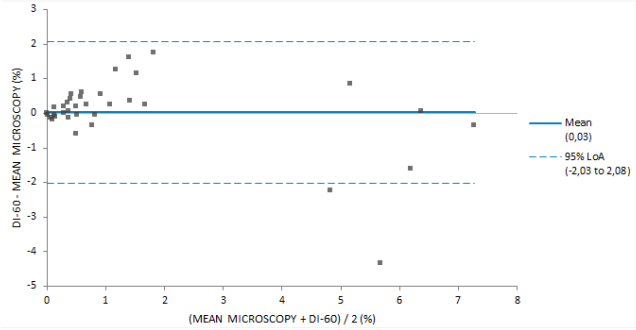
N	52

	Minimum	Maximum
MEAN MICROSCOPY	0,050	39,300
DI-60	0,200	42,100

Correlation - r	0,933

Figure 6: Elliptocytes



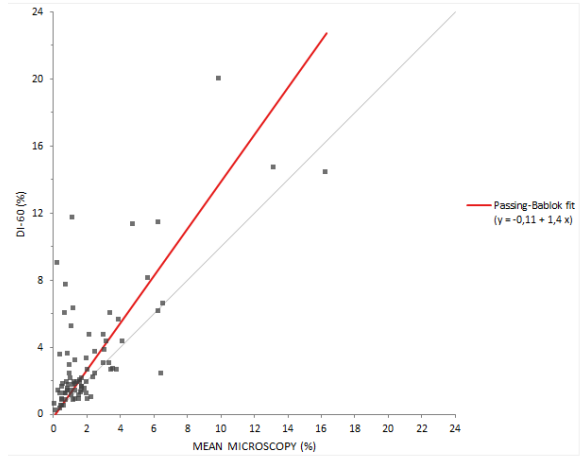


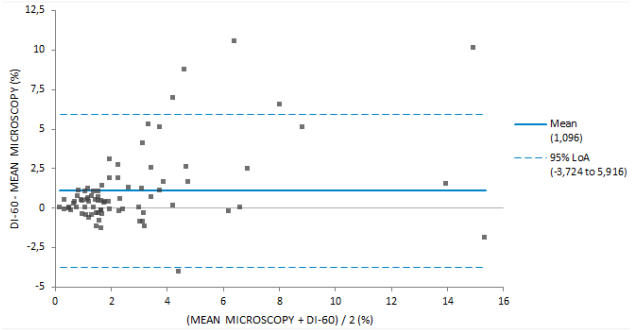
N	36

	Minimum	Maximum
MEAN MICROSCOPY	0,00	7,85
DI-60	0,00	7,10

Correlation - r	0,901

Figure 7: Schistocytes



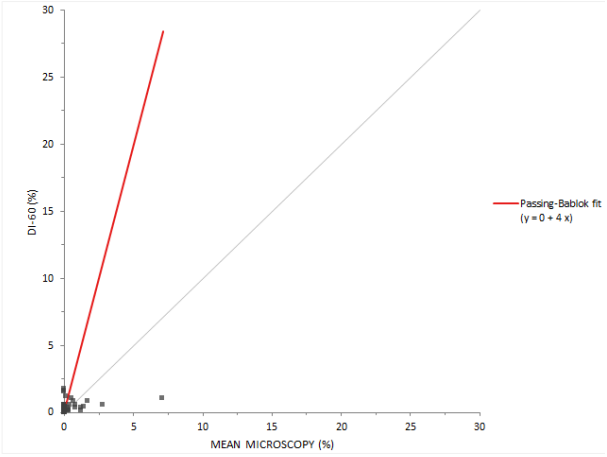


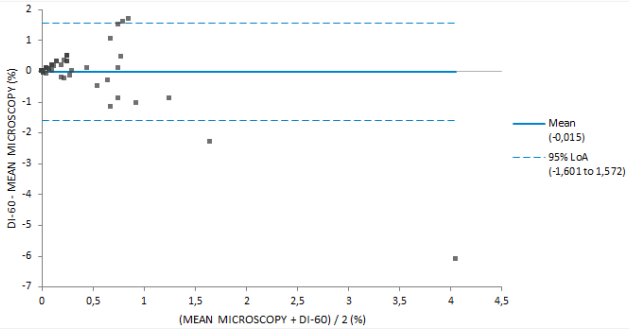
N	80

	Minimum	Maximum
MEAN MICROSCOPY	0,100	16,300
DI-60	0,200	20,000

Correlation - r	0,740

Figure 8: Howell-Jolly bodies



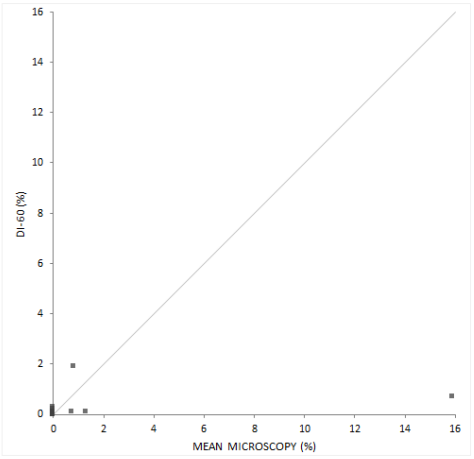


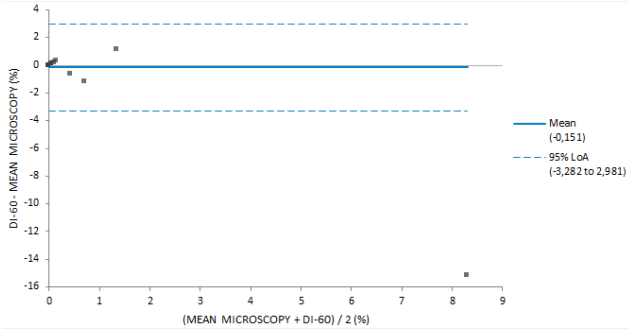
N	89

	Minimum	Maximum
MEAN MICROSCOPY	0,000	7,100
DI-60	0,000	1,700

Correlation - r	0,328

Figure 9: Malaria parasites



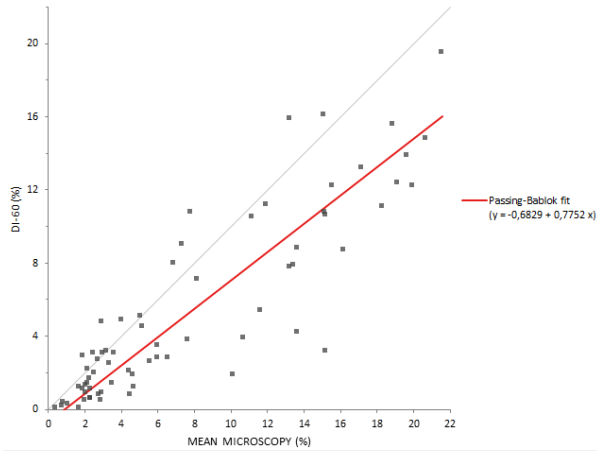


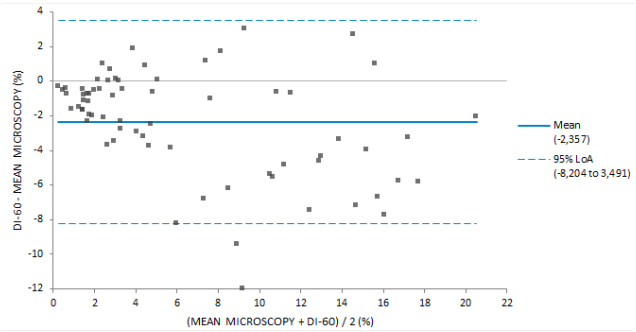
N	92

	Minimum	Maximum
MEAN MICROSCOPY	0,000	15,900
DI-60	0,000	1,900

Correlation - r	0,367

Figure 10: Ovalocytes



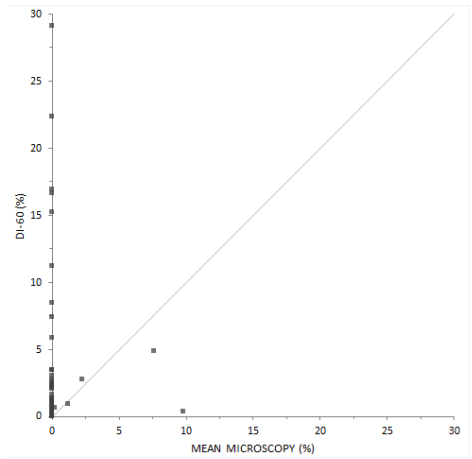


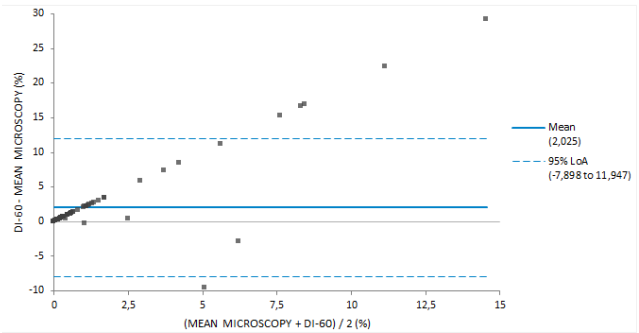
N	69

	Minimum	Maximum
MEAN MICROSCOPY	0,400	21,550
DI-60	0,100	19,500

Correlation - r	0,882

Figure 11: Pappenheimer bodies



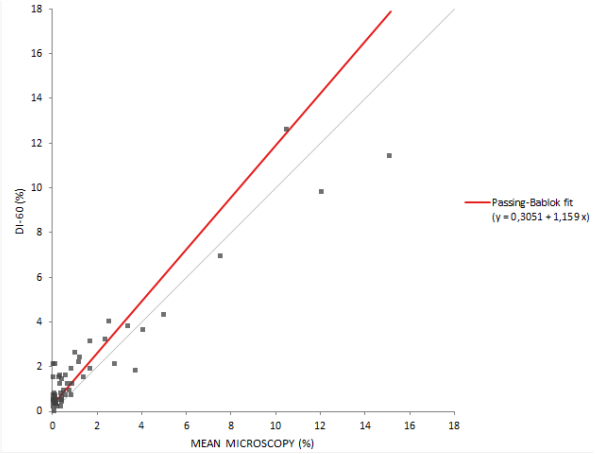


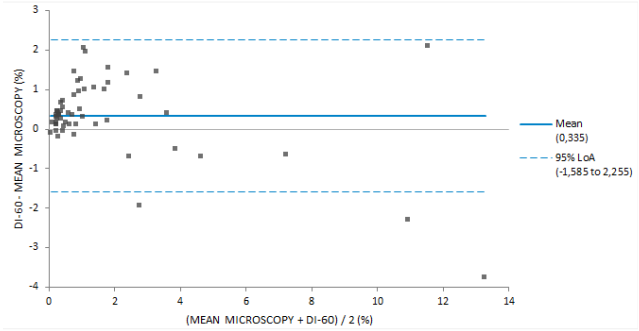
N	89

	Minimum	Maximum
MEAN MICROSCOPY	0,000	9,850
DI-60	0,000	29,100

Correlation - r	-0,002

Figure 12: Polychromasia



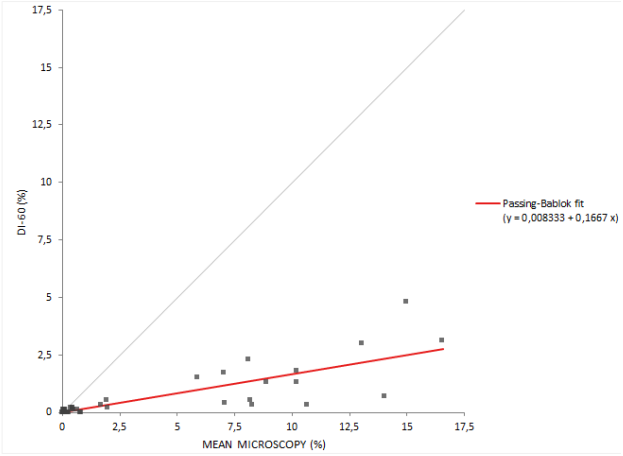


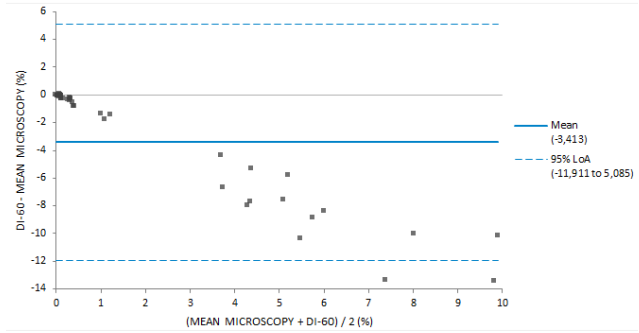
N	56

	Minimum	Maximum
MEAN MICROSCOPY	0,050	15,150
DI-60	0,000	12,600

Correlation - r	0,951

Figure 13: Spherocytes



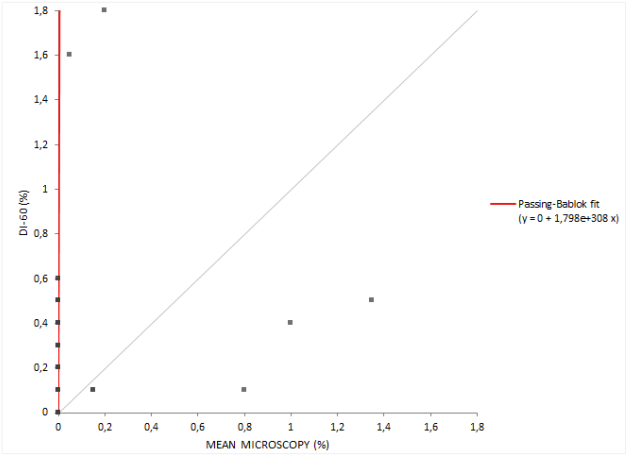


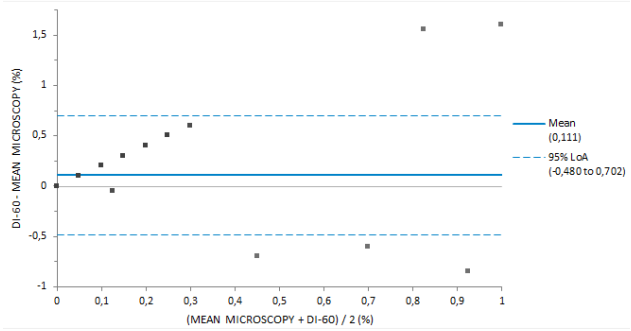
N	38

	Minimum	Maximum
MEAN MICROSCOPY	0,000	16,550
DI-60	0,000	4,800

Correlation - r	0,804

Figure 14: Sickle cells



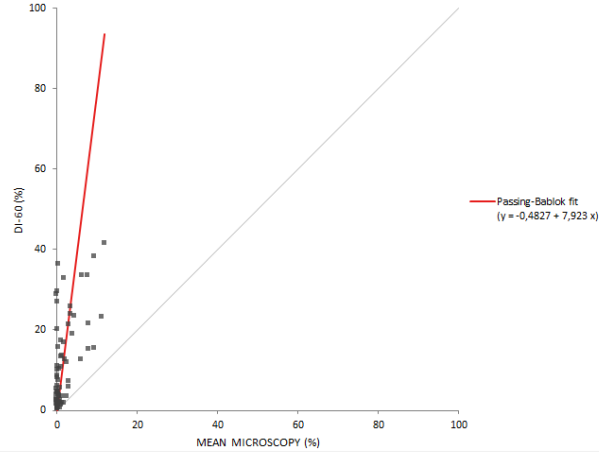


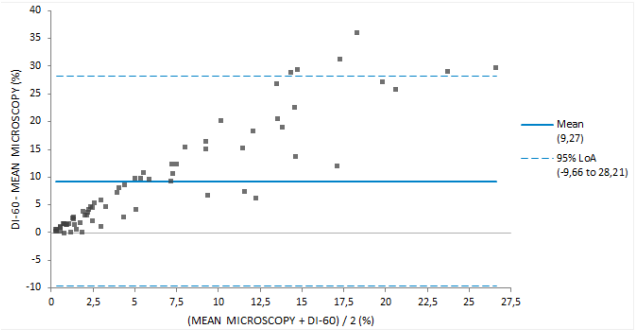
N	98

	Minimum	Maximum
MEAN MICROSCOPY	0,000	1,350
DI-60	0,000	1,800

Correlation - r	0,211

Figure 15: Stomatocytes



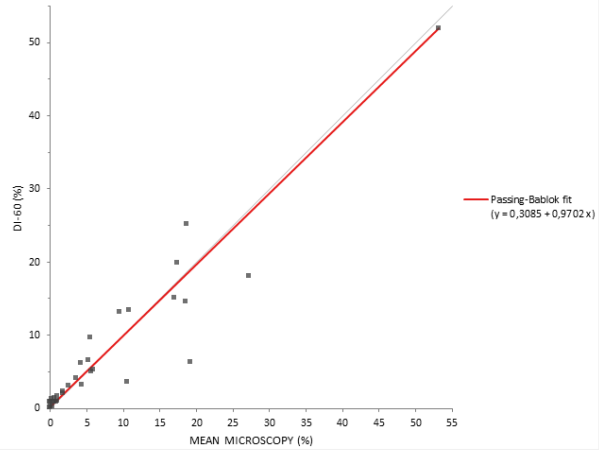


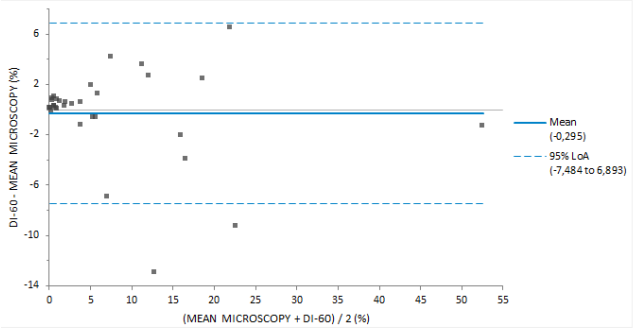
N	70

	Minimum	Maximum
MEAN MICROSCOPY	0,05	11,85
DI-60	0,40	41,40

Correlation - r	0,598

Figure 16: Target cells



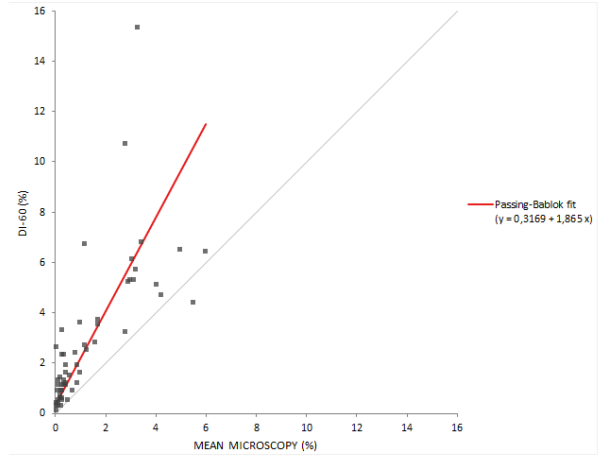


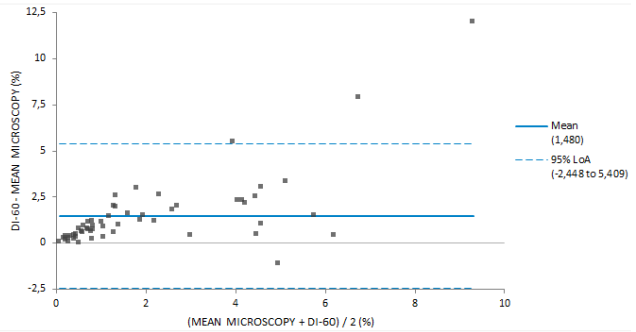
N	32

	Minimum	Maximum
MEAN MICROSCOPY	0,000	53,200
DI-60	0,100	51,900

Correlation - r	0,944

Figure 17: Teardrop cells





N	56

	Minimum	Maximum
MEAN MICROSCOPY	0,050	6,000
DI-60	0,100	15,300

Correlation - r	0,728

Table 6: calculated cut-off values with corresponding sensitivity and specificity

	New settings DI-60 (%)				Sensitivity	Specificity	review by MLT?	Remarks
	negative		*positive*					
semi-quantitative	**0**	**+**	**++**	**+++**				
Acanthocytes	0	0,1	7,2	21	89%	100%	review not necessary	More high positive samples (≥ 21%) need to be included.
Basophilic stippling	0	0,1		3	-	-	review if +++ and adjust grading	No true positive samples (≥ 5%) included. Follow-up necessary.
Echinocytes	0	0,1	9,2	31	92%	98%	review not necessary	
Elliptocytes	0	0,1	3,5	15	97%	100%	review not necessary	More high positive samples (≥ 15%) need to be included.
Howell-Jolly bodies	0	0,1		0,5	100%	86%	review if +++ and adjust grading	Only 2 true positive samples (≥ 2%). Follow-up necessary. With cut-off for positivity of 0,5% there is only 44% sensitivity, but 90% specificity for splenectomy.
Ovalocytes	0	0,1	3,2	14,7	89%	91%	review not necessary	
Pappenheimer bodies	0	0,1		0,3	100%	29%	review if +++ and adjust grading	Only 3 true positive samples (≥ 2%). Follow-up necessary.
Polychromasia	0	0,1	4,1	18	100%	100%	review not necessary	More high positive samples (≥ 18%) need to be included.
Spherocytes	0	0,1		0,3	100%	92%	review if +++ and adjust grading	
Sickle cells	0			0,1	100%	52%	review if +++ and adjust grading	Only 3 true positive sample (diagnosis of sickle cell anemia). Follow-up necessary.
Stomatocytes	0	0,1		12,7	100%	74%	review if +++ and adjust grading	
Target cells	0	0,1	3,3	19,8	100%	89%	review not necessary	
Teardrop cells	0	0,1		3,8	100%	79%	review if +++ and adjust grading	Only 3 true positive samples (≥ 5%). Follow-up necessary.
	negative	*positive*						
semi-quantitative	**0**	**+**	**++**	**+++**				
Schistocytes	0	0,5		0,9	98%	30%	review if +++ and adjust grading	
Malaria parasites	0			0,1	100%	80%	review if +++ and adjust grading	Only 4 true positive samples (diagnosis of malaria infection). Low positive samples have between-run variability of 53%, therefore sensitivity is probably lower than reported. Follow-up necessary.