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# The assessment of the utility of novel peripheral blood morphology parameters, including reticulocytic, in the diagnosis of iron deficiency and sideropenic anemia

## Ocena użyteczności nowych parametrów morfologii krwi obwodowej, w tym retikulocytarnych, w diagnostyce niedoboru żelaza i niedokrwistości syderopenicznej

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**Keywords**

morphology, reticulocytes, sideropenic anemia, diagnostic tests

**Słowa kluczowe**

morfologia, retikulocyty, niedokrwistość syderopeniczna, badania diagnostyczne

S u m m a r y

**Introduction.** A sideropenic anemia (IDA) account for 80% of all cases of anemia and diagnostically it belongs to microcytic and non-pigmented anemia. In order to differentiate IDA and chronic disease anemia (ACD) which is accompanied by functional iron deficiency, both routine blood morphology and biochemical parameters are used.

**Aim.** The aim of the study was to the assessment of the utility of novel peripheral blood morphology parameters, including reticulocytic, in the diagnosis of iron deficiency and sideropenic anemia.

**Material and methods.** The material included 275 results of peripheral blood counts with reticulocytes obtained from the Sysmex XN 2000 analyzer from patients of the Independent Public Central Clinical Hospital in Warsaw. The material was divided into 2 groups: group I – 275 samples with a measured CRP concentration, established as a criterion for diagnosis of inflammation, which were further divided into samples with CRP < 5 mg/l CRP ≥ 5 mg/l, group II – 67 samples with absolute iron deficiency, without the influence of inflammatory component (transferrin saturation < 16% and CRP concentration < 5 mg/l). All of the measurements were made on the material from the simultaneous collection.

**Results.** The highest AUC value in the diagnosis of absolute iron deficiency was obtained for: UIBC – 0.939, S-RET-He – 0.926, Fe – 0.915, S-% MicroR – 0.898 and S% HYPO-He – 0.896 respectively, while the values of the area under the curve for the "routine" morphology parameters, which are the basis of IDA diagnosis and differentiation, are: S-HGB – 0.721 and S-MCV – 0.872. The calculated AUC values show that both S-% MicroR and S-% HYPO-He have more diagnostic power than e.g. MCV which is the parameter commonly used for the differentiation of sideropenic anemia. In addition, we found a statistically significant negative correlation between the concentration of CRP and S-RET-He, S-% HYPO-He, S-Delta-He, in both groups of patients.

**Conclusions.** The study showed that novel parameters of peripheral blood morphology, including reticulocytic ones, are useful in the diagnosis of iron deficiency. However, they are not able to differentiate between absolute and functional deficiencies, since they are influenced by the acute phase.

S t r e s z c z e n i e

**Wstęp.** Niedokrwistość syderopeniczna (IDA) stanowi 80% wszystkich niedokrwistości. Pod względem diagnostycznym należy do grupy niedokrwistości mikrocytowych, niedobarwliwych. Do diagnostyki różnicowej IDA i niedokrwistości chorób przewlekłych (ACD), której towarzyszy funkcjonalny niedobór żelaza, wykorzystywane są zarówno rutynowe parametry morfologii krwi, jak i parametry biochemiczne.

**Cel pracy.** Celem pracy była ocena użyteczności nowych parametrów krwi obwodowej, w tym retikulocytarnych, w diagnostyce niedoboru żelaza i niedokrwistości syderopenicznej, z uwzględnieniem wpływu komponenty zapalnej.

**Conflict of interest**
**Konflikt interesów**

None
Brak konfliktu interesów

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**Materiał i metody.** Materiał stanowiło 275 wyników morfologii krwi obwodowej z retikulocytami uzyskanych z analizatora Sysmex XN 2000. Próbki pochodziły od pacjentów Samodzielnego Publicznego Centralnego Szpitala Klinicznego w Warszawie. Materiał badawczy podzielono na dwie grupy: grupa I – 275 próbek z oznaczonym stężeniem CRP, traktowanym jako kryterium rozpoznania stanu zapalnego, a wśród nich próbki z CRP $< 5$ mg/l i z CRP $\geq 5$ mg/l, grupa II – 67 próbek z bezwzględnym niedoborem żelaza, bez wpływu komponenty zapalnej (saturacja transferyny $< 16\%$ i stężenie CRP $< 5$ mg/l). Wszystkie oznaczenia wykonano w materiale pochodzącym z jednoczasowego pobrania.

**Wyniki.** Największą wartość AUC w diagnostyce bezwzględnego niedoboru żelaza uzyskano odpowiednio dla: UIBC – 0,939, S-RET-He – 0,926, Fe – 0,915, S-%MicroR – 0,898 i S-%HYPO-He – 0,896, podczas gdy wartości pola pod krzywą dla „rutynowych" parametrów morfologii, na podstawie których diagnozuje i różnicuje się IDA, wynoszą: S-HGB – 0,721 i S-MCV – 0,872. Z wyliczonych wartości AUC wynika, że zarówno S-%MicroR, jak i S-%HYPO-He mają większą moc diagnostyczną niż np. powszechnie stosowany parametr do różnicowania niedokrwistości syderopenicznej, jakim jest MCV. Ponadto, wykazano ujemną, istotną statystycznie korelację pomiędzy stężeniem CRP i S-RET-He, S-%HYPO-He, S-Delta-He zarówno w grupie pacjentów ze stanem zapalnym, jak i bez stanu zapalnego.

**Wnioski.** W pracy wykazano, że nowe parametry morfologii krwi obwodowej, w tym retikulocytarne, są użyteczne w diagnostyce niedoboru żelaza. Nie umożliwiają jednak różnicowania niedoboru bezwzględnego i funkcjonalnego, gdyż wykazują zależność od stanu ostrej fazy.

## INTRODUCTION

Reticulocytes are precursors of red blood cells produced during erythropoiesis in the bone marrow. A multistage process of differentiation takes place, originate with the stem cells which transforms into proerythroblast, then into basophilic erythroblast, followed by polychromatophilic erythroblast and acid-absorbing erythroblast (orthochromatic). When the last one extrudes the cell nucleus, it becomes a reticulocyte which further matures in the bone marrow for two days. After release into the peripheral blood, it becomes a mature erythrocyte within approximately 24 hours. The number of reticulocytes in whole blood reflects the erythropoietic efficiency of the bone marrow and the level of eytropoiesis intensity (1). Figures 1-5 present an image of erythropoiesis cells. The erythroblasts were stained with the May-Grunwald-Giemsa method and the reticulocytes were stained intravitally with methylene blue. The photos were obtained from Central Laboratory of Independent Public Central Clinical Hospital in Warsaw (SP CSK) archive. Currently the vast majority of medical laboratories have an analyzer equipped with an automatic reticulocyte determination module. However, before these methods became so widely available, reticulocytes were counted manually. In the whole blood sample stained intravitally with alkaline dyes (e.g. methylene blue, gentian violet, brilliant blue FCF), the number of reticulocytes was determined for at least 1000 erythrocytes. The result was given in percent or per mil in relation to the number of erythrocytes. When compared to manual methods the automatic are have a smaller error, with better repeatability and take less time since the counting is made in about 1 minute. Additionally automatisation of the counting process allowed for obtaining additional parameters that characterize the reticulocytes population (2). The so called reticulocyte parameters obtained by automated methods include: the number reticulocytes relative to the erythrocyte count (Ret%), the absolute reticulocyte count (Ret #), fraction of unmatured reticulocytes – which is the sum of HFR and MFR (IRF), reticulocyte fraction based on low (LFR), mean(MFR) and high fluorescence (HFR), mean reticulocyte volume (MRV) and the reticulocyte hemoglobin equivalent (RET-He).

The factors which may interfere with automatic methods of measuring both the reticulocytes count and parameters include: Howell-Jolly bodies, intracellular parasites, basophilic stippling of erythrocytes and dyes used in angiography. The main indications for the determination of reticulocyte parameters are: differential diagnosis of anemia, monitoring of iron, vitamin $B_{12}$, folic acid and erythropoietin therapies or monitoring of the patient's condition after bone marrow or stem cell transplantation. The number of reticulocytes is presented in both relative and absolute values. The greater clinical significance is attributed to absolute values since they directly reflect the erythropoiesis activity. A particularly important parameter when monitoring the renewal of bone marrow function after transplantation is the IRF, since it reflects the changes in the red cell rejuvenation system most rapidly. The IRF increases

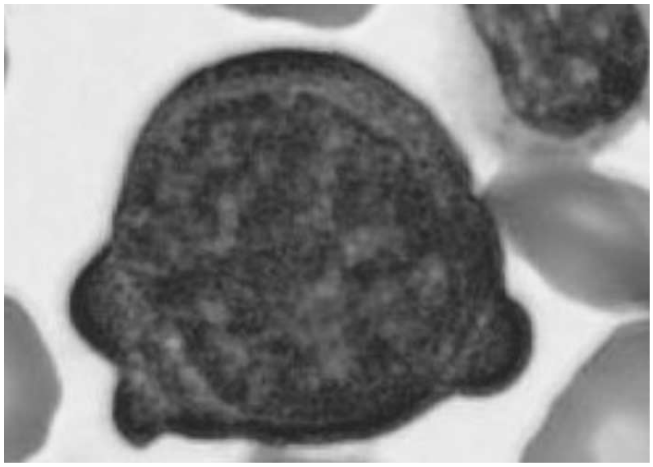

**Fig. 1.** Proerythroblast

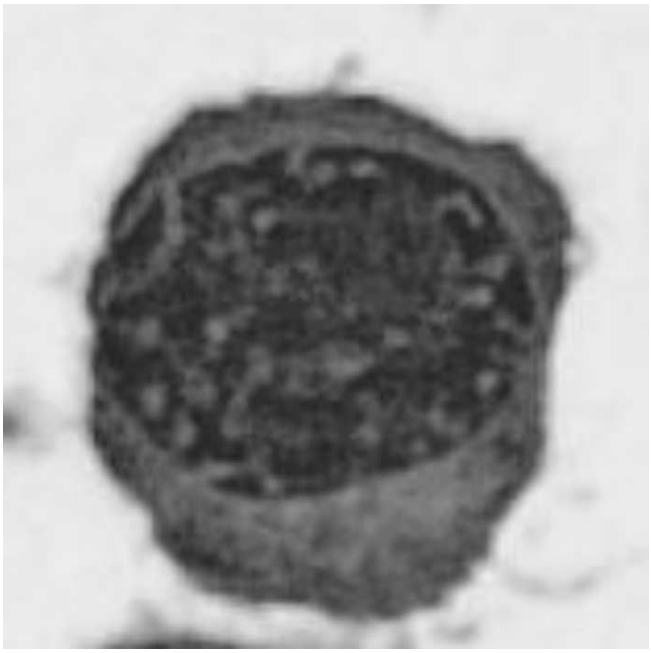

**Fig. 2.** Basophilic erythroblast

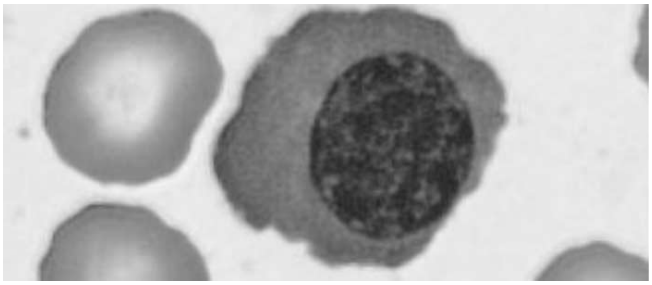

**Fig. 3.** Polychromatophilic erythroblast

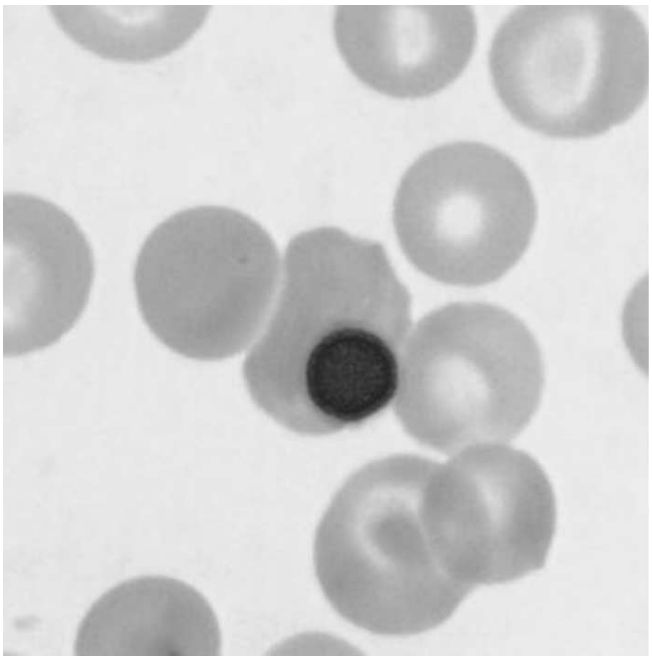

**Fig. 4.** Eosinophilic erythroblast

just after a few hours from bone marrow or stem cell transplantation, while the total number of reticulocytes increases only after 2-3 days. LFR, MFR and HFR are distinct reticulocyte fractions divided by the degree of

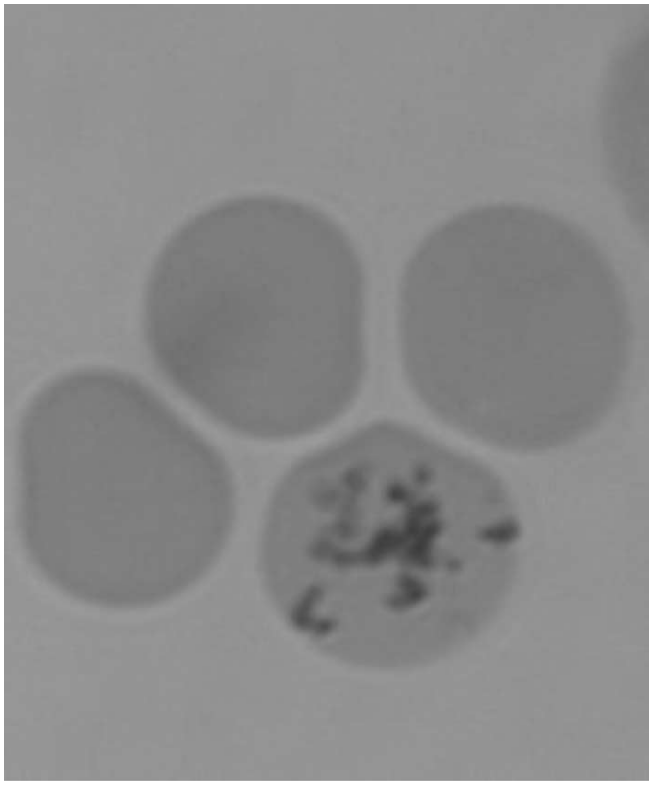

**Fig. 5.** Reticulocyte

their maturity. They are used to monitor both erythropoietin treatment (in dialysis patients) and anemia therapy. Each manufacturer of blood analyzers establishes its own "gating" method for the reticulocytic (scategram) which is then used to divide reticulocytes into individual populations.

The reticulocyte hemoglobin equivalent (RET-He) reflects current iron resources available for erythropoiesis. Therefore when there is a gradual decrease in body stores of iron, RET-He shows changes much earlier than the classical parameters e.g. concentration of hemoglobin. It is used mostly to monitor chronically dialyzed patients and when treating IDA sideropenic anemia.

Parameters included into the "research" group are not yet considered and approved for routine measurements. The assessment of their usefulness requires further research which will confirm their role, both diagnostic and clinical. The following should be mentioned: hemoglobin delta (S-Delta-He), percentage of microcytes (S-% MicroR) and percentage of hypochromic erythrocytes (S-% HYPO-He). S-Delta-He is the difference between the hemoglobin concentration in reticulocyte (S-RET-He), and the hemoglobin concentration in mature red blood cells (S-RBC-He). Delta-He reflects the level of erythropoiesis intensity dependent on the systemic availability of iron. If the value of this parameter increases, the "efficiency" of erythropoiesis increases. When the parameter decreases in value or drops below zero (negative delta-He) it may mean that there is a gradual reduction of iron availability (both in absolute and functional deficiency e.g. in the course of

inflammation and chronic diseases) and the verge of developing anemia. S-Delta-He is described not only as a marker of iron availability for erythropoiesis, but also a potential marker of inflammation (3). According to the manufacturers of blood analyzers, the S-% MicroR is intended to narrow the potential causes of anemia during differential diagnosis (2). S-% MicroR is the percentage of microcytes, or erythrocytes with a volume less than 80 fl (4). S-% HYPO-He is a calculated parameter and it is the percentage of hypochromic red blood cells, defined as the ones with a hemoglobin content below 17 pg (4). It reflects the systemic availability of iron for erythropoiesis and is a reliable marker of functional and absolute iron deficiency (5).

The biochemical parameters determined in the serum and used to assess the iron metabolism are: iron concentration (Fe), transferrin concentration (Transf), transferrin saturation (TfS), ferritin (Ferryt), total iron-binding capacity (TIBC) and unsaturated iron-binding capacity (UIBC) and soluble transferrin receptor (sTfR) (tab. 1).

**Tab. 1.** Reference ranges of biochemical parameters of iron metabolism applicable for Laboratorium Centralne SP CSK in Warsaw

| Parameter | Women | Men | Unit |
|---|---|---|---|
| Fe | 37-158 | | μg/dl |
| Transf | 250-320 | | mg/dl |
| TfS | 15-50 | | % |
| Ferryt | 13-150 | 30-400 | ng/ml |
| TIBC | 149-504 | | μg/dl |
| sTfR | 1.79-4.63 | | mg/l |

Iron is a micronutrient crucial for erythropoiesis. The systemic reserves contain about 4 g of which about 2.5-3 g are stored in erythrocytes and their precursors. The recommended daily intake is estimated for 1-2 mg daily. Fe can be absorbed mainly in the duodenum in both heme (meat containing hemoglobin and myoglobin) and the ferrous ions form (hydroxides, citrates, iron oxalates). Fe in the heme form is absorbed independently of the gastric juice pH, whereas non-heme Fe requires a low pH to be fully absorbed.

Therefore, during Fe supplementation, it is recommended to eat foods rich in vitamin C and to reduce antacids. The malabsorption of Fe may be caused by the damaged stomach mucosa, ulcers or shortened intestinal transit. The iron is imported form the apical membrane by the divalent metal transporter DMT1 (in order for the non-heme iron to be absorbed, it must first be reduced by the ferrireductase, the enzyme which is present in the duodenum), then passes through the cell membrane to the basal membrane and from there to the plasma (Fe is exported from enterocytes and macrophages by ferroportin-1). In the plasma the Fe is bound with transferrin which is a transport protein. The highest amount of Fe is stored in macrophages of the liver and the regulation of its release is regulated by hepcidin. On the surface of hepatocytes the transferrin receptors are located, which allows for rapid Fe release when it's needed urgently. The main Fe storage protein in the body is ferritin, however some part of Fe is stored in hemosiderin (Fe accumulated in hemosiderin is hardly available for erythropoiesis). Everyday with exfoliated intestinal, bladder and epidermis cells about 1-2 mg Fe is lost with additional amount being lost with menstrual bleeding. So far, the mechanism of active removal of Fe from the body has not been examined.

The vast the majority of the iron being built in the heme comes from "recycling", meaning when the erythrocyte is destroyed the iron which was accumulated in it is phagocytized, to be later incorporated into the new red blood cell (6). The process is regulated by macrophages of the retinal endothelial system (RES) (1, 7). Iron measured in the blood serum is a subject to large circadian fluctuations. According to the guidelines, the sample should be collected from the patient in the morning on an empty stomach. The result of Fe measurements are significantly influenced by inflammation, which results in redistribution of iron in the body and lowering of iron concentration in serum (1). Contrary to that after eating a meat meal, while taking dietary supplements and in hemolyzed samples the results are elevated. Ferritin and transferrin are proteins produced in the liver. Ferritin is localized mainly intracellularly, in body fluids and in low concentrations in the blood. It is an iron storage protein, which is able to store up to 4500 iron atoms (usually 2000) and it is a positive protein of acute phase. The decrease in its concentration indicates the systemic iron deficiency. Values < 12 μg/l with 50% sensitivity and 90% specificity indicate an absolute Fe deficiency. However elevated concentration does not necessarily mean iron overloading, since it is a positive acute phase protein and during inflammation the concentrations increases (8). The concentration of ferritin also increases in hyperthyroidism, in the elderly patients, in liver diseases, alcoholism and during taking oral contraception. The false decrease of ferritin level is characteristic for vitamin C supplementation, hypothyroidism and after physical exercise. The increase in serum ferritin may be caused by the damage of the liver, spleen and bone marrow cells. Transferrin is an iron transporting protein; it can bind up to two atoms of iron. It attaches to the surface of the cell where the transferrin receptor is localized and in the process of endocytosis goes inside the cell. The serum transferrin concentration does not show circadian fluctuations (9). Iron deficiency stimulates transferrin synthesis in order to bind as much Fe as possible from the limited resources. Transferrin is a negative acute phase protein, which means that during inflammation, independently of systemic Fe resources, its concentration decreases. Increased transferrin concentration occurs in pregnancy and during taking the oral contraceptives. Therefore a more useful parameter than the transferrin concentration itself is the transferrin saturation with iron (so-called

transferrin saturation). TfS is the ratio of Fe concentration and serum transferrin concentration. TfS values lower than 16% indicate an absolute or functional deficiency of Fe, therefore it is possible that this might be the effect of an inflammatory component, e.g. during ACD. TfS < 10% strongly indicates IDA. Values > 45% are characteristic for iron overload conditions (congenital and secondary hemochromatosis) (1).

TIBC is the total iron binding capacity, which reflects the total amount of Fe that can be bound to transferrin. The reduced TIBC value indirectly provides information about the transferrin concentration. The lower there is the concentration of Fe in the serum, the higher the TIBC value is. The unsaturated iron binding capacity (UIBC) is the difference between TIBC and iron concentration; it reflects the amount of iron needed to completely saturate transferrin. Both ferritin and transferrin are acute phase proteins. During the differential diagnosis of functional and absolute deficiency of Fe, the concentration of C-reactive protein (CRP) should be additionally measured. Its concentration increases during bacterial infections, inflammation, trauma, cancer or heart attack and in other conditions (8). The transferrin receptor (TfR) is a transmembrane glycoprotein which has a high affinity for iron-saturated transferrin. TfR receptors take part in the process of iron endocytosis into cells. TfR serum concentration depends on the need of aforementioned cells for Fe and erythropoietic activity of cells in the bone marrow, both of which importantly are independent of the acute phase. Immunochemical methods made it possible to measure the proteolytically cleaved fragment of the external receptor; the so-called soluble part of TfR soluble transferrin receptor – sTfR, which is an indicator of tissue iron deficiency. sTfR is elevated in iron deficiency (in the latent stage) and during increased erythropoiesis (e.g. acute hemorrhagic anemia). In the acute phase as a reliable iron deficiency parameter the quotient of the ratio of sTfR and the logarithm of transferrin concentration is used. Values > 1.5 indicate a shortage of iron, especially the values > 2 (1).

The golden standard for assessing iron resources is staining bone marrow with Prussian blue revealing the presence of iron and sideroblasts. However, as it is an invasive method it's rarely used now in the differential diagnosis of sideropenic anemia (1).

The World Health Organization (WHO) defines anemia as a reduction in hemoglobin(HGB) concentration below:

- 11 g/dl in children from 6 months to 6 years old,
- 12 g/dl in children from 6 to 14 years old,
- 13.5 g/dl in adult men,
- 11.5 g/dl in adult women,
- 11.0 g/dl in pregnant women.

Sideropenic anemia (iron deficiency anemia) accounts for 80% of all anemia and belongs to the group of microcytic, non-pigmented anaemias (9). Iron deficiency leads to impaired erythropoiesis, which then results in a decrease in hemoglobin. Anemia can be either caused by the iron deficiency (IDA) or by a restriction of its availability for erythropoiesis: functional iron deficiency (FID) e.g. during acute phase (10).

Diagnosing the iron deficiency anemia is performed with the laboratory criteria: a greater decrease in hemoglobin concentration than the number of erythrocytes; microcytosis; hypochromia; anisocytosis; poikilocytosis (with the presence of pencil and elliptical erythrocytes) (fig. 6); RDW increase – indication of anisocytosis (one of the first symptoms); reticulocyte percentage below the reference range or within these values; reduction of Fe and ferritin, decrease in transferrin saturation; increase in transferrin concentration, increase in TIBC and UIBC.

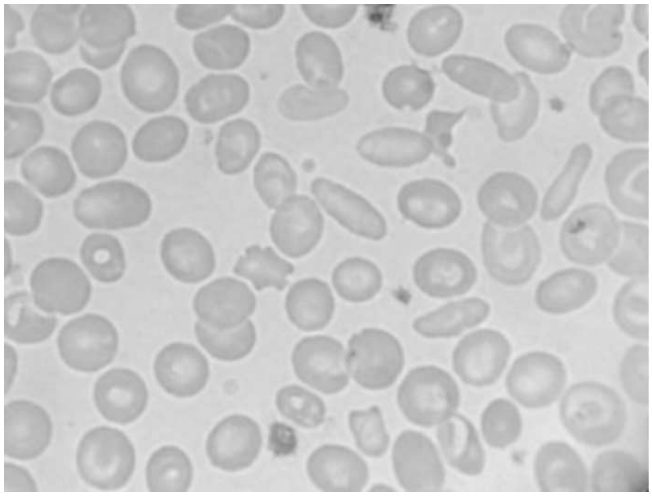

**Fig. 6.** Peripheral blood smear in IDA anemia (microcytosis, poikilocytosis, hypochromia). The photographs come from the Central Laboratory of SP CSK archive

There are three stages of iron deficiency (9): (1) iron depletion – Fe concentration in the reference range, correct blood count, decrease in ferritin – stage caused by depletion of iron stored in the system; (2) iron deficiency – decrease in serum Fe concentration, increase in transferrin concentration, UIBC, TIBC, at this stage there is insufficient amount of iron in relation to the needs of erythropoiesis; (3) iron deficiency anemia – decreased hemoglobin, hematocrit, MCV and MCH values, clinical symptoms, the further deterioration of previously described deviations.

IDA should be differentiated with other microcytic anaemias (e.g. thalassemia) and ACD. Table 2 shows the IDA, ACD and FID differentiation schemes.

**Tab. 2.** IDA, FID and ACD differentiation

| Parameter | IDA | FID | ACD |
|---|---|---|---|
| Fe | ↓ | N | ↓ |
| TIBC | ↑ | ↑ | ↓ |
| TfS | < 20%, peculiarly < 10% | < 30% | < 20% |
| Ferryt | < 100 ng/ml, peculiarly < 30 g/dl | 100 ng/ml | 100 ng/ml |
| HCT/HGB | ↓ | ↓ | ↓ |
| CRP | – | – | ↑ |

Anemia of chronic disease (ACD) is the most common anemia type among hospitalized patients (11), occurring simultaneously with other diseases e.g. other chronic infections, inflammations, tumors or connective tissue diseases in which the increased secretion of proinflammatory cytokines (Il-6, INF-γ, INF-β,) which leads to the shortening of the survival time of erythrocytes (< 10%); insufficient Fe release from RES; decreased production of erythropoietin and deterioration of bone marrow response to the release of EPO which directly inhibit the erythropoiesis (8).

In ACD, normochronic normocytes usually are present in the peripheral blood smear, however sometimes hypochromic microcytes may also appear, similarly to iron deficiency anemia (12). The ACD characteristic findings are: occurrence of underlying disease, markers of inflammation (↑ ESR, ↑ CRP), N/↑ Ferrite, ↓ Fe, N sTfR, N Transf, TfS > 16%, also slight hypochromia is possible in the microscope image with a normal bone marrow image.

## AIM

The aim of the study was to the assessment of the utility of novel peripheral blood morphology parameters, including reticulocytic, in the diagnosis of iron deficiency and sideropenic anemia.

## MATERIAL AND METHODS

The material included 275 results of peripheral blood counts with reticulocytes obtained from the Sysmex XN 2000 analyzer from patients of the Independent Public Central Clinical Hospital in Warsaw. The material was divided into 2 groups:

1. Group I: 275 samples with measured CRP concentration, established as a criterion for diagnosis of inflammation, among them samples were divided for those with CRP < 5 mg/l (N = 121) and those with CRP ≥ 5 mg/l (5.2-295.4 mg/l) (N = 154). In both groups correlations between the established parameters of sideropenic and iron deficiency anemia were calculated, including: hemoglobin concentration, MCV, iron concentration, TIBC, UIBC, transferrin saturation, transferrin concentration, ferritin and novel peripheral blood parameters that may be markers of iron deficiency: RET-He, S-Delta-He, P-MRV, S-% HYPO-He, S-% MicroR.
2. Group II: 67 samples with absolute iron deficiency, without the influence of inflammatory (transferrin saturation < 16% and CRP concentration < 5 mg/l), in which ROC (Receiver Operating Characteristic) curves were determined, areas under the curve were calculated (AUC) and the proposed cut-off points in the diagnosis of iron deficiency regarding: HGB, MCV, Delta-He, hemoglobin content in reticulocyte (RET-He), S-% HYPO-He, S-% MicroR and iron, transferrin and ferritin concentrations were presented.

All of the measurements were made on the material from the simultaneous collection. Peripheral blood morphology and reticytocyte parameters were measured in whole blood collected on K3EDTA, reticulocyte parameters by fluorescence flow cytometry. Biochemical parameters were measured from the venous blood with the use of the Cobas 6000 analyzer from Roche. Statistica 12 (StatSoft) was used for statistical analysis. The means, standard deviations, Spearman rank correlation coefficients were calculated and the ROC curve was determined and the area under the AUC curve was calculated. The P value < 0.05 was considered statistically significant.

## RESULTS

The values of correlation coefficients (r) for selected red cell parameters, reticulocytic parameters determined on the Sysmex XN 20000 analyzer and biochemical parameters of the iron metabolism evaluation are presented in table 3.

ROC curves graphs assessing the diagnostic power of red blood cell parameters, reticulocytes and biochemical parameters of iron metabolism in the diagnosis of iron deficiency in group II are presented in figure 7 and table 4.

The lowest values of the area under the curve were obtained for S-Delta-He (AUC = 0.685, Po = 1.4 pg) and S-HGB (AUC = 0.721, Po = 9.1 g/dl). The area under the curve values were calculated with the proposed cut-off points for the following parameters: Transf (AUC = 0.799, Po = 246 mg/dl), TIBC (AUC = 0.831, Po = 255 μg/dl), S-MCV (AUC = 0.872, Po = 84.9 fl), Ferrite (AUC = 0.895, Po = 45 ng/ml), S-% HYPO-He (AUC = 0.896, Po = 2.5%), S-% MicroR (AUC = 0.898, Po = 4.1%). The highest area under the curve values were obtained for UIBC (AUC = 0.939, Po = 212 μg), S-RET-He (AUC = 0.926, Po = 27.9 pg) and Fe (AUC = 0.915, Po = 39 μg/dl).

## DISCUSSION

According to the WHO data, anemia affects 2 billion people worldwide (13). The gigantic scale of the problem motivates manufacturers of hematological analyzers to expand the blood count panel with new parameters, improving both the diagnosis process and monitoring of the anemia treatment. The clinical usefulness of some of them e.g. the content of hemoglobin in reticulocyte, has been repeatedly confirmed; other parameters from the "research" group, must be subjected to a wider comparative analysis (4, 11).

The aim of this study was the assessment of the diagnostic capacity of novel parameters of peripheral blood morphology and reticulocyte compared to established iron deficiency markers. The highest AUC value in the diagnosis of absolute iron deficiency was obtained for: UIBC (0.939), S-RET-He (0.926), Fe (0.915), S-% MicroR (0.898) and S-% HYPO-He (0.896). While the area under the curve values for "routine" morphology parameters, which are the basis for the IDA is diagnose and differentiation, are: S-HGB (0.721) and S-MCV (0.872). The calculated AUC values show that both S-% MicroR

**Tab. 3.** Values of correlation coefficients (r) for selected red cell parameters, reticulocytic parameters determined on the Sysmex XN 20000 analyzer and biochemical evaluation parameters of the iron metabolism

| Parameter | All | | CRP < 5 | | CRP ≥ 5 | |
|---|---|---|---|---|---|---|
| | r | N | r | N | r | N |
| S-RET-He and S-% MicroR | -0.6036* | 270 | -0.6555* | 119 | -0.5602* | 151 |
| S-RET-He and S-% HYPO-He | -0.6333* | 269 | -0.7036* | 118 | -0.6055* | 151 |
| S-RET-He and Fe | 0.6116* | 109 | 0.7289* | 49 | 0.5043* | 60 |
| S-RET-He and TIBC | -0.4800* | 89 | -0.7395* | 42 | -0.1066 | 47 |
| S-RET-He and UIBC | -0.3243* | 54 | -0.7355* | 23 | -0.1076 | 31 |
| S-RET-He and TfS | 0.7125* | 88 | 0.7440* | 42 | 0.6458* | 46 |
| S-RET-He and Transf | -0.4595* | 52 | -0.6449* | 28 | -0.0784 | 24 |
| S-RET-He and Ferryt | 0.0960 | 106 | 0.5753* | 52 | 0.0021 | 54 |
| S-RET-He and CRP | -0.1382* | 270 | 0.0797 | 119 | -0.2037* | 151 |
| S-% MicroR and S-% HYPO-He | 0.9146* | 269 | 0.9362* | 118 | 0.8576* | 151 |
| S-% MicroR and Fe | -0.3131* | 110 | -0.4404* | 49 | -0.2944* | 61 |
| S-% MicroR and TIBC | 0.5761* | 89 | 0.6991* | 42 | 0.1913 | 47 |
| S-% MicroR and TfS | -0.4497* | 88 | -0.4770* | 42 | -0.4092* | 46 |
| S-% MicroR and Transf | 0.5395* | 52 | 0.6300* | 28 | 0.0966 | 24 |
| S-% MicroR and Ferryt | -0.1200 | 106 | -0.3950* | 52 | -0.0401 | 54 |
| S-Delta-He and CRP | -0.2472* | 269 | -0.0883 | 118 | -0.2424* | 151 |
| S-% HYPO-He and Fe | -0.3350* | 108 | -0.4878* | 48 | -0.3511* | 60 |
| S-% HYPO-He and TIBC | 0.6054* | 88 | 0.7162* | 41 | 0.2368 | 47 |
| S-% HYPO-He and UIBC | 0.3620* | 54 | 0.4796* | 23 | 0.1244 | 31 |
| S-% HYPO-He and TfS | -0.4759* | 87 | -0.5191* | 41 | -0.5107* | 46 |
| S-% HYPO-He and Transf | 0.5176* | 51 | 0.6161* | 27 | 0.0588 | 24 |
| S-% HYPO-He and Ferryt | -0.1050 | 105 | -0.3993* | 51 | 0.0227 | 54 |
| S-% HYPO-He and CRP | -0.0038 | 269 | -0.0927 | 118 | 0.1765* | 151 |

r – value of the correlation coefficient; N – sample size; S-RET-He – hemoglobin equivalent in reticulocyte; S-% MicroR – microcytes percentage; S-Delta-He – hemoglobin delta; S-% HYPO-He – hypochromic erythrocytes percentage; Fe – iron concentration; TIBC – total iron binding capacity; UIBC – unsaturated iron binding capacity; TfS – transferrin saturation with iron; Transf – transferrin concentration; ferrite – ferritin concentration; CRP – C-reactive protein concentration
*P < 0.05 – statistically significant

**Tab. 4.** The results of ROC curves analysis in the group II

| Parameter | AUC | Proposed cut-off points | P |
|---|---|---|---|
| S-HGB | 0.721 | 9.1 | < 0.0001 |
| S-Delta-He | 0.685 | 1.4 | 0.0002 |
| S-RET-He | 0.926 | 27.9 | < 0.0001 |
| S-% HYPO-He | 0.896 | 2.5 | < 0.0001 |
| S-% MicroR | 0.898 | 4.1 | < 0.0001 |
| Fe | 0.915 | 39 | < 0.0001 |
| TIBC | 0.831 | 255 | < 0.0001 |
| UIBC | 0.939 | 212 | < 0.0001 |
| Transf | 0.799 | 246 | < 0.0001 |
| Ferryt | 0.895 | 45 | < 0.0001 |
| S-MCV | 0.872 | 84.90 | < 0.0001 |

and S-% HYPO-He have more diagnostic power than e.g. MCV which is the commonly used parameter for differentiation of sideropenic anemia.

The hemoglobin delta (S-Delta-He) is the difference between hemoglobin concentration in reticulocyte and erythrocyte. In the literature, it is indicated as a potential marker of systemic iron availability for erythropoiesis. It is also suggested that it is a marker of inflammation (3). The acute phase state causes the redistribution of plasma iron, which limits its accessibility to erythropoiesis and results in a decrease of reticulocytes hemoglobin concentration. The effect of the aforementioned relationships is also a decrease of S-Delta-He value, which during inflammation often goes into negative values. In a studies conducted by the team of Danielson from Karolinska Institute in Sweden, they showed a negative correlation between Delta-He and inflammatory markers. In the study, the correlation coefficient between Il-6 and Delta-He was r = -0.45 (p < 0.001), and between Delta-He and CRPr = -0.36 (P < 0.001) (3). In our study the negative and statistically significant S-Delta-He correlation with CRP was observed for both all samples and CRP ≥ 5 mg/l samples. The subject of many analyzes is the evaluation of the usefulness of the "research" group parameters which is, among others, the percentage of hypochromic erythrocytes – S-% HYPO-He (1, 8,

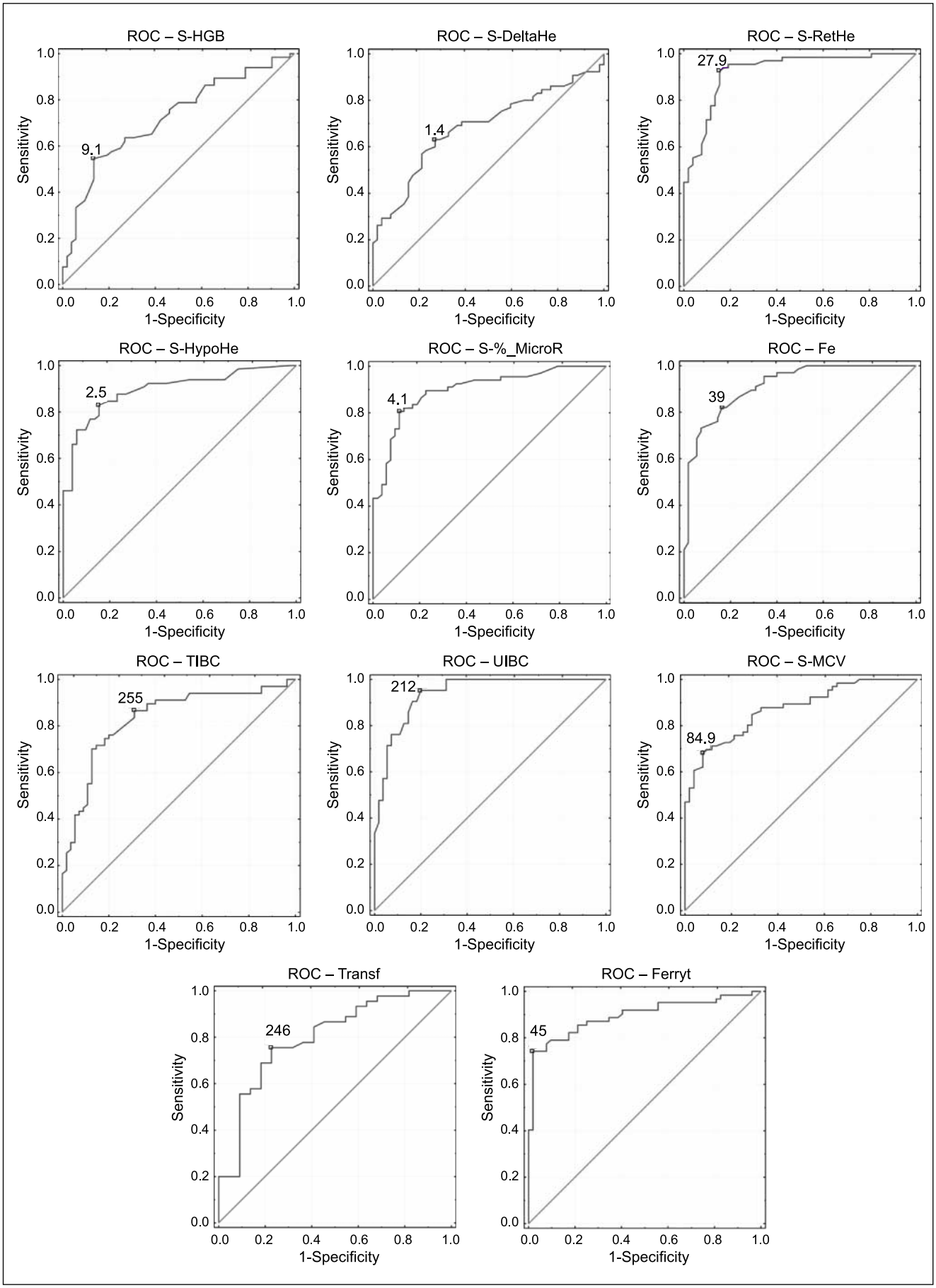


**Fig. 7.** ROC curves graphs of red blood cell parameters, reticulocytic parameters and biochemical evaluation parameters of iron metabolism in samples with transferrin saturation < 16% and CRP < 5 mg/l

11-16). A brazilian team led by Torino from Universidade Estadual de Campinas found this parameter to be the most reliable in the diagnosis of absolute iron deficiency in patients with ACD with the AUC = 0.785 in the 95% confidence interval (0.661-0.909) and the cut-off point to be 1.8% (11). In our study, the following values were obtained for samples with absolute iron deficiency: AUC = 0.896 and a proposed cut-off point was 2.5%. Therefore, the percentage of S-HypoHe > 2.5% is a positive predictor of the actual iron deficiency. On the other hand, a study by Bovy from CHU Sart-Tilman in Belgium found the percentage of hemoglobin deficient red blood cells to be the best predictor of functional iron deficiency in hemodialyzed patients, with a cutoff point > 6% (17). At the same time, the group paid special attention to the necessity of clinical evaluation of the discussed parameter in the phase of induction of treatment with human recombinant erythropoietin, since there might be a potential independence of % HYPO-He from erythropoietic activity (18).

The higher utility of % HYPO-He, in comparison to the biochemical parameters of the iron metabolism evaluation, as a marker of iron deficiency in the hemodialyzed patients was demonstrated by Butarello from the University of Padova, while establishing a threshold value of 2.7%. In discussed the studies, it was proved that the stability of the sample in the case of% Hypo-He measurements, was is 8 hours from the material collection (13).

According to the Spanish team of Urrechaga S-% HYPO-He and S-RET-He provide information on individual cell characteristics, therefore the quantitative measurement of hypochromic erythrocytes measures the possible improvement in erythropoiesis and the status of iron metabolism (5). The ROC analysis for the diagnosis of the actual iron deficiency (the criterion for inclusion to the iron deficiency group was sTfR above 21 nmol) was: S-RET-He AUC = 0.935 with a proposed cut-off of 29.8 pg and for S-% HYPO-He AUC = 0.925 and proposed cut-off point was the value of 3.5%. In our study, the inclusion criterion for real iron deficiency group was transferrin saturation below 16% and CRP concentration < 5 mg/L. The AUC value for S-RET-He was 0.926 with the cut-off point of 27.9 pg and for the S-% HYPO-He AUC was 0.896 with the cut-off point of 2.5%.

Rehu et al. (19) from the University of Eastern Finland emphasized the usefulness of both erythrocyte iron deficiency percentage and hemoglobin equivalent in reticulocyte for the diagnosis of IDA and ACD. The areas under the ROC curve obtained by them were: IDA vs. control – 0.99; ACD vs. control – 0.85 and IDA vs. ACD – 0.88. Therefore, they found that the % HYPO-He value is higher and the RET-He value is lower in patients with IDA and ACD compared to the control. However, at the same time, the authors indicated that the described direction of changes is not only present in absolute iron deficiency, but may also appear when there is a limited accessibility of iron to erythropoiesis e.g. inflammation as previously described. Muusze et al. (20) in an article from 2008 stated that RET-He is practically independent from inflammation. In our own study we found a negative, statistically significant correlation between S-RET-He and CRP and S-% HYPO-He and CRP which was demonstrated both for all patients and for a separate group of patients with known inflammation (CRP ≥ 5). Therefore, it has been proved that both S-RET-He and S-% HYPO-He are parameters dependent on inflammation. Joosteni et al. Stated that RET-He does not provide any better information compared to classical parameters of the MCH and MCHC, based on which we could distinguish patients with IDA and ACD (21). Urrechaga et al. (22) in a paper from 2009 claims that RET-He is a proper marker of functional iron deficiency and reveals reduced iron availability during treatment with erythropoietin in the group of chronic kidney disease patients. RET-He is also assigned a role in the early detection of abnormal hemoglobinization of erythrocytes in honorary blood donors (23), in patients after orthopedic surgery (24) and in oncological cancer centers (25).

Technological progress allows for the new parameters to be introduced, most of which currently still belong to the “research” group. Their great advantage is the high diagnostic power in the diagnosis of iron deficiency and IDA anemia, and that the additional measurements do not involve collecting another blood sample from the patient and do not generate high costs. However, the some influence of the state of the acute phase on their level was shown, therefore none of them clearly differentiates the absolute and functional deficiency of iron.

## CONCLUSIONS

The study showed that novel parameters of peripheral blood morphology, including reticulocytic ones, are useful in the diagnosis of iron deficiency. However, they do not allow for differentiation between absolute and functional deficiencies, because of a high influence of the state of acute phase.

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