Comparison of erythrocyte and reticulocyte indices for the diagnosis of iron deficiency

Primerjava eritrocitnih in retikulocitnih parametrov pri ugotavljanju pomanjkanja železa

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Abstract

Background: The increased prevalence of iron deficiency anemia represents a global public health issue which can be reduced by early diagnosis of pre-iron deficient states. Clinical utility of biochemical indices for iron deficiency diagnosis is limited due to the influence of inflammation. Erythrocyte and reticulocyte indices, reticulocyte hemoglobin content, and percentage of hypochromic red cells have been extensively used in diagnosing different iron-deficient conditions. The aim of our study was to compare the utility of erythrocyte and reticulocyte indices provided by different hematology analyzers for the diagnosis of iron deficiency.

Methods: 186 people, 25 patients with iron deficiency anemia, 103 patients with chronic kidney disease, and 58 healthy donors were included in our study. Their whole blood samples were analyzed using two different automated analyzers, XN-1000, Sysmex and Advia 120, Siemens Bayer Diagnostics, to compare reticulocyte and erythrocyte indices.

Results: Linear correlation between indices for hemoglobin reticulocyte content (Ret-He and CHr) has been confirmed while correlation between both erythrocyte indices (Hypo-He and %HYPO) can be described by 2nd degree polynomial. Cut-off value for the diagnosis of iron deficiency for Ret-He was established at < 28.2 pg and for Hypo-He at > 1.6 % and showed high diagnostic sensitivity (Ret-He = 76 %; Hypo-He = 72 %) and specificity (100 %).

Conclusions: Reticulocyte indices Ret-He and CHr are directly comparable and can be used for latent iron deficiency evaluation. Although erythrocyte indices Hypo-He and %HYPO are not exchangeable, both can be used for long-term iron deficiency evaluation. Additionally, we determined reference intervals for Hypo-He and Ret-He.

Izvleček

Izhodišča: Naraščajoča pojavnost anemije zaradi pomanjkanja železa je globalni javnozdravstveni problem, ki ga lahko zmanjšamo z zgodnjim odkrivanjem pomanjkanja železa. Uporaba biokemijskih parametrov za oceno pomanjkanja železa se omejuje na stanja, ko vnetje ni prisotno. Eritrocitni in retikulocitnih parametri, količina hemoglobina v retikulocitih ter delež hipokromnih eritrocitov izkazujejo široko uporabnost v različnih bolezenskih stanjih. Namen naše raziskave je primerjati uporabnost eritrocitnih in retikulocitnih parametrov dveh različnih hematoloških analizatorjev pri ugotavljanju pomanjkanja železa.

Metode: V raziskavo smo vključili skupno 186 oseb, med katerimi je bilo 25 bolnikov z anemijo zaradi pomanjkanja železa, 103 bolniki s kronično ledvično boleznijo ter 58 zdravih oseb. Njihove vzorce polne krvi smo analizirali na dveh hematoloških analizatorjih, XN-1000, Sysmex in Advia 120, Siemens Bayer Diagnostics, da bi primerjali vrednosti eritrocitnih in retikulocitnih parametrov.

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Rezultati: Potrdili smo dobro linearno ujemanje parametrov za oceno količine hemoglobina v retikulocitih (Ret-He in CHr) in povezavo, ki ustreza polinomu drugega reda parametrov za oceno deleža hipokromnih eritrocitov (Hypo-He in %HYPO). Določili smo mejne vrednosti za oceno pomanjkanja železa, Ret-He < 28,2 pg in Hypo-He > 1,6 %, z visoko diagnostično občutljivostjo (Ret-He = 76 %; Hypo-He = 72 %) in specifičnostjo (100 %). Značilne razlike med povprečnimi vrednostmi eritrocitnih in retikulocitnih parametrov potrjujejo njihovo uporabnost pri diagnosticiranju anemije zaradi pomanjkanja železa in anemije zaradi kronične ledvične bolezni.

Zaključki: Retikulocitna parametra Ret-He in CHr sta povsem primerljiva in lahko enakovredno služita za oceno latentnega pomanjkanja železa. Zaradi drugačnega načina določanja eritrocitna parametra Hypo-He in %HYPO nista primerljiva neposredno, čeprav lahko oba z ustreznim preračunom primerljivo služita za oceno dolgotrajnejšega pomanjkanja železa. Določili smo tudi okvirni referenčni interval za Hypo-He in Ret-He.

Introduction

Iron deficiency anemia (IDA) is the top-ranking cause of anemia worldwide. IDA develops as a primary disease or accompanies other diseases. Secondary causes of IDA refer to chronic inflammation, common in chronic inflammatory bowel disease, malignancies and chronic kidney disease (CKD) (1-4). Chronic inflammation increases hepcidin production, which prevents iron mobilization from iron stores in the presence of increased demands, and leads to functional iron deficiency. Anemia of chronic kidney disease (ACKD) is a special type of anemia of chronic disease, caused by decreased erythropoietin production. Patients with ACKD should receive iron supplementation and erythropoiesis-stimulating agents for managing the physiological iron level and erythrocyte production. The insufficient iron therapy increases the risk for the development of functional iron deficiency that eventually leads to IDA, which may coexist with ACKD. To achieve the optimal therapy for iron deficiency in patients with CKD, effective differential diagnosis of functional iron deficiency, IDA and ACKD is required (5).

Laboratory diagnosis of iron deficiency is currently based on the measurements of transferrin saturation percentage (TSAT%), and serum concentrations of ferritin (S_{FER}) and iron (S_{Fe}), and application of erythrocyte and reticulocyte indices. In the most challenging cases, the assessment of iron stores in the bone marrow is required for final confirmation of iron store depletion and iron deficiency (6). The guidelines for the assessment of iron deficiency in the presence of inflammation recommend the application of erythrocyte and reticulocyte indices and determination of C-reactive protein (CRP) level (4,7,8). Conventional erythrocyte indices (MCV, MCH, MCHC) determine the cell size and cellular amount of hemoglobin, and help distinguish between hypochromic microcytic IDA and normochromic macrocytic ACKD. Differential diagnosis based on the conventional erythrocyte indices is limited and helpful only in combination with other indices for iron deficiency determination due to the high impact of pre-analytical factors in their determination. Nowadays, the application of alternative erythrocyte and reticulocyte indices is required in the differential diagnosis of anemia (9-12), their application, however, depends on currently available automated flow cytometric systems, provided by different manufactures, which have patented the nomenclature and the method of determining

the indices (7,13). Reticulocyte hemoglobin content (CHr) and the percentage of hypochromic erythrocytes with hemoglobin concentration < 280 g/L (%HYPO) provided by Siemens analyzers were the first alternative indices used in the diagnosis of anemia. The determination of CHr reflects the status of iron incorporation into hemoglobin in reticulocytes, and provides the information about iron availability in the bone marrow for erythropoiesis within the last two days. Decreased CHr levels indicate imbalanced iron availability and erythrocyte production considerably before the reduced hemoglobin concentration, therefore CHr could be used as an early marker of iron deficiency. A late marker of iron deficiency is %HYPO, which reflects iron deficiency during the last 3 months. In 1997, CHr was first included in the clinical practice for iron deficiency determination in the USA. The same year, the European guidelines for managing ACKD also recommended the application of CHr for iron deficiency determination in patients with CKD. Updates in 2004 added %HYPO to the European guidelines for managing ACKD (4,7,13). The Ret-He parameter is a calculated value of reticulocyte hemoglobin equivalent, provided by Sysmex analyzers. These systems also enable determination of percentage of hypochromic erythrocytes with hemoglobin content < 17 pg (Hypo-He).

In 2005, the American Food and Drug Administration (FDA) confirmed the clinical utility of Ret-He for managing iron deficiency due to several reports, which confirmed the linear correlation between CHr and Ret-He (13-19). Erythrocyte indices, Hypo-He and %HYPO, differ in the method of determination and are not directly comparable, however, recalculated values of Hypo-He showed a linear correlation with %HYPO and could be used for easier comparison between the indices (20).

Despite various reports confirming equal clinical utility of Ret-He and CHr, and %HYPO and Hypo-He, the clinical guidelines for the diagnosis and management of ACKD currently do not include Ret-He and Hypo-He determination. Slovenian clinical laboratories at different healthcare levels use different automated flow cytometric systems, which provide different reticulocyte and erythrocyte indices. Our aim was to determine the clinical utility of Ret-He and Hypo-He indices in the diagnosis of iron deficiency in patients with IDA and ACKD, and compare their values with CHr and %HYPO indices. Our next aim was to verify the reference values for Ret-He and Hypo-He due to different reference values for these indices reported in the literature.

Patients and methods

The study population consisted of 186 subjects; their whole blood samples were analyzed within 15 minutes of collection on hematology analyzer XN-1000, Sysmex and Adiva 120, Siemens Bayer Diagnostcis. We were focused on the evaluation of hemoglobin (Hb) and CRP levels, and the values of Ret-He, Hypo-He, CHr and %HYPO. We divided the subjects into three groups according to the values of blood count analysis. In the reference group of healthy individuals (N = 58) all the values of blood count analysis were within the reference intervals. In the IDA group (N = 25), the patients with confirmed IDA by biochemical indices (TSAT%, S_{FER} and S_{Fe}) were included on the basis of Hb values (women: Hb 120 g/L, men: Hb < 130 g/L). The presence of inflammation was excluded by measuring CRP (CRP < 5 mg/L), and so were other accompanying diseases. In the

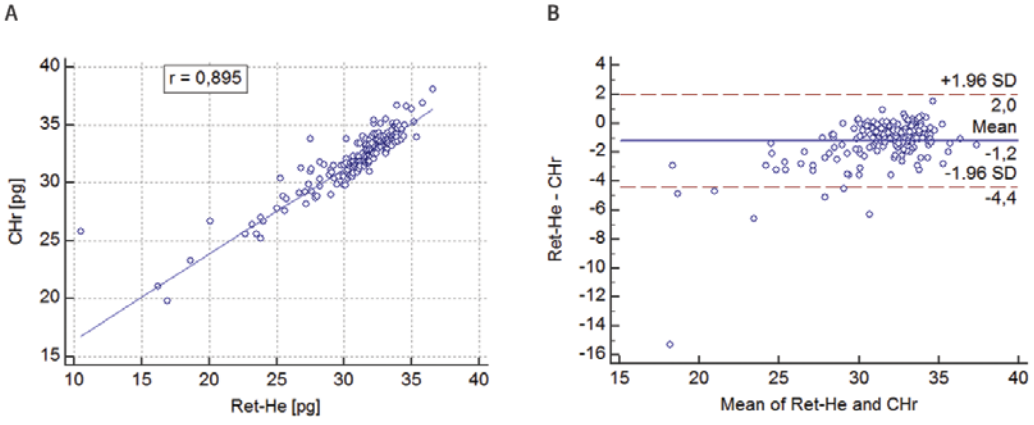

Figure 1: A) Correlation between reticulocyte indices Ret-He and CHr (N=186). B) Bland-Altman plot of Ret-He and CHr measurements.

CKD group, the patients with confirmed CKD on dialysis were included (N = 103) and divided into subgroups according to biochemical indices (TSAT%, S_{FER} and S_{Fe}). In 15 of the 103 CKD patients anemia was not found present (Hb > 120 g/L); the remaining 88 were divided into ACKD 1 (N = 50), if inflammation was not present (CRP < 5 mg/L), ACKD 2 (N = 7), if IDA was simultaneously present (CRP < 5 mg/L, TSAT% < 20 %, S_{FER} < 200 µg/L), and ACKD 3 (N = 31), if inflammation was present (CRP > 5 mg/L). All CKD patients had stage 5 CKD, except 6 who had stage 4 CKD (2 in group ACKD 1, and 4 in group ACKD 3).

All collected data were statistically analyzed with MedCalc 14.8.1. software (MedCalc Software, Ostend, Belgium).

Results

We evaluated the correlation between reticulocyte indices (Ret-He and CHr), which reflect the hemoglobin content in reticulocytes. The Kolmogorov-Smirnov test confirmed an asymmetric distribution of our data (P < 0.05). We found a linear correlation between Ret-He and CHr indices (r = 0.895). The median value of Ret-He was slightly lower (31.7 pg, 95 % CI = 31.3–31.9 pg) compared to CHr (32.3 pg; 95 % CI = 31.9–32.8 pg). The

Table 1: Median values of erythrocyte and reticulocyte indices in patients with SDA, AKLB and in healthy individuals.

Parameter	Healthy individuals (N = 58)		SDA (N = 25)		AKLB (N = 88)		AKLB 1 (N = 50)		AKLB 3 (N = 31)	
	Med	CIM	Med	CIM	Med	CIM	Med	CIM	Med	CIM
Ret-He [pg]	31,9	31,5–32,1	25,6	23,5–28,2	32,1	31,6–32,5	32,7	32,2–33,1	30,2	28,1–31,3
CHr [pg]	32,1	31,6–32,5	27,8	26,4–29,6	33,4	31,7–34,1	33,7	33,1–33,9	31,7	30,9–33,1
Hypo-He [%]	0,2	0,1–0,2	10,5	1,7–17,4	0,4	0,2–0,9	0,3	0,3–0,4	0,8	0,4–1,7
%HYPO [%]	0,9	0,7–1,4	15,8	3,8–20,0	1,9	1,0–5,3	1,4	1,1–1,7	5,0	2,6–9,3

Med – median; CIM – confidence interval for a median

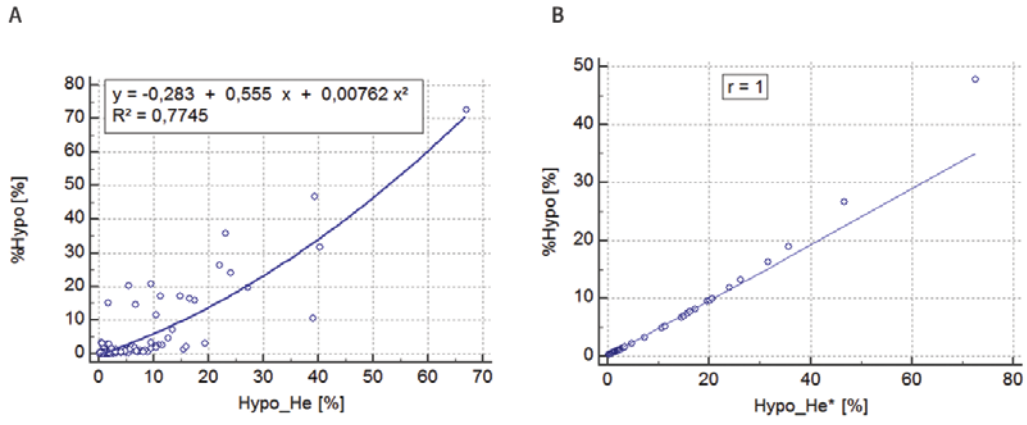

Figure 2: A) Second degree polynomial correlation between Hypo-He and %HYPO. B) Linear correlation between recalculated Hypo-He* and %HYPO.

Bland-Altman plot showed good agreement between the measurements of Ret-He and CHr within ± 2 SD differences, with few outliers at concentrations < 30 pg (except for one outlier that appeared at > 30 pg). The low mean difference between measurements (1.2 pg) also showed a good correlation between Ret-He and CHr. Although the Bland-Altman plot showed outliers at lower Ret-He concentrations, the Spearman's rank correlation coefficient confirmed an overall good agreement between Ret-He and CHr indices (r = 0.766, N = 45).

The comparison of Hypo-He and %HYPO indices reflecting the percentage of hypochromic erythrocytes confirmed a second degree polynomial correlation (Figure 2. A), which has been demonstrated by other authors (20). Using polynomial equation found in the literature (20) (Hypo-He* = 0.2016 + 0.3981 × %HYPO + 0.00356 × %HYPO^2), we recalculated Hypo-He into Hypo-He* and found a linear correlation between Hypo-He* and %HYPO. Linear correlation enables easier identification of agreement between these two indices (Figure 2.B).

Clinical utility of erythrocyte and reticulocyte indices was statistically evaluated by analysis of differences between the mean values of tested indices (Table 1). In the analysis we included the reference group of healthy individuals, tested groups of patients with IDA, patients with ACKD (Hb < 120 g/L), classified into subgroups ACKD 1 and ACKD 3 (Table 1). The ACKD 2 subgroup was excluded from the analysis due to low sample number. Nonparametric Mann-Whitney test of two independent variables with 95 % confidence interval was used to compare the mean values of Ret-He and

Table 2: Cut off values for iron deficiency identification in iron deficiency anemia.

Parameter	Cut-offvalue	Sensitivity/ Specificity [%]	Determined normal values	Reference values[21]
Ret-He	<28,2 [pg]	76/100	29,6–34,1 [pg]	32,1–38,8 [pg]
Hypo-He	>1,6 [%]	72/100	0,1–1,5 [%]	0,1–1,1 [%]
CHr	<29,7 [pg]	72/98		
%HYPO	>2,7 [%]	74/90		

Hypo-He within the tested groups. We found significantly ($P < 0.0001$) reduced values of hemoglobin content in reticulocytes (Ret-He and CHr), and significantly ($P < 0.0001$) increased values of the percentage of hypochromic erythrocytes (Hypo-He and %HYPO) in patients with IDA compared to healthy individuals. The comparison of mean values of hemoglobin content in reticulocytes did not show significant differences between the patients with ACKD and healthy individuals, whereas increasing percentages of hypochromic erythrocytes were found in the patients with ACKD, mostly in the subgroup ACKD 3.

Using ROC analysis, we determined the cut-off values for Ret-He, CHr, Hypo-He and %HYPO for IDA identification. The cut-off values were determined considering high diagnostic sensitivity and specificity (Table 2). According to the area under the curve values (AUC), we found a better predictive power of Ret-He (AUC = 0.893) and Hypo-He (AUC = 0.938) compared to CHr (AUC = 0.856) and %HYPO (AUC = 0.737) indices for iron deficiency identification in IDA. We also defined reference intervals of Ret-He and Hypo-He in healthy individuals, using the percentile method. The lower reference limit was placed at 2.5 percentile and the higher reference limit was placed at 97.5 percentile of the measured values with a 95 % confidence interval. The determined reference intervals were similar to those found in the literature (21).

Discussion

Different modern hematology analyzers enable determination of reticulocyte and erythrocyte indices. Their nomenclature, unlike other parameters in blood picture, depends on the type of analyzer, which introduces ambiguity in interpretation in clinical practice. Our aim was to demonstrate the correlation between reticulocyte and erythrocyte indices available on two different analyzers, and widely used in laboratory diagnosis at the UMC Ljubljana and in other Slovenian laboratories.

The clinical usefulness of measuring hemoglobin content in reticulocytes has been demonstrated in the identification of iron deficiency and IDA in adults and children, with the advantage of a noninvasive method of measuring current iron stores in bone marrow. Acute or chronic inflammation does not affect the values of reticulocyte and erythrocyte parameters, as is the case with conventional biochemical parameters, which provides an important advantage in the diagnosis of anemia of chronic disease and IDA. The guidelines for monitoring iron levels in patients with CKD recommend using CHr for the evaluation of iron availability. Incorrect dosage of epoetins and iron supplements in ACKD treatment leads to IDA and functional iron deficiency or adverse iron overload (3,13,22). We have confirmed a good correlation between the Ret-He and CHr indices (Figure 1), which has also been demonstrated in other studies showing a linear correlation and good agreement between reticulocyte indices for iron deficiency identification (15,17,18,23).

A lower number of patients with IDA may be attributed to the slightly weaker correlation between reticulocyte indices in the range of reduced Ret-He values (< 30 pg). Therefore, it would be relevant to restrict the comparison only to the subjects with very low Ret-He values. It should be noted that values of reticulocyte indices normalize rapidly after the beginning of the treatment, which is why we observed only a few patients with IDA among patients with CKD. The biggest differences between reticulocyte

indices were observed in extremely low levels (< 25 pg) without false normal values among the subjects.

Hypo-He and %HYPO erythrocyte indices reflect long-term iron deficiency, since the lifespan of erythrocytes in the blood is 120 days. In clinical practice, Hypo-He and %HYPO are used in the differential diagnosis of IDA, ACKD and anemia of chronic disease. Several studies have demonstrated their clinical usefulness in monitoring the treatment of dialysis patients. Current guidelines recommend the application of %HYPO with CHr/Ret-He for monitoring the treatment of ACKD (5,7,14,24,25). In line with previous findings, we have confirmed a second degree polynomial correlation between Hypo-He and %HYPO indices. Using the previously described mathematical equation of Hypo-He, we have demonstrated a linear correlation between Hypo-He* and %HYPO (20). A different method of determination of Hypo-He and %HYPO prevents their intersection, however, the recalculated values of Hypo-He* enable a direct comparison with a widely used %HYPO parameter (Figure 2).

We have evaluated the application of Ret-He and Hypo-He in healthy individuals, patients with IDA, and patients with CKD, in whom the iron level is routinely monitored using CHr and %HYPO. As expected, we have found significantly lower values of erythrocyte and reticulocyte indices in patients with IDA (Table 1). The patients with CKD showed well monitored iron supplementation, especially ACKD1 patients without inflammation, which reflected comparable values of erythrocyte and reticulocyte indices to those found in healthy individuals. Less favorable values of erythrocyte and reticulocyte indices were found in ACKD 3 patients with inflammation present. However, even in this group, we did not find a patient, in whom the tested parameters would exceed the reference values. The comparison of the erythrocyte indices (%HYPO and Hypo-He) has shown a significant difference in ACKD 3 patients with a median %HYPO value of 5 %. The cut off value of 5 % is also used for the identification of functional iron deficiency. In the diagnosis of anemia, different cut off values are used for iron deficiency identification: values > 5 % confirm the presence of functional iron deficiency, whereas the values > 10 % indicate an absolute iron deficiency, which requires immediate treatment (4). The median value of Hypo-He in ACKD3 patients was slightly lower (0.8 %) as expected due to the cut off value 2.7 %. Therefore, erythrocyte and reticulocyte indices are absolutely comparable and important in the diagnosis of anemia in clinical practice.

We have determined the cut off values for Ret-He (< 28.2 pg) and CHr (29.7 pg) to have high diagnostic sensitivity and specificity for iron deficiency identification in patients with IDA, wherein the Ret-He cut off value shows higher predictive values (Table 2). Similar findings have also been found for the cut off values of Hypo-He (> 1.6 %) and %HYPO (>2.7 %) indices. The determined reference values for Ret-He and Hypo-He parameters in healthy individuals are consistent with those in the literature, however, it is worth noting that in clinical practice cut-off values are preferentially used. We have confirmed that the cut off value of Ret-He at 28.2 pg indicates reduced iron availability, which is in line with previous findings (4). Our results have confirmed that the values of Ret-He and CHr < 28 pg reliably indicate iron deficiency. Reference values of Hypo-He, which reflects a functional iron deficiency, were slightly lower than the determined cut off value 1.6 %. Howev-

er, functional iron deficiency usually increases %HYPO > 5 % and corresponds to Hypo-He 2.3 % after polynomial transformation, which confirms effective iron deficiency diagnosis at the cut off point of 2.7 % for Hypo-He.

Conclusions

Since manufacturers have protected tags, reference intervals and the method of determination of erythrocyte and reticulocyte indices, it is important that there are no disturbances in the diagnosis of anemia and in the monitoring of patients treated with iron supplementations, chemotherapy and epoetins due to the replacement of laboratory equipment. Therefore, it is crucial that in clinical practice directly comparable parameters, such as CHr and Ret-He, as well as those that are not, Hypo-He and %HYPO, are correctly used. Our results have confirmed high diagnostic sensitivity and specificity of the erythrocyte and reticulocyte indices in iron deficiency identification. Many Slovenian laboratories use analyzers, which enable reticulocyte analysis complementing other hemogram parameters and biochemical indices in clinical practice, therefore, using reticulocyte analysis has an important clinical value in the diagnosis of anemia.

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