CME/SAM

The Role of Automated Measurement of RBC Subpopulations in Differential Diagnosis of Microcytic Anemia and β-Thalassemia Screening

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Upon completion of this activity you will be able to:
- list the analytical characteristics of thalassemia.
- discuss how the discriminant indices take advantage of the different values of individual parameters in thalassemia and iron deficiency.
- describe how to combine these parameters in formulas to improve recognition of each disease.
- describe how the quantification of RBC subsets might provide complementary information useful for the differential diagnosis of microcytic anemia and for screening for thalassemia.

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Questions appear on p 476. Exam is located at www.ascp.org/ajcpcme.

Abstract

Cell counter–based formulas have been used in the differential diagnosis of microcytic anemia. The measurement of RBC subpopulations is now available on the Sysmex XE 5000 analyzer (Sysmex, Kobe, Japan). We describe the new formulas: % microcytic – % hypochromic; and % microcytic – % hypochromic – red cell distribution width (RDW), derived from the percentages of microcytic and hypochromic RBCs. The present study aimed to prospectively evaluate the reliability of these new formulas in the differential diagnosis of microcytosis and β-thalassemia screening compared with already published indices. The indices were calculated for a set of 250 iron-deficient patients and 270 β-thalassemia carriers. Independent samples t *test and receiver-operating characteristics analysis were applied.*

The % microcytic – % hypochromic – RDW, % microcytic – % hypochromic, and Green and King indices provided higher areas under the curve. The % microcytic – % hypochromic – RDW was the most reliable index evaluated, with 100% sensitivity and 92.6% specificity. This index can be used to efficiently screen patients with microcytosis for further hematologic studies to confirm β-thalassemia.

Microcytic anemia is frequently due to iron deficiency anemia (IDA) or thalassemia. The differentiation between thalassemic and nonthalassemic microcytosis has important clinical implications.[1] The World Health Organization has recently highlighted its growing concern about anemia, affecting an estimated 2 billion people, 50% of the cases caused by iron deficiency.[2]

Iron deficiency is one of the leading risk factors for disability and death worldwide, with a high prevalence in the developing world, and has substantial health and economic costs, including poor pregnancy outcome,[3,4] impaired school performance,[5] and decreased productivity. Anemia in elderly persons may derive from a variety of potential contributing causes: nutritional, chronic inflammation, and renal insufficiency.[6]

Microcytic anemia in the case of thalassemia results from impaired globin chain synthesis and decreased hemoglobin (Hb) synthesis.[7] Thalassemia syndromes are among the most common genetic disorders worldwide, with 1.7% of the world's population carrying thalassemic genes.[8] Thalassemia is prevalent in some parts of the world (Mediterranean regions, up to 8%; countries of the Middle East, up to 10%; India, 3%-15%; Southeast Asia, up to 9%), where it represents a major public health problem. However, nonendemic countries such as in Northern Europe and North America are also involved in thalassemia-related problems as a result of demographic changes caused by migration of ethnic minority groups with a high frequency of thalassemic mutations.[9,10]

The diagnosis of β-thalassemia involves measuring the HbA_2 concentration of lysed RBCs via high-performance liquid chromatography, with an ion-exchange column or

electrophoresis methods.[11,12] This measurement is considered the "gold standard" and is useful for assessing the sensitivity and specificity of other testing methods.

On the basis of classic hematologic parameters, subjects with IDA are inappropriately discriminated from subjects with anemia owing to thalassemia or chronic disease.[13] As a state of iron deficiency proceeds, the mean cell volume (MCV), mean cell hemoglobin (MCH), and RBC count tend to decline, but results in both microcytic anemias overlap.

A number of formulas and indices have been proposed to differentiate IDA from heterozygous β-thalassemia using formulas that incorporate at least 2 of the RBC parameters provided by the modern automated hematologic analyzers (MCV, MCH, RBC count, red cell distribution width [RDW]) and Hb in various combinations.[14-18] These indices, defined to quickly discriminate IDA and β-thalassemia, can be effective for use as preliminary screening tools to allow "reflex" HbA_2 analysis when a proper cutoff is chosen.

Automated blood cell counters have changed substantially during the last 20 years. Hematology analyzers, based on principles of flow cytometry, can provide information about individual cell characteristics, identifying small subpopulations of RBCs within the total RBC population. The quantification of the percentages of microcytic and hypochromic RBCs has proved its clinical usefulness in the differential diagnosis of microcytic anemia,[19-22] but, to date, the measurement of the RBC subpopulations has been restricted to the analyzers of a single manufacturer, the ADVIA series (Siemens Medical Solutions Diagnostics, Tarrytown, NY).

The Sysmex XE 5000 analyzer (Sysmex, Kobe, Japan) is a fully automated hematology analyzer that provides CBC and WBC differential counts. The flow fluorescence cytometry technology incorporated enables independent measurement of the volume and Hb content of individual RBCs. Derived from this technology, 4 new RBC extended parameters are now available in this analyzer: (1) % Hypo-He, the percentage of hypochromic RBCs with an Hb content equivalent to less than 17 pg; (2) % Hyper-He, the percentage of hyperchromic RBCs with an Hb content equivalent to more than 49 pg; (3) % MicroR, the percentage of microcytic RBCs with a volume less than 60 fL; and (4) % MacroR, the percentage of macrocytic RBCs with a volume greater than 120 fL.

We propose different mathematical formulas, calculated from these new indices: % MicroR – % Hypo-He (M-H) and % MicroR – % Hypo-He – RDW (M-H-RDW).

This study was designed to prospectively evaluate the reliability of these new indices, which can be calculated from RBC extended parameters included on the Sysmex XE 5000 instrument, in the differential diagnosis of microcytic anemia and β-thalassemia screening and to compare their discriminant efficiency with the conventional published indices from Sirdah et al,[23] Ehsani et al,[24] England et al,[14] Green and King,[15] Mentzer,[16] Ricerca et al,[17] Shine and Lal,[7] and Srivastava and Bevington.[18]

Materials and Methods

Criteria for Selecting Groups of Patients

Only adults were included in the present study. None of them received a transfusion or had an acute bleeding episode in the previous month.

The samples were obtained in the course of routine analysis and collected in EDTA anticoagulant tubes (Vacutainer, Becton Dickinson, Rutherford, NJ). Tests were run in the Sysmex XE5000 analyzer within 6 hours of collection.

Reference Subjects

Samples were obtained from 45 male and 45 female healthy adult subjects with no clinical symptoms of disease. The results of blood cell counts and biochemical iron test results were within the reference ranges.

Subjects With Mild IDA

The group consisted of 250 patients with Hb levels less than 12.0 g/dL (120 g/L), MCV less than 80 μm^3 (80 fL), serum iron level less than 42 μg/dL (7.5 μmol/L), transferrin saturation less than 20%, and serum ferritin level less than 50 ng/mL (112 pmol/L). Patients with Hb levels less than 9.0 g/dL (90 g/L) were excluded because these cases of severe anemia are not confused with β-thalassemia carriers in daily practice.

β-Thalassemia Carriers

Samples were extracted from 270 patients with a previous diagnosis of the disease.

β-Thalassemia screening is routinely performed in our laboratory by means of the measure of RBC parameters. Samples with erythrocytosis (RBC count >5.5 × 10^6/μL [5.5 × 10^{12}/L]) and microcytosis (MCV <80 μm^3 [80 fL]) are selected for HbA_2 quantification (HPLC HA 8160, Menarini Diagnostics, Florence, Italy). An increased level of HbA_2 (>3.5%) is considered confirmatory for β-thalassemia trait. Molecular analysis is performed if genetic counseling is required. Molecular characterization of mutations is performed with allele-specific oligonucleotide polymerase chain reaction techniques.[25,26]

Biochemical and hematologic data for the groups are summarized in **Table 1**.

Data for a second group (validation group) were analyzed. The group included 297 patients with mild microcytic anemia: 154 with IDA, 82 with pure β-thalassemia, and 61 thalassemia carriers with other disease (43 with inflammation or infection and 18 with iron deficiency).

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Statistical Evaluation of Analytic Results

Discrimination indices used in the evaluation were calculated. **Table 2** summarizes these mathematical formulas.

The sensitivity, specificity, positive predictive value, and negative predictive value were calculated as follows:

$$\text{Sensitivity} = [\text{True-Positive}/(\text{True-Positive} + \text{False-Negative})] \times 100$$

$$\text{Specificity} = [\text{True-Negative}/(\text{True-Negative} + \text{False-Positive})] \times 100$$

$$\text{Youden Index} = (\text{Sensitivity} + \text{Specificity}) - 100$$

The statistical software package, SPSS version 17.0 for Windows (SPSS, Chicago, IL), was applied for statistical analysis of the results. An independent samples *t* test was performed to detect statistical deviations between the groups of patients. *P* values less than .05 were considered statistically significant. Receiver operating characteristic curve analysis was used to illustrate the diagnostic performance for thalassemia screening of the indices studied. Mathematical formulas and cutoffs were those defined by authors in original published reports.[7,14-18,23,24]

Results

An independent samples *t* test was applied, and, although the differences between the 2 groups of anemic patients were statistically significant ($P < .001$), the central 95th percentile ranges showed considerable overlap for RBCs, Hb, MCV, MCH, mean cell hemoglobin concentration, and RDW. In the β-thalassemia trait group, the RBC count was higher (5.79×10^6/μL [5.79×10^{12}/L]) than in healthy subjects (4.95×10^6/μL [4.95×10^{12}/L]; $P < .001$) and subjects with IDA (4.6×10^6/μL [4.6×10^{12}/L]; $P < .001$), while MCV and MCH values were lower ($P < .001$).

The RDW was increased in the 2 patient populations with respect to the healthy subjects, higher in IDA (18.0%) than in β-thalassemia (16.1%; $P < .001$). The % MicroR was much more increased in thalassemia (37.8%) than in IDA (19.1%; $P < .001$).

Table 3 shows the results of the receiver operating characteristic curve analysis of the indices studied for β-thalassemia screening.

In this study, the Shine and Lal[7] and Ricerca et al[17] indices provided the best sensitivity, 100%, but had low specificities. This means a high number of false-positive samples undergoing HbA_2 quantification. The Srivastava and Bevington index[18] had a good specificity, 91.3%, but the 70.8% sensitivity represents about 29% of β-thalassemia carriers being wrongly recognized. The same is true for the Green and King,[15] England et al,[14] Sirdah et al,[23] and Ehsani et al[24] indices, providing good specificities (99.1%, 98.4%, 97.9%, and 89.9%, respectively) but lower sensitivities (91.0%, 78.6%, 81.3%, and 87.2%, respectively). The Mentzer,[16] M-H, and M-H-RDW indices showed better sensitivities (94.3%, 97.4%, and 98.1%, respectively) than specificities (84.2%, 96.0%, and 97.1% respectively).

The M-H-RDW and M-H indices together with the Green and King index[15] showed good diagnostic efficiency, with the

Table 1
Hematologic, Biochemical, and Morphologic Data for a Healthy Group, β-Thalassemia Carriers, and Patients With IDA*

	Healthy (n = 90)	Thalassemia (n = 270)	IDA (n = 250)
RBCs ($\times 10^{12}$/L)	4.95 (0.37)	5.79 (0.52)	4.6 (0.4)
Hb (g/L)	151 (9)	120 (11)	105 (10)
MCV (fL)	90.9 (2.9)	64.9 (3.6)	72.7 (4.5)
MCH (pg)	30.5 (0.9)	20.8 (1.2)	22.3 (1.8)
MCHC (g/L)	335 (9)	320 (6.7)	303 (10)
RDW (%)	13.1 (0.6)	16.1 (1.0)	18.0 (2.9)
% MicroR	1.1 (0.44)	37.8 (11.4)	19.1 (9.7)
% Hypo-He	0.3 (0.16)	11.9 (7.2)	15.7 (11.5)
M-H	0.8 (0.4)	25.9 (7.6)	3.4 (5.6)
M-H-RDW	–12.3 (0.7)	9.7 (7.3)	–15.2 (6.5)
Iron (μmol/L)	17.1 (2.3)	17.2 (6.1)	4.8 (2.8)
Transferrin (μmol/L)	3.02 (0.37)	2.95 (0.4)	3.89 (0.82)
Ferritin (ng/mL)	103 (54)	139 (94)	15 (18)
Transferrin saturation (%)	28 (5.9)	29 (9.9)	6.5 (4.3)
Elliptocytes, % PF	0	2.9 (8)	18.0 (45)
Target cells, % PF	0	98.8 (267)	62.8 (157)
Basophilic stippling, % PF	0	11.1 (30)	0

Hb, hemoglobin; % Hypo-He, percentage of hypochromic RBCs; IDA, iron deficiency anemia; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; M-H, % MicroR – % Hypo-He; M-H-RDW, % MicroR – % Hypo-He – RDW; % MicroR, percentage of microcytic RBCs; RDW, RBC distribution width.

* Data are given as mean (SD) except for the peripheral blood morphologic data (elliptocytes, target cells, and basophilic stippling), which are expressed as the percentage of patients with positive findings (% PF). Selected laboratory values are given in Système International units; conversions to conventional units are as follows: RBCs ($\times 10^6$/μL), divide by 1; Hb (g/dL), divide by 10; MCV (μm^3), divide by 1; MCHC (g/dL), divide by 10; iron (μg/dL), divide by 0.179; and transferrin (mg/dL), divide by .0.0123. Ferritin values are given in conventional units; to convert to SI units (pmol/L), multiply by 2.247.

Table 2
Indices Evaluated[7,14-18,23,24]*

Indices	Iron Deficiency Anemia	Thalassemia
E = MCV – (10 * RBC)	>15	<15
E&F = MCV – RBC – 5 * Hb – 3.4	>0	<0
G&K = MCV^2 * RDW/100 * Hb	>65	<65
M = MCV/RBC	>13	<13
R = RDW/RBC	>4.4	<4.4
S&L = MCV^2 * MCH * 0.01	>1,530	<1,530
Si = MCV – RBC – 3 * Hb	>27	<27
S = MCH/RBC	>3.8	<3.8
M-H	<11.5	>11.5
M-H-RDW	< –5.1	> –5.1

E, Ehsani et al; E&F, England et al; G&K, Green and King; Hb, hemoglobin; % Hypo-He, percentage of hypochromic RBCs; M, Mentzer; MCH, mean cell hemoglobin; MCV, mean cell volume; M-H, % MicroR – % Hypo-He; M-H-RDW, % MicroR – % Hypo-He – RDW; % MicroR, percentage of microcytic RBCs; R, Ricerca et al; RDW, RBC distribution width; S, Srivastava and Bevington; Si, Sirdah et al; S&L, Shine and Lal.

* Mathematical formulas and cutoffs are depicted as defined in the original published reports. Asterisks indicate multiplication.

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Table 3
Receiver Operating Characteristic Curve Analysis Results and Predictive Value of Evaluated Indices for the Differential Diagnosis of Microcytic Anemia and Thalassemia Screening*

Indices	Area Under the Curve	Cutoff	Sensitivity (%)	Specificity (%)	Youden Index (%)
Ehsani et al[24]	0.961	15	87.2	89.9	77.1
England et al[14]	0.975	0	78.6	98.4	77.0
Green and King[15]	0.991	65	91.0	99.1	90.1
Mentzer[16]	0.958	13	94.3	84.2	78.5
Ricerca et al[17]	0.976	4.4	100	13.7	13.7
Shine and Lal[7]	0.887	1,530	100	13.3	13.3
Sirdah et al[23]	0.978	27	81.3	97.9	79.2
Srivastava and Bevington[18]	0.924	3.8	70.8	91.3	62.1
M-H	0.994	11.5	97.4	96.0	93.4
		7.3	100	78.2	78.2
M-H-RDW	0.997	–5.1	98.1	97.1	95.2
		–7.6	100	92.6	92.6

% Hypo-He, percentage of hypochromic RBCs; M-H, % MicroR – % Hypo-He; M-H-RDW, % MicroR – % Hypo-He – RDW; % MicroR, percentage of microcytic RBCs.
* Cutoffs were those defined by the authors in the original published reports; for the M-H and M-H-RDW indices, both cutoffs (highest Youden index and sensitivity of 100%) are reported.

highest area under the curve of all indices evaluated at 0.997, 0.994, and 0.991, respectively.

The cutoff providing the best Youden index for M-H was 11.5, while a sensitivity of 100% was obtained with a cutoff 7.3 (specificity, 78.2%). The cutoff providing the best Youden index for M-H-RDW was –5.1, while a sensitivity of 100% was obtained with a cutoff of –7.6 (specificity, 92.6%).

The cutoffs that provided the best Youden index were used to confirm the diagnostic performance of the new indices in the validation group.

When the M-H was calculated, 144 cases of IDA (93.5%) were correctly classified, as were 82 (100%) of the pure β-thalassemia cases, 43 (100%) of thalassemia carriers with inflammation, and 15 (83%) of 18 thalassemia carriers with IDA, so 97.2% (139/143) of the β-thalassemia carriers were recognized. When the M-H-RDW was calculated, 96.0% of the IDA cases were correctly diagnosed, as were 82 (100%) of the pure β-thalassemia cases, 43 (100%) of thalassemia carriers with inflammation, and 14 (78%) of 18 thalassemia carriers with IDA; this means that 138 (96.5%) of 143 β-thalassemia carriers were correctly classified.

Discussion

Differentiation between thalassemic and nonthalassemic microcytosis has important clinical implications because each has an entirely different cause, pathogenesis, prognosis, and treatment. Thalassemia testing should be done on all patients with microcytic anemia who are not iron deficient and do not respond to iron therapy. HbA_2 must be quantified to confirm the presence of the disease.

Although thalassemia is endemic to the Mediterranean basin and countries of the Far East, owing to migration of populations in recent decades, there is virtually no country in the world in which thalassemia does not affect some percentage of the inhabitants. As in all chronic diseases, prevention is important in the overall management of the disease. The real danger of nondiagnosis or misdiagnosis of carriers of the thalassemia trait is the potential homozygous offspring. Appropriate screening, detection of patients, and counseling of couples at risk are the most important procedures for the reduction of morbidity and mortality.[27]

A discriminant formula or index based on RBC parameters, derived from automated blood cell analyzers, with a high level of specificity and sensitivity for detecting the thalassemia trait would be a useful tool in the investigation of microcytic anemia, a matter of great interest in geographic areas where nutritional deficiencies and thalassemia are present with a high prevalence.[28] The fact that 2 new indices have been recently published demonstrates the interest in the matter.[23,24]

The usefulness of the indices is to detect patients with a high probability to be β-thalassemia carriers, so the best index must have as high a sensitivity as possible to detect almost all β-thalassemia patients. On the other hand, specificity must be good enough to avoid the measurement of HbA_2 in a high number of samples that should not undergo further analysis (false-positives).

"Suspicious" samples can be selected for HbA_2 analysis to confirm the presumptive diagnosis of the disease. β-Thalassemia can be diagnosed with confidence when an increased HbA_2 level, erythrocytosis, microcytosis, and a normal serum ferritin level are present.

To be useful in selecting microcytic samples for β-thalassemia testing, the index must detect the maximum number of thalassemic patients (high sensitivity) while eliminating as many nonthalassemic as possible (high

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specificity). Table 3 summarizes the clinical usefulness of β-thalassemia screening of the discriminant formulas included in our evaluation.

The results obtained in this study agree with recent evaluations of the published discriminant formulas. The Green and King[15] results are the most reliable of them, with the highest area under the curve, 0.991, among the published indices.[29,30]

The reason for the discrepancies among the diverse studies could be the fact that, in the present study, only cases of mild anemia were selected, those that provide the greatest diagnostic dilemma, while in other studies, cases with a more severe anemic status and some thalassemia carriers with concomitant iron deficiency were included.

The erythrocyte indices MCV, MCH, and mean cell hemoglobin concentration, included in the published formulas, represent the mean values obtained for the total RBC population. In contrast, modern hematology analyzers, based on principles of flow cytometry, provide information about individual cell characteristics, detecting small changes in the number of RBCs with inadequate Hb content and/or volume.

Taking into account the potential usefulness of the parameters derived from this technology, we joined together the parameters % MicroR and % Hypo-He, and the formula % MicroR – % Hypo-He (M-H) was proposed. The results obtained suggest that it could be considered a useful tool in differential diagnosis of microcytosis,[31] and results have been confirmed in the present study.

The best sensitivity, 100%, is the goal for a test used for screening purposes. It is reached at a cutoff of 7.3, with a specificity of 78.2%; this means 21.8% of the cases are false-positives. In an attempt to improve the diagnostic performance of the M-H index for thalassemia screening, we calculated the % MicroR – % Hypo-He – RDW. The introduction of the RDW in the formula improves the specificity up to 92.6%.

The M-H-RDW index has proven to be the most reliable index evaluated. The optimal cutoff, –7.6, provides a sensitivity of 100%, which means 0% false-negatives; thus, all thalassemia carriers could be recognized. According to the results of this study, an M-H-RDW value higher than –7.6 is a highly suspicious feature of β-thalassemia trait, and the HbA_2 level must be quantified to confirm the presence of the disease. Specificity is also high, 92.6%; it is assumed that it would mean 7.4% false-positives, but these samples, with HbA_2 levels within the reference range, could be correctly diagnosed.

Conclusions

None of the formulas provides 100% sensitivity and 100% specificity for discrimination purposes, so further confirmatory testing must be performed before a case can be correctly diagnosed. From that point of view, we concluded that the new M-H-RDW, derived from RBC extended parameters provided by the Sysmex XE 5000 analyzer, could be used as a laboratory-based criterion for the selection of samples for thalassemia testing, with a high degree of accuracy. Findings from further studies should confirm our results on prospectively recruited populations of patients with microcytic anemia and assess the pattern of microcytic and hypochromic RBC measurements in microcytic anemia due to other causes.

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