Efficacy of Advanced Discriminating Algorithms for Screening on Iron-Deficiency Anemia and β-Thalassemia Trait

A Multicenter Evaluation

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Key Words: Microcytic anemia; Screening algorithm; Iron-deficiency anemia (IDA); β-thalassemia; %MicroR; %HypoHe

Abstract

For many years, application of RBC indices has been recommended for discriminating between subjects with iron deficiency from those with thalassemia. However, application of the algorithms resulted in only 30% to 40% of subjects being appropriately classified. The aim of the study was to establish the efficacy of algorithms for anemia screening including new hematologic parameters such as percentage of hypochromic and microcytic RBCs and hemoglobin content of reticocytes. Subjects with iron deficiency anemia (IDA) (n = 142) and subjects with β-thalassemia (n = 34) were enrolled in a European multicenter study. Apparently healthy subjects were used as a reference group (n = 309). Hemocytometric investigations were performed on a Sysmex XE5000 hematology analyzer. The algorithms for IDA discrimination yielded results for area under the curve, sensitivity, specificity, and positive and negative predictive values of 0.88, 79%, 97%, 74%, and 98%, respectively. The algorithms for β-thalassemia discrimination revealed similar results (0.86, 74%, 98%, 75%, and 99%, respectively). We conclude that the advanced algorithms, derived from extended RBC parameters provided by the Sysmex XE5000 analyzer, are useful as laboratory anemia screening devices.

The application of RBC indices has been recommended for discriminating between subjects with iron deficiency and subjects with thalassemia.1-4 However, application of the England and Fraser formula (mean corpuscular volume [MCV] – 5 × hemoglobin [Hb] – RBC) and Mentzer formula (MCV/RBC) resulted in only 30% to 40% of subjects being appropriately classified.

Additional application of protoporphyrin content in RBCs was recommended for classifying microcytic RBC disorders.4-11 Multivariant discriminant analysis of algorithms including MCV, mean corpuscular hemoglobin (MCH), RBC, and red cell distribution width (RDW) was useful for the differential diagnosis of α- or β-thalassemia and iron-deficiency, but resulted in an inconclusive diagnosis in several cases.4,12 Measurement of the percentage of hypochromic and microcytic RBCs (%HypoHe and %MicroR) has demonstrated usefulness for detecting small changes in the amount of RBCs with inadequate hemoglobinization.13-15

Iron deficient erythropoiesis was characterized by the production of RBCs with decreased hemoglobin content resulting in an increased result for %HypoHe.16,17 As severity of anemia progresses, results for %MicroR will increase. However, subjects with β-thalassemia showed erythrocytosis and a high score for microcytosis. RBCs in case of subjects with β-thalassemia have a decreased volume because of impaired hemoglobin synthesis.

We decided to study the efficacy of new hematologic parameters for RBCs, like %HypoHe and %MicroR, and parameters for hemoglobinization of reticulocytes (Ret-He and δ-He) to validate the application of discriminating algorithms for screening subjects for iron deficiency anemia (IDA) and β-thalassemia.
The study objectives included (1) establishing the sensitivity and specificity of new algorithms in a cohort of subjects with IDA, a group of subjects confirmed to have β-thalassemia, and a control group of healthy subjects, and (2) comparing the algorithms with current existing formulas for discrimination.

**Materials and Methods**

**Study Design**

Our European multicenter study included 3 subject groups, namely, subjects with IDA, subjects with β-thalassemia, and apparently healthy hospital employees.

**Hemocytometry**

Blood samples were collected in Vacutainer tubes with K$_2$EDTA as anticoagulant (Becton Dickinson, Plymouth, England) and analyzed within 4 hours after collection. For hemocytometric investigations, we used the Sysmex XE5000 automated hematology analyzer (Sysmex, Kobe, Japan). Samples were selected from the daily workload and analyzed with full hemocytometric parameter profile (CBC + differential count + nucleated RBCs + reticulocytes) to ensure that results of all parameters were available. Parameters of particular interest were hemoglobin, RBC, MCV, RDW–standard deviation (SD), RDW–coefficient of variation (CV), reticulocytes Ret–hemoglobin equivalent (He), and δ-He. In addition, %HypoHe and %MicroR were quantified, indicating the percentage of hypochromic RBCs with a hemoglobin content of less than 17 pg and the percentage of microcytic RBCs with a volume of less than 60 fl.

**ZPP/Hb Ratio**

Zinc protoporphyrin (ZPP/Hb ratio) was measured with front surface illumination fluorometry using a dedicated hematofluorometer (Aviv Biomedical, Lakewood, NJ). Hemoglobin Electrophoresis

Hemoglobin electrophoresis was used to diagnose subjects with β-thalassemia. Increased HbA$_2$ content (>3.2%) was considered to confirm β-thalassemia. In case of an abnormal hemoglobin variant and an increased fetal hemoglobin fraction in the electropherogram (≥1.5%), the sample was excluded from the study.

**Statistical Evaluation**

The statistical software package MedCalc, version V11.5.1 for Windows, was applied for statistical analysis of results (MedCalc, Mariakerke, Belgium). Receiver operating characteristic (ROC) curve analysis, including calculation of the area under the curve (AUC), was used to evaluate the diagnostic performance of the algorithms. Cut-off values were established based on the optimal combination of sensitivity and specificity. Sensitivity (sens%), specificity (spec%), positive predictive value (PPV), and negative predictive value (NPV) were calculated as follows:

- Sensitivity = \[\frac{\text{true positive}}{\text{true positive} + \text{false negative}}\] \times 100
- Specificity = \[\frac{\text{true negative}}{\text{true negative} + \text{false positive}}\] \times 100
- Positive predictive value = \[\frac{\text{true positive}}{\text{true positive} + \text{false positive}}\] \times 100
- Negative predictive value = \[\frac{\text{true negative}}{\text{true negative} + \text{false negative}}\] \times 100

Mathematical formulas and cut-offs were previously published elsewhere.**

**Results**

**Subjects With IDA**

One hundred forty-two subjects with IDA (6 male and 135 female) were enrolled in the study based on suspect results for MCV (n = 132 MCV ≤80 fl.; n = 9 MCV 80-86 fl.) and ZPP/Hb ratio greater than or equal to 100 µmol per mol of heme. All selected subjects demonstrated results for %MicroR of ≥3 or higher. This limit was used as the first precondition step for discrimination using microcytic erythropoiesis.

The subjects were subsequently divided into 3 groups with normal to slightly decreased MCV (<85 and ≥75 fl), moderately decreased MCV (<75 and ≥65 fl), and severely decreased MCV (<65 fl). These subgroups were used in the second precondition step.

In addition, for the subgroup with MCV (<85 and ≥75 fl), ROC curve analysis (AUC) for %MicroR demonstrated that the optimal cut-off for %MicroR was 5. As shown in Table 1, new algorithms, including conventional and advanced hematologic parameters, were created for these subgroups. Results of ROC curve analysis (AUC) were applied to establish optimal cut-offs for concerning parameters.

**Subjects With β-Thalassemia**

Thirty-four subjects with β-thalassemia (18 male and 16 female) were enrolled in the study. We used an increased HbA$_2$ content (>3.2%) to confirm β-thalassemia. Subjects with results for iron metabolism markers beyond the reference range were excluded. All subjects demonstrated %MicroR results of 3 or higher. This limit was used as the first precondition step for discrimination of microcytic erythropoiesis.

The subjects were subsequently divided into 3 subgroups as follows: (1) normal to slightly decreased MCV (<85 and ≥75 fl), RDW-SD values less than 44.7 fl, and RBC
values greater than or equal to \(3.50 \times 10^{12}/L\); (2) moderately decreased MCV (<75 and \(\geq 65\) fL) and RBC values greater than or equal to \(3.50 \times 10^{12}/L\); and (3) severely decreased MCV (<65 fL). These subgroups were used in the second precondition step (Table 1, numbers 4-6).

As shown in Table 1 (numbers 4-6) new algorithms, including conventional and advanced hematologic parameters, were created for these subgroups. Results of ROC curve analysis (AUC) were applied to establish optimal cut-offs for concerning parameters.

To clarify the decision-making process for screening for IDA and \(\beta\)-thalassemia, we used the preselection criteria and algorithms depicted in Figure 1. Our algorithms for IDA and \(\beta\)-thalassemia discrimination, including the cut-off levels, were compared with previously published discriminating algorithms listed in Table 2.

### Apparentely Healthy Subjects

Three hundred nine apparently healthy hospital employees (133 male and 176 female, 16-63 years of age) without any clinical symptoms of disease were used as a reference group. Of these subjects, 3 scored a positive result for one of the IDA algorithms. These subjects demonstrated ferritin results of less than 10 µg/L. All other subjects showed results

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**Table 1**

<table>
<thead>
<tr>
<th>No.</th>
<th>Precondition 1</th>
<th>Precondition 2</th>
<th>Algorithm</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>%MicroR (\geq 3)</td>
<td>MCV &lt;85 and (\geq 75) and %MicroR (\geq 5)</td>
<td>%MicroR/%HypoHe &lt;4 and ([MCV^2 \times RDW-CV/(Hb*100)] (\geq 75) and Ret &lt;0.08)</td>
<td>IDA</td>
</tr>
<tr>
<td>2</td>
<td>%MicroR (\geq 3)</td>
<td>MCV &lt;75 and (\geq 65)</td>
<td>%MicroR/%HypoHe &lt;3.4 and ([MCV^2 \times RDW-CV/(Hb*100)] (\geq 77) and Ret &lt;0.08)</td>
<td>IDA</td>
</tr>
<tr>
<td>3</td>
<td>%MicroR (\geq 3)</td>
<td>MCV &lt;65</td>
<td>%MicroR – %HypoHe – RDW-CV &lt; –5.2</td>
<td>IDA</td>
</tr>
<tr>
<td>4</td>
<td>%MicroR (\geq 3)</td>
<td>MCV &lt;85 and (\geq 75) and RDW-SD &lt;44.7 and RBC (\geq 3.50)</td>
<td>([MCV^2 \times RDW-CV/(Hb<em>100)] (\geq 75) and ([MCV-RBC –3.4 – (5</em>Hb)] &lt;4 and Ret &lt; 0.08) and (\delta)-He (\geq 0)</td>
<td>Thal</td>
</tr>
<tr>
<td>5</td>
<td>%MicroR (\geq 3)</td>
<td>MCV &lt;75 and (\geq 65) and RBC (\geq 3.50)</td>
<td>([MCV^2 \times RDW-CV/(Hb*100)] (\geq 77) and %MicroR/%HypoHe (\geq 2.0)</td>
<td>Thal</td>
</tr>
<tr>
<td>6</td>
<td>%MicroR (\geq 3)</td>
<td>MCV &lt;65</td>
<td>(%MicroR – %HypoHe – RDW-CV) (\geq –5.2)</td>
<td>Thal</td>
</tr>
</tbody>
</table>

CV, coefficient of variance; Hb, hemoglobin; IDA, iron-deficiency anemia; MCV, mean corpuscular volume; \%MicroR, percentage of microcytic RBCs; \%HypoHe, percentage of hypochromic RBCs; RDW, red cell distribution width; Ret, reticulocytes; SD, standard deviation.

* Parameters used in the algorithms are expressed in the following units: RBC (\(\times 10^{12}/L\)), Hb (g/dL), MCV (fL), RDW-SD (fL), RDW-CV (%), Ret (\(\times 10^{12}/L\)).
within the reference ranges for hemocytometric parameters, including %MicroR less than 3, and iron metabolism markers (data not shown).\textsuperscript{19} None of the subjects scored a positive result with the algorithms for $\beta$-thalassemia.

The new algorithms for IDA discrimination demonstrated results for AUC, sensitivity, specificity, and positive and negative predictive values of 0.88, 79\%, 97\%, 74\%, and 98\%, respectively. The algorithms for $\beta$-thalassemia discrimination showed comparable results of 0.86, 74\%, 98\%, 75\%, and 99\%, respectively \textit{Table 3}.

**Discussion**

Application of RBC indices has been recommended for discriminating between subjects with IDA and subjects with thalassemia.\textsuperscript{1-3} In this study, application of these formulas resulted in only 30\% to 40\% of subjects with $\beta$-thalassemia in a proper classification.

More recently, Urrechaga et al\textsuperscript{17,20,21} reported discriminating formulas with novel hematologic parameters such as %HypoHe and %MicroR combined with RDW-CV. Measurement of %HypoHe and %MicroR was demonstrated to be useful for detecting small changes in the amount of RBCs with inadequate hemoglobinization.

Because of the lifespan of circulating mature RBCs, %HypoHe yields information on iron status in the preceding 2 to 3 months; this parameter is shown to be a sensitive indicator for detecting functional iron deficiency.

In subjects with $\beta$-thaslesmia, %HypoHe results are below the reference range because of ineffective erythropoiesis resulting from reduced production of intact hemoglobin. %HypoHe results in subjects with $\beta$-thaslesmia are not different from those observed in subjects with IDA.

Results for %MicroR are increased in $\beta$-thaslesmia subjects compared with those with IDA.

A European multicenter evaluation was performed to establish advanced algorithms for anemia discrimination. The study revealed algorithms with advanced hematologic parameters, such as %HypoHe, %MicroR, Ret-He, and $\delta$-He, for discrimination of IDA and $\beta$-thaslesmia. The algorithms demonstrated excellent diagnostic efficacy, compared with conventional discriminating formulas. In our study, application of the Urrechaga formulas resulted in about 50\% of

**Table 3**

Efficacy of the Newly Developed Algorithms Compared With the Previously Published Conventional Formulas\textsuperscript{a}

<table>
<thead>
<tr>
<th>Reference</th>
<th>AUC</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>AUC</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>England and Fraser\textsuperscript{1}</td>
<td>0.696</td>
<td>97</td>
<td>45</td>
<td>10</td>
<td>90</td>
<td>0.918</td>
<td>51</td>
<td>96</td>
<td>36</td>
<td>98</td>
</tr>
<tr>
<td>Green and King\textsuperscript{2}</td>
<td>0.691</td>
<td>97</td>
<td>56</td>
<td>15</td>
<td>90</td>
<td>0.945</td>
<td>64</td>
<td>97</td>
<td>44</td>
<td>99</td>
</tr>
<tr>
<td>Mentzer\textsuperscript{3}</td>
<td>0.615</td>
<td>94</td>
<td>47</td>
<td>4</td>
<td>90</td>
<td>0.854</td>
<td>48</td>
<td>95</td>
<td>30</td>
<td>99</td>
</tr>
<tr>
<td>Urrechaga\textsuperscript{20}</td>
<td>0.701</td>
<td>76</td>
<td>83</td>
<td>53</td>
<td>93</td>
<td>0.806</td>
<td>84</td>
<td>68</td>
<td>10</td>
<td>99</td>
</tr>
<tr>
<td>Urrechaga et al\textsuperscript{21}</td>
<td>0.828</td>
<td>90</td>
<td>68</td>
<td>54</td>
<td>93</td>
<td>0.893</td>
<td>69</td>
<td>89</td>
<td>21</td>
<td>99</td>
</tr>
<tr>
<td>Algorithm 1-3 for discrimination of IDA</td>
<td>0.878</td>
<td>79</td>
<td>97</td>
<td>74</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Algorithm 4-6 for discrimination of $\beta$-thaslesmia</td>
<td>0.860</td>
<td>74</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75</td>
<td>99</td>
</tr>
</tbody>
</table>

AUC, area under the curve; IDA, iron-deficiency anemia; NPV, negative predictive value; PPV, positive predictive value.
subjects with IDA and only 10% to 20% of subjects with β-thalassemia being appropriately classified.17,20,21

The purpose of using formulas in anemia discrimination is to detect subjects who have a high probability of requiring appropriate follow-up to reduce unnecessary investigations and costs. In future, screening and reporting results of various parameters will be insufficient. Reduction of healthcare budgets and increasing numbers of parameters available in laboratory hematologic analyses make it necessary to provide support and interpretation for a correct clinical diagnosis. Algorithms for discrimination purposes must have high sensitivity scores to detect the maximum number of subjects of interest. On the other hand, adequate screening algorithms should be able to eliminate as many “other” subjects (high specificity) as possible to avoid further analysis (false positives). Using our recently developed algorithms for anemia screening, about 75% of subjects with IDA and subjects with β-thalassemia were classified properly. High sensitivity and specificity scores were demonstrated compared with conventional formulas.1-3 The high sensitivity and specificity scores of the algorithms demonstrate that the discriminating algorithms are appropriate devices for microcytic anemia screening.

None of the algorithms had 100% sensitivity and 100% specificity in discriminating between subjects with iron deficiency from subjects with β-thalassemia. Therefore, after screening with the 6 algorithms, confirmatory testing should be performed for proper diagnosis. The efficacy of the algorithms should be confirmed in a subsequent study with prospectively selected subjects with microcytic anemia because of other causes such as α-thalassemia or combinations of IDA with thalassemia.

We conclude that the advanced algorithms, derived from extended RBC parameters provided by the Sysmex XE5000 analyzer, are useful as laboratory devices for anemia screening.

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References