Verification of 20 Mathematical Formulas for Discriminating Between Iron Deficiency Anemia and Thalassemia Trait in Microcytic Anemia

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Laboratory Medicine 2020;51:628-634

DOI: 10.1093/labmed/lmaa030

ABSTRACT

Background: Currently, more than 45 mathematical formulas based on simple red blood cell (RBC) parameters have been proposed for differentiating between iron deficiency and thalassemia in microcytic anemia, of which 20 are relatively new and have not been thoroughly independently verified. The study goal was to verify these 20 new formulas and to identify which RBC parameters have a decisive impact on the performance of those formulas.

Methods: A database containing laboratory and diagnostic data from 2788 subject individuals with microcytic anemia was used for assessing performance by receiver operating characteristic (ROC) analysis.

Results: The new Index26 had excellent performance, equivalent to the Green and King, Jayabose, and Janel formulas previously identified in the literature. The discriminant power of nearly all newer formulas was lower in our study than that claimed by the original authors. We discovered that a well-performing formula requires mean cell volume (MCV), RBC distribution width (RDW), and RBC measurements, whereas hemoglobin measurements appeared not to be essential.

Conclusions: Only the new Index26 performed at a level comparable to the very strongest established formulas. All other new formulas had lower performance than was claimed in the original publications, underscoring that independent verification of new formulas is indispensable.

Keywords: hematology, clinical pathology, thalassemia, microcytic anemia, iron deficiency anemia, discriminant formula, diagnostic performance

Microcytic anemia continues to have a high prevalence worldwide, especially in resource-limited countries. iron deficiency anemia (IDA) and thalassemia trait are the 2 major causes of microcytic anemia.[1] Traditionally, thalassemia was endemic in the "thalassemia belt" that extends from the Mediterranean basin via the Middle East, the Arabian peninsula, and the Indian subcontinent to Southeast Asia. However, global migration has now spread the disease through all continents.[2] Whereas IDA is an acquired disorder that can be treated with iron therapy relatively easily, thalassemia is an inherited disease, wherein iron is not indicated. Therefore, it is essential to make a correct diagnosis. Also, because differential diagnostic possibilities are limited in low-resource areas where microcytic anemia is particularly ubiquitous, the need has arisen for simple, inexpensive, effective screening methods.

In the early 1970s, various hematologists[3–5] pioneered the development of mathematical formulas that could successfully differentiate between thalassemia and IDA, using only basic and readily available red blood cell (RBC) parameters. Since those days, approximately 1 new discriminant formula (DF) per year was invented; currently, more than 45 such simple DFs are available. Moreover, other diagnostic pathways based on more-advanced RBC parameters or methods have been described in the literature.[6] Still, even if their diagnostic performance is sometimes high, we will not consider those other pathways in this article because they

Abbreviations

IDA, iron deficiency anemia; RBC, red blood cell; DF, discriminant formula; AENOR, Asociación Española de Normalización y Certificación; ROC, receiver operating characteristic; AUC, area under the curve; HCT, hematocrit; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, RBC distribution width; MPV, mean platelet volume; … , nonapplicable; PLT, platelet

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require analyzers and methods that are not widely available in regions with limited healthcare resources.

The fact that so many formulas have been devised simply means that the ideal, universally applicable DF still remains to be discovered. Researchers continue to report formulas with purportedly excellent diagnostic performance. However, many new DFs were developed using small numbers of patients, and often, independent validation of a new DF has not been performed. Previously, a verification study of 25 DFs was reported.[7] Since that time, a considerable number of newer DFs have been published or otherwise came to our attention,[8–18] all of which are still awaiting independent verification.

In the second part of the study, we performed an investigation of how the DFs are constructed and which RBC parameters in the formulas are major determinants of the discriminant performance. To this end, we rearranged DFs by substituting all calculated parameters to only incorporate primary, directly measured RBC parameters.

The present study had 2 aims. First, our goal was to present the results of an independent verification of 20 newer DFs for the discrimination of thalassemia trait from IDA. Second, we attempted to find a relationship between DF performance and the RBC parameters applied in the DF. For both goals, we used a large database containing information on well-defined subjects with microcytic anemia.

Materials and Methods

Database

Our database of information on individuals with microcytic anemia comprises data from the daily workload specimens in the Core Laboratory of the hospital. When applicable, residual specimens were used after the requested tests had been completed. Laboratory results were supplemented with diagnosis and clinical data from the hospital medical-records system. The database was built using measurement results by various hematology analyzers, most recently from the Sysmex XN-9000 (Sysmex Corporation) and Mindray BC-6800Plus (Mindray Bio-Medical Electronics Co, Ltd) analyzers. These analyzers were calibrated and controlled using standard procedures, as advised by the respective manufacturers, following Good Laboratory Practice and the national AENOR (Asociación Española de Normalización y Certificación) accreditation requirements. The study was performed in accordance with the usual ethical and scientific principles and in accordance with the guidelines established by the local Ethics Committee.

Diagnostic Performance of DF

For each of the 20 new DFs, we applied the formulas, as described in **Table 1**. Where necessary, we transformed parameters to comply with internationally accepted units of expression. Three DFs that were found to have the highest performance in another study were added, for the purposes of comparison—namely, the Green and King, Jayabose, and Janel formulas.[19–21] We used receiver operating characteristic (ROC) curve analysis for assessing the test performance of each DF, with the area under the curve (AUC) being the main outcome variable of performance. To compare the performances of these DFs with those from studies that did not report ROC analysis, we also calculated the *Youden index* ([sensitivity + specificity] – 1), at the optimal cutoff value.

Formula Composition and Discriminant Performance

For this part of the study, we substituted all calculated RBC parameters in the original formulas by directly measured parameters: HCT (hematocrit) was replaced by MCV (mean cell volume) x RBC, MCH (mean corpuscular hemoglobin) by Hb (hemoglobin)/RBC, MCHC (mean corpuscular hemoglobin concentration) by Hb/(RBC x MCV), and relative RDW (RBC distribution width) was multiplied by MCV for obtaining an estimate of RDW-SD,[22] and constants in the formulas were omitted for simplicity. Moreover, we also indicated the position of the primary parameters in the formulas.

Statistics

The diagnostic performance of each DF was investigated by ROC analysis using the MedCalc statistical software package (version 19.2.1; MedCalc Software bvba). This program was also used for comparing ROC curves. Statistical significance was assumed at P values less than .05.

Table 1. Discriminant Formulas Used for Distinguishing IDA from Thalassemia in Patients with Microcytic Anemia[a]

Discriminant Formula (Reference Publication)	Calculation	Thalassemia Cutoff Value	AUC	Youden Index
Trivedi[8]	RDW-SD = RDW-% × MCV	<46	…	0.849
Sargolzaie[9]	125.6 + (44.3 × RBC) – (20.9 × Hb) – (2.5 × MCV) + (20.3 × MCH) – (12.18 × MCHC)	<0.5	0.998	0.950
Hisham[10]	MCH × RDW / RBC	<67	…	0.875
Hameed[10]	MCH × HCT × RDW / (RBC × Hb)^2	<220	…	0.880
Chandra[11]	RBC × MCHC × MPV / RDW × PLT	>0.22	0.808	0.505
Matos and Carvalho[12]	1.91 × RBC + 0.44 × MCHC	>23.85	0.950	0.760
Ravanbakhsh-F1[13]	MCV / HCT	<2.0	…	0.745
Ravanbakhsh-F2[13]	RDW – 3 × RBC	<1.5	…	0.670
Ravanbakhsh-F3[13]	MCV × RDW – (100 × RBC)	<600	…	0.789
Ravanbakhsh-F4[13]	MCV × Hb / RDW × RBC	<10	…	0.643
Zaghloul-1[14]	Hb + HCT + RBC	>52.5	0.888	0.627
Zaghloul-2[14]	Hb + HCT + RBC – RDW	>37.1	0.877	0.586
Kandhro-1[15]	RBC / HCT + 0.5 × RDW	<8.2	1.000	1.000
Kandhro-2[15]	RDW × 5 / RBC	<16.8	1.000	1.000
Merdin-1[16]	RDW × RBC / MCV	>1.27	…	0.947
Merdin-2[16]	RDW × RBC × Hb / MCV	>14.7	…	0.896
Alparslan[16]	^{10}log (MCH × MCHC × RDW / RBC)	<3.34	…	0.947
Roth (SVM)[17]	1.45 × (MCV – 82.8) / 10.28 + 0.66 × (MCH – 27.0) / 3.9 + 0.98	<0.0	…	0.870
Cruise[18]	MCHC + 0.603 RBC + 0.523 RDW	≥42.63	0.747	0.493
Index26[18]	Combination of scores from 26 indices	≥16	0.858	0.719
Green and King[19, b]	MCV^2 × RDW / 100 Hb	<65	…	…
Jayabose[20, b]	MCV × RDW / RBC	<220	…	0.840
Janel[21, b]	Combination of scores from 11 indices	≥8	0.947	0.832

IDA, iron deficiency anemia; AUC, area under the curve; RDW, red-blood-cell distribution width; MCV, mean corpuscular volume; … , not available; RBC, red blood cell; Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; HCT, hematocrit; MPV, mean platelet volume; PLT, platelet.
[a]The cutoff values favoring thalassemia, AUC, and Youden index are shown as reported in the original publications.
[b]Older formula known to have excellent performance, which was added to the 20 newer formulas in the analysis.

Results

Study Subjects

At the time of our analysis, the database contained records of 2788 subjects with microcytic anemia, defined as Hb of less than 13.0 g per dL and MCV of less than 80 fL. In total, 60% of the subject individuals were women (median age, 50.0 y; range 18–95 y) and 40% were men (median age, 58.0 y; range, 18–91 y). Of these individuals, 1218 had been diagnosed with "pure" thalassemia trait (980 β- and 238 α-thalassemia), 1402 with IDA, 72 with concomitant IDA and thalassemia (34 β- and 38 α-thalassemia), 29 with simultaneous thalassemia and anemia of chronic disease (20 β- and 9 α-thalassemia), 13 with complex thalassemia (7 Lepore β-thalassemia, 5 δ-β thalassemia, and 1 β-thalassemia + sickle trait), and 54 subjects with other types of microcytic anemia.

Diagnostic Performance

The discriminant performance of the 20 newer DFs is shown in Table 2, along with the 3 best-performing DF identified elsewhere.[7] It appears that the new Index26 is part of a group of 4 formulas with the highest AUC and Youden index of all DFs examined; the AUC was not significantly different from the Jayabose or the Green and King formulas and was marginally higher than the Janel formula ($P < .03$). The other 19 newer formulas all had significantly lower AUC than these top-3-performing formulas ($P \leq .004$ or lower). The Youden index, a composite measure of sensitivity and specificity, ran reasonably well in parallel to the AUC order (Table 2).

Formula Composition and Discriminant Performance

Table 3 summarizes an in-depth analysis of the composition of the overall 20 best-performing DFs, with 13 having been

Table 2. Diagnostic Performance of 20 Newer Discriminant Formulas, Compared with 3 Best Performing DFs Identified Elsewhere[a]

Discriminant Formula[a]	AUC (95% CI)	Optimal Cutoff	Sensitivity	Specificity	Youden Index
Index26	0.952 (0.943 – 0.960)	>14	0.881	0.929	0.809
Jayabose[b]	0.950 (0.941 – 0.958)	≤238	0.902	0.900	0.803
Green and King[b]	0.950 (0.941 – 0.958)	≤74.5	0.893	0.899	0.792
Janel[b]	0.949 (0.940 – 0.957)	≥8	0.850	0.957	0.807
Hisham	0.944 (0.934 – 0.953)	≤74.9	0.889	0.881	0.771
Ravanbakhsh-F3	0.941 (0.931 – 0.950)	≤690	0.902	0.882	0.784
Hameed	0.936 (0.926 – 0.945)	≤4.43	0.886	0.846	0.732
Alparslan	0.927 (0.917 – 0.937)	≤3.39	0.887	0.841	0.728
Matos and Carvalho	0.918 (0.907 – 0.928)	>23.63	0.811	0.869	0.680
Kandhro-2	0.917 (0.906 – 0.928)	≤16.9	0.880	0.828	0.708
Ravanbakhsh-F1	0.917 (0.906 – 0.927)	≤2.00	0.845	0.850	0.694
Ravanbakhsh-F2	0.915 (0.904 – 0.925)	≤1.80	0.877	0.826	0.704
Trivedi	0.911 (0.900 – 0.922)	≤11.8	0.869	0.828	0.692
Roth	0.865 (0.851 – 0.877)	≤–1.19	0.863	0.738	0.601
Sargolzaie	0.845 (0.831 – 0.858)	≤5.0	0.701	0.860	0.561
Zaghloul-2	0.827 (0.812 – 0.841)	>34.4	0.779	0.730	0.510
Merdin-2	0.787 (0.770 – 0.802)	>11.7	0.864	0.605	0.469
Kandhro-1	0.778 (0.761 – 0.794)	≤8.62	0.815	0.657	0.472
Zaghloul-1	0.775 (0.759 – 0.791)	>49.8	0.805	0.608	0.413
Chandra	0.760 (0.735 – 0.784)	>0.29	0.836	0.625	0.461
Merdin-1	0.729 (0.712 – 0.746)	>1.18	0.681	0.704	0.386
Cruise	0.583 (0.564 – 0.602)	≤44.5	0.849	0.323	0.172
Ravanbakhsh-F4	0.577 (0.557 – 0.596)	≤11.4	0.891	0.280	0.171

AUC, area under the curve; CI, confidence interval.
[a]*Arranged in order of decreasing AUC.*
[b]*A best performing DF, as identified in another study.*[7]

Table 3. The 20 Overall Best-Performing Discriminant Formulas in which All Calculated RBC Parameters were Substituted by Directly Measured Parameters

Discriminant Formula[a]	Calculation After Substitution	Sensitivity	Specificity	Youden Index	Hb[b]	RBC[b]	MCV[b]	RDW[b]
Index26[18]	Combination of scores from 26 indices	0.881	0.929	0.809	$A_5N_{10}D$	A_4ND_{16}	$A_6N_9D_4$	A_3N_7
Janel[21]	Combination of scores from 11 indices	0.850	0.957	0.807	A_2N_6D	A_3ND_5	A_3N_6	N_4
Jayabose[20]	MCV × RDW / RBC	0.902	0.900	0.803	…	D	N	N
Green and King[19]	MCV^2 × RDW / 100 Hb	0.893	0.899	0.792	D	…	N	N
Ravanbakhsh-F3[13]	MCV × RDW – (100 × RBC)	0.902	0.882	0.784	…	A	N	N
Wongprachum[28]	(MCV × RDW / RBC) – 10 Hb	0.890	0.884	0.774	A	D	N	N
Sirdah[26]	MCV – RBC – (3 Hb)	0.883	0.891	0.774	A	A	A	…
Hisham[10]	Hb × MCV × RDW / RBC^2	0.889	0.881	0.771	N	D	…	N
Mentzer[4]	MCV / RBC	0.898	0.862	0.760	…	D	N	…
Ehsani[27]	MCV – (10 RBC)	0.864	0.891	0.755	…	A	A	…
Kerman-2[25]	10 MCV^2 / RBC	0.888	0.863	0.751	…	D	N	…
England & Fraser[3]	MCV – RBC – (5 Hb) – 3.4	0.870	0.866	0.736	A	A	A	…
Das Gupta[24]	1.89 RBC – 0.33 RDW – 3.28	0.847	0.888	0.734	…	A	…	A
Hameed[10]	MCV^2 × RDW / (RBC^2 × Hb)	0.886	0.846	0.732	D	D	N	N
Alparslan[16]	^{10}log (Hb^2 × RDW / RBC^3 × MCV)	0.887	0.841	0.728	N	D	D	N
Srivastava[5]	Hb / RBC^2	0.901	0.817	0.718	N	D	…	…
Kerman-1[25]	MCV × Hb / RBC^2	0.881	0.836	0.717	N	D	N	…
Ricerca[23]	RDW / RBC	0.880	0.828	0.708	…	D	…	N
Ravanbakhsh-F2[13]	RDW – 3 × RBC	0.877	0.826	0.704	…	A	…	A
Ravanbakhsh-F1[13]	Hb / (MCV × RBC)	0.845	0.850	0.694	N	D	D	…

RBC, red blood cell; Hb, hemoglobin; MCV, mean corpuscular volume; RDW, red-blood-cell distribution width; … , nonapplicable; A, addition; N, numerator; D, denominator.
[a]*The DFs are arranged in decreasing order of Youden index. Formulas in italics were not among the 20 newer DF but were reported earlier in the literature.*
[b]*Uppercase letters denote the position in the substituted formula: For the Index26 and Janel formulas, the subscript numbers indicate how many directly measured RBC parameters make up the formula.*

already mentioned herein and 7 previously reported.[23–28] Table 3 makes clear that the common features accompanying high AUC are MCV and RDW in the numerator of the DF and RBC in the denominator. Only a few of the best-performing DFs incorporate Hb, whereas many formulas that include Hb have lower performance. DFs containing platelet parameters were not represented in the group of 20 best-performing formulas.

Discussion

Multiple published studies presenting a new formula for distinguishing between thalassemia and IDA in microcytic anemia have one or more limitations: some DFs were developed using relatively small patient numbers (a median of only 249 in the newer DFs cited herein), the patient cohorts were sometimes not representative due to selection bias (inclusion of healthy, normocytic, and nonanemic normal control groups; inclusion of patients with severe anemia; and exclusion of α- and complex thalassemia cases). Also, independent verification was nearly always absent: only 2 studies included a validation group.[12,15]

To fully appreciate the diagnostic performance of DFs, we consider independent verification to be mandatory. However, independent verification studies are scarce. In 2015, Hoffmann and colleagues[6] presented a meta-analysis on the discriminant power of 12 DFs that had been independently verified in 5 or more studies. These authors also reported on a single-center verification study of 25 DFs[7] that had been published in the literature by that time. In all parts, among the conclusions from the microcytic cohort investigated, practically none of the DFs was as sensitive and specific as the DFs suggested by the original authors.[6,7]

In the present study, we report on 20 newer DFs, most of which have not been evaluated by other investigators to date. Our major finding is that 1 newer formula—namely, the Index26 formula—outperformed the other 19 DFs.[18] Its performance was closely comparable to the 3 best DFs that were identified in the literature—the Green and King, Jayabose (RDW index), and Janel (11T) formulas—whereas all other 19 newer DFs had significantly lower performance than the 4 formulas discussed in this paragraph (Table 2). Despite the excellent performance of the top-4 DFs, none of them is strong enough to use in making a final diagnosis, mainly because various cases of α-thalassemia go undetected by any of the DFs. However, the formulas can be used effectively for selecting those specimens that require confirmatory diagnostic testing if a probable diagnosis of thalassemia is indicated by 1 or more DF.

When comparing the discriminant performance, expressed as AUC or Youden index, that was reported in the original DF publications (Table 1) with those from the present study (Table 2), we usually discovered lower performance in the latter. The exceptions were for the Ravanbaksh-F3 and -F2 formulas, which yielded similar Youden indices to those originally reported,[13] and the Index26 which, in our investigations, yielded even higher AUC and Youden index than those given in the original report.[18] So, the high discriminant performance claimed by some authors in describing a new DF may be true generally for their local population; however, it is often too optimistic for generalized use in other populations. These results underline the need for independent verification, preferably also in other regions.

Collating the data reported by the original authors (Table 1) with those from our patient group (Table 2) reveals that the optimal cutoff values are generally slightly different. We believe that this finding can be explained by differences in hematologic parameters between populations, due to variations in the genetic background in various regions of the world, as described earlier in the literature.[9,21] Differences in hematology analyzers are known to have limited, if not negligible, influence.[6]

Another observation is that existing DFs are sometimes presented as novel formulas. We have now established that the Kandhro-2 formula[15] is identical to the Ricerca index;[23] also, the Keikhaei DF[30] is a duplication of the Jayabose RDW index,[20] and the Sehgal index[29] is identical to the Kerman-2 formula.[25] We see 2 possible explanations for these duplications: the original journals may be difficult to access, and authors sometimes use calculated RBC parameters in their "new" DFs that obscure identity with directly measured parameters. Also, the inventors of Index26 have apparently overlooked this issue, so their formula actually consists of 24 unique indices;[18] however, the performance of Index24 is essentially identical to that of Index26 (not shown herein), so the 2 duplicates are not of strong importance.

We were intrigued that no less than 15 of the 20 newer DFs contain RDW, whereas only 9 out of 25 "older" DFs incorporate RDW. Most likely, the inventors of new

formulas better appreciate the power of RDW as part of a successful discriminant index, as we discuss in the coming paragraph.

In the final part of the study, we examined how well-performing DFs are constructed, in an attempt to identify the RBC parameters that exert major influence on the diagnostic performance of a DF. To simplify the comparison, we eliminated all parameters that are calculated by the hematology analyzer (HCT, MCH, and MCHC) and replaced them with directly measured RBC parameters. We were not surprised to discover that the best-performing DFs include MCV, RBC and RDW, whereas Hb seems to be unimportant for achieving a high performance. This finding is succinctly exemplified by the Wongprachum formula: its Youden index is marginally lower than that of the Jayabose formula, and the only difference between those formulas is that the former contains Hb and the latter does not (**Table 3**). For high performance it looks essential that MCV and RDW are in the numerator of the DF, whereas RBC is in the denominator (or the inverse, of course). Again, the position of Hb does not seem to have much effect (**Table 3**).

The critical role of MCV, RDW, and RBC is easy to understand in the light of the common knowledge that MCV in thalassemia is similar or slightly lower than in IDA, RDW is definitely lower in thalassemia, and RBC tends to be higher in thalassemia than in IDA. Therefore, the quotient better emphasizes the difference between both disorders, and power functions further enhance this effect (**Table 3**). Also, because Hb concentrations in patients with IDA and those that carry thalassemia are highly similar, we understand that Hb does not essentially contribute to DF performance.

Two research groups[11,31] have advocated using platelet parameters in the DF. In the results of our analysis, both formulas had significantly lower AUC than the overall 20 best DFs (0.806 and 0.564, respectively); therefore, those values are not included in **Table 3**. Despite our finding that median platelet counts are significantly higher and mean platelet volume (MPV) is lower in our IDA group than in our thalassemia-carrying group (results not shown), these differences did not translate into added value for discriminating between IDA and thalassemia.

The present study has certain potential limitations. Probably the most important of these is that the patient database we have used is generated in Vizcaya, a region in the north of Spain, where the diversity of thalassemia genes might be different than in areas elsewhere in the world. By nature, thalassemia belongs to the genetic background in Spain as part of the Mediterranean "thalassemia belt", and the native population is seemingly relatively homogeneous. During recent decades, however, increased global migration patterns have brought new thalassemia genes into the area, resulting in increased heterogeneity.[32] Still, the genetic makeup in our population may be different from that in other endemic regions, which can result in differences in DF performance between areas.

Another potential limitation is that the database was built during a period of more than 10 years, and the laboratory had operated different types of hematology analyzers during that period. However, the basic RBC parameters, as applied in DF formulas herein (other than RDW), are internationally standardized and are highly comparable between analyzers, making the type of hematology analyzer a factor of minor, if not negligible, importance.[6]

Finally, comparing our findings with those from other researchers is challenging because published evaluations of the 20 newer DFs are virtually unavailable. The single exception is the Matos and Carvalho index, which was evaluated in 4 reports,[18,33–35] with the Youden index ranging between 0.393 and 0.859. The Youden index discovered in our present study (0.680) fits well within this range.

In summary, of the 20 newer DFs, only the Index26 was verified to have relatively high performance in our patient cohort, even higher than originally described in other reports. In contrast, the discriminant power of all other newer DFs appeared to be lower than their inventors had reported. Also, we identified MCV, RDW, and RBC as the predominant RBC parameters that affect DF performance, whereas inclusion of Hb into a formula does not seem necessary for highly discriminant performance. **LM**

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