Original paper

Karbala Formula to Differentiate Beta-Thalassemia Trait from Iron Deficiency Anemia

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Article information:
Received: 2022-04-22
Accepted: 2022-06-07
Vol. 15, No. 1, DEC, 2022.

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Abstract

Background: Hypochromic microcytic red blood cells are common findings in routine laboratory work in all age and ethnic groups. The differentiation between iron deficiency anemia and β-thalassemia trait represents a challenge in populations with limited access to costly lab investigations
Aim: To create a new mathematical formula using RBC hematological parameters to discriminate between iron deficiency anemia and β-thalassemia trait applicable to Karbala population.
Methods: A descriptive cross-sectional study was done at Karbala hereditary blood disease center. The sample of this study was selected from couples attending the premarital screening clinics in Karbala governorate, Iraq. All subjects were adults above 18 years old diagnosed with β-thalassemia trait or iron deficiency anemia. New formula (Karbala) was developed using binary logistic regression (stepwise backward elimination). The new formula and 28 previously published formulas were evaluated using receiver operator curve ROC analysis.
Results: 1380 subjects were included in this study. The new Karbala discriminant formula showed superior performance in ROC curve analysis within Karbala population. Karbala formula has a significantly higher AUC of 0.921 (0.905 – 0.935), followed by England Fraser, MDHL, Matos Carvalho, and Hameed.
Conclusion: Hematological discriminant formulas should be tailored to fit the local population of β-thalassemia trait. Karbala discriminant formula best fits our population.
Keywords: Hematological discriminant formula, β- thalassemia trait, iron deficiency anemia, binary logistic regression, Karbala formula, ROC curve, AUC, Youden index, accuracy, sensitivity, specificity, cut-off value.

Introduction

Finding hypochromic microcytic red blood cells (RBC) is common in routine laboratory work in all age and ethnic groups. The leading causes are iron deficiency anemia (IDA), hemoglobinopathies, or the coexistence of the two. Anemia of chronic diseases, sideroblastic anemia, and lead poisoning are less frequently seen. Iron deficiency anemia is prevalent, especially in young females, because of menstrual blood loss and nutritional insufficiency [(1)].
In this study, β-thalassemia trait was chosen among other hemoglobinopathies because it has the highest prevalence in Karbala/Iraq (3.8%) [(2)]. It is asymptomatic in most cases apart from mild anemia, especially in situations of stress or pregnancy. Iron overload, oligohydramnios, and intrauterine growth retardation are other modes of presentation [(3)]. Despite the mild clinical nature of β-thalassemia trait, it risks producing thalassemia syndrome if two carriers of the defective gene (or genes) are married according to the Mendelian mode of inheritance [(4)]. Transfusion-dependent thalassemia is a lifelong disease that costs a lot for patients and their families in terms of financial, psychological, and social aspects and carries a significant burden on the ministry of health [(5)]; subsequently, premarital prevention of this type of marriage is of paramount importance.
Hypochromia, microcytosis, or both are conditions for referral to the hereditary blood disease center to rule out hemoglobinopathies. The diagnostic tests are mainly serum ferritin and hemoglobin electrophoresis or high performance liquid chromatography HPLC analysis of hemoglobin subtypes. A lower-than-normal ferritin level indicates an iron deficiency, and The first step in carrier identification is to measure the Hb A2 level in patients with microcytosis [(6)]. The aforementioned confirmative tests are costly, especially when done over a large-scale project like premarital screening program. It is worth

using a low-cost, reliable test to differentiate between the two diagnoses. This test will guide further investigations to save time and money.

Individuals with average or high RBC Count, low mean corpuscular volume (MCV), low mean corpuscular hemoglobin (MCH), normal red cell distribution width (RDW), and low normal hemoglobin (Hb) level or even mild anemia are suspected of being heterozygous for thalassemia; but the mean corpuscular hemoglobin concentration (MCHC) is normal in contrast to the decreased MCHC seen in advanced iron deficiency anemia [6]. It is not always the case, and the situation will become more difficult in childbearing age females with a lower normal Hb level than males of comparable age and ethnicity. For these reasons, screening Discriminant RBC mathematical formulas are suggested by many researchers using the complete blood count (CBC) parameters read by the well-controlled automated CBC Counters. RBC hematological discriminant formulas with high sensitivity and specificity would be valuable for differentiating thalassemia trait from iron deficiency anemia, especially in populations endemic with thalassemia disease and nutritional deficiency.

This study is a trial to evaluate 28 previously published formulas calculated from CBC (Table 1). An attempt was made to create a new formula that fits the data of Karbala population. Automated CBC counters obtained RBC parameters from Karbala population by studying adult people attending premarital clinics from 2015 to 2021. In most published literature discussing Discriminant RBC formulas, the authors' evaluation was based on receiver operating characteristic ROC curve performance.

The ROC curve is obtained by calculating the sensitivity and specificity of a test at every possible cut-off point and plotting sensitivity against 1-specificity [7] (figure1). The curve may be used to select optimal cut-off values for a test result, assess the diagnostic accuracy, and compare the usefulness of different tests. ROC curve is independent of disease prevalence since it is based on sensitivity and specificity [8]. Summary measures can be obtained to assess the validity of diagnostic tests, such as the area under the curve AUC and Youden index. The Youden Index (J statistic) is a well-known measurement for the ROC curve to measure clinical diagnostic ability. It is defined as ***Youden index = sensitivity + specificity –1*** [9]. Sensitivity is the probability of correctly identifying the diseased, while specificity is the probability of correctly identifying the non-diseased subjects [8].

Material and Methods

A cross-sectional descriptive study was conducted at Karbala Hereditary Blood Disease Center. The sample of this study was selected from couples referred to the center from January 2015 to August 2021 from the premarital screening clinics in Karbala governorate, Iraq. The premarital screening program for hemoglobinopathies was started in Karbala governorate on June 13, 2012, under the support of the Iraqi Ministry of Health. Five premarital clinics were established, three in peripheral districts, Al-Husseiniya, Al-Hindiya, and EinAltamr, while two clinics were established in Karbala center, Al-Hussein, and Obstetric hospitals (2). The total number of individuals referred to the hereditary blood disease center from the clinics from January 2015 to August 2021 was 3912 individuals (1956 couples). Subjects included in the study were adults above 18 years old and diagnosed with β-thalassemia trait or iron deficiency anemia. Subjects included should have full hematological parameters (RBC, Hb, HCT, MCV, MCH, MCHC, and RDW), serum ferritin, and hemoglobin electrophoresis were done for them. Subjects were excluded from the study if they had missing data or did not fulfill the definition of IDA or β-thalassemia trait. Subjects with IDA should have hemoglobin less than 12 and 13 g/dL for females and males, respectively (10). MCH and MCV should be less than 27 pg and 80 fL, respectively, and serum ferritin less than 12.0 µg/dL for both sexes (11). Subjects with β-thalassemia trait (βTT) should have MCV less than 80 fL and hemoglobin A2 more than 3.5 %. The ethical committee of the Karbala health directorate approved the study, and subjects gave signed consent before enrollment in the study. Descriptive analysis of hematological variables (mean and standard deviation) was assessed. Normality was tested using Shapiro-Wilk test and Mann-Whitney U test used to compare two groups (βTT and IDA).

Twenty eight Discriminant formulas (Table1) from published literature were calculated using blood count hematological parameters. The new formula (Karbala) was developed using binary logistic regression (stepwise backward elimination) with SPSS 24 software. Logistic regression analysis of the main

hematological parameters HGB, HCT, MCV, and LNRDW (natural logarithm of RDW) as independent variables.

Table 1. previously published Mathematical equations for the discriminant formulas, and cut-off value for the diagnosis of β-thalassemia minor

Discriminant formula (reference)	Equation	βTT Cut–off
England Fraser (14)	MCV – RBC – (5 Hb) – 3.4	< 0.00
MDHL (15)	MCH × RBC/MCV	> 1.75
Matos Carvalho (16)	1.91 RBC + 0.44 MCHC	> 23.9
Hameed (17)	(MCH×HCT×RDW)/(RBC×Hb)2	< 220
RBC (18)	RBC	> 5
Sirdah (19)	MCV − RBC − (3 Hb)	< 27
Das Gupta (20)	1.89 RBC − 0.33 RDW − 3.28	> 1.7
Wongprachum (21)	(MCV × RDW/RBC) – 10 HB	< 104
Green King (22)	(MCV2 × RDW)/(100 HB)	< 65
Rahim Keikhaei (23)	(Hb × RDW × 100)/(RBC2 × MCHC)	< 21
Sirachainan (24)	1.5 HB – 0.05 MCV	> 14
RDW index (25)	(MCV × RDW)/RBC	< 220
Mentzer (26)	MCV/RBC	< 13
Ehsani (27)	MCV − (10 RBC)	< 15
Ricerca (28)	RDW/RBC	< 4.4
Hisham (17)	(MCH×RDW)/RBC	< 67
Srivastava (29)	MCH/RBC	< 3.8
Kerman II (30)	(MCV × MCH × 10)/(RBC × MCHC)	< 85
Sehgal (31)	MCV2/RBC	< 972
Kerman I (30)	(MCV × MCH)/RBC	< 300
Nishad (32)	0.615 MCV + 0.518 MCH + 0.446 RDW	< 59
Huber Herklotz (33)	(MCH × RDW/10 RBC) + RDW	< 20
Amendolia (34)	Single Vector Analysis	< 0
Shine Lal (35)	MCV × MCH × 0/01	< 1530
Sargol-zaie_Moghaddam (36)	125.643+44.304×RBC-20.932×Hb-2.501×MCV+20.302×MCH-12.183×MCHC	< 0.5
Bordbar (37)	|80−MCV| × |27−MCH|	>44.76
D Onofrio (38)	MCV/MCH	< 0.9
MCHD (15)	MCH/MCV	> 0.34

Multivariate analysis is used in many research fields, given its ability to explore multiple independent variables [12]. Binary logistic regression helps understand the relationship between multiple independent variables and a binary response variable [13]. The logistic regression model is a construction of the relationship between *P*, the probability of an event of interest (probability of the diagnosis of thalassemia trait given value 1), and a linear combination of independent variables (Xs) (hematological parameters HGB, HCT, MCV, and LNRDW). The logit link function is the natural log of the odds ratio (the ratio between the probability of occurrence of an event of interest,

(if occurred p, and if not occurred 1 − p), (thalassemia trait = 1, IDA = 0) as shown in the equation:

$$\boldsymbol{logit(p) = ln\left(\frac{p}{1-p}\right) = \beta 0 + \beta 1 X1 + \beta 2 X2 + \beta 3 X3 + \cdots\cdots} \quad (13)$$

where $\boldsymbol{P}$ is the probability of having the outcome, $\boldsymbol{P / (1\text{-}P)}$ is the odds of the outcome, $\boldsymbol{\beta_0}$ is the intercept, ($\boldsymbol{\beta_1, \beta_2, \beta_3}$ ....) are a regression coefficient for each $\boldsymbol{X}$ which is the independent variable that can be continuous, discrete, or categorical variables, The dependent binary variable was the probability of diagnosing thalassemia trait (given value 1) or iron deficiency anemia (given value 0). Final regression equation can be written as:

$$\boldsymbol{ln\ (p\ TT/(1\text{-}pTT)) = \beta 0 - \beta_1 * RBC + \beta_1 * HGB + \beta_3 * HCT + \beta_4 MCV + \beta_5 * ln\ (RDW) + \beta_6 * MCV + \beta_7 * HCT}$$

Receiver Operator Characteristics (ROC) analysis to evaluate the performance of the discriminant formulas and pairwise comparison of top five ROC curves with Karbala Discriminant formula was done using MedCalc® v.19 statistical software.

Results

The total number of subjects included in this study was 1380 (568 males and 812 females). Subjects with IDA, N= 802 (198 males and 604 females). Subjects with βTT, N= 578 (370 males and 208 females). The hematological parameters differ significantly between IDA and βTT subjects (Table 2). (Table 3) shows the binary logistic regression coefficients and Odds ratio. The model's overall fit offers 86.1% correct classifications (Table 4). The new probability equation of thalassemia trait **TT**

$$\boldsymbol{ln\ (pTT/(1\text{-}pTT)) = 19.6 - 2.72 * RBC + 1.106 * HGB - 1.492 * LN(RDW) - 0.467 * MCV + 0.502 * HCT}$$

In order to have (0.00) cut-off value of the equation, (0.38) was added to the equation's constant, making it (19.6) instead of (19.22). ROC curve analysis of discriminant formulas calculated AUC with 95% CI, Youden index, and cut-off value for the diagnosis of β-thalassemia trait (Table 5) showed that Karbala formula has the highest AUC 0.921 (0.905 – 0.935), followed by England Fraser 0.918 (0.901 – 0.932), MDHL 0.916 (0.899 – 0.931), Matos Carvalho 0.909

(0.890 – 0.924), and Hameed 0.907 (0.889 – 0.923). Pairwise comparison of the top five AUCs following Karbala formula showed that the AUC of Karbala formula was significantly higher than the other formulas (Table 6). Youden index for the upper three indices with the higher AUC, Karbala, England Fraser, and MDHL was the same (0.71), followed by lower index values for the remaining formulas. Karbala formula is associated with a cut-off value (0.00). The cut-off values for other discriminant formulas calculated using the study database differed slightly or remarkably from published results. Performance analysis (Table 7) shows Accuracy, Sensitivity, Specificity, PPV, and NPV. The accuracy of Karbala formula (85.6%) was slightly higher than MDHL (85.5%), followed by England Fraser (85%), Hameed (84.9%), and Matos Carvalho (84.5%), but notably higher than the remaining indices.

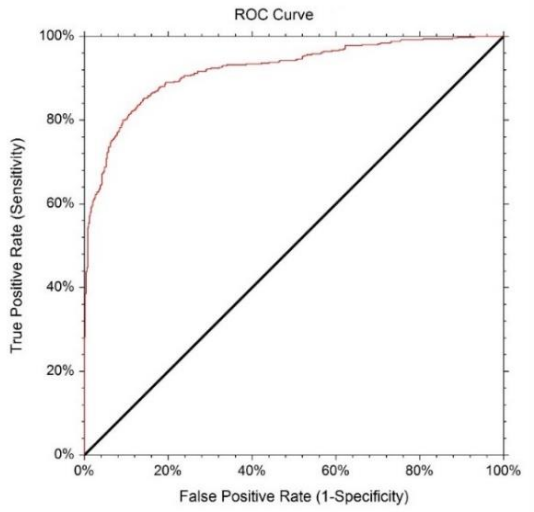

Figure 1. ROC curve of Karbala Discriminant formula

Table 2. Summary statistics of the hematological values of subjects with iron deficiency anemia and Beta thalassemia trait

	Iron deficiency anemia	Beta-thalassemia Trait	
	Mean ± SD (N=802)	Mean ± SD (N=578)	P-value
RBC	4.70 ± 0.56	5.93 ± 0.75	<0.001
HGB	10.1 ± 1.42	12.3 ± 1.70	<0.001
HCT	31.7 ± 4.07	37.6 ± 4.83	<0.001
MCV	67.8 ± 6.82	63.7 ± 6.22	<0.001
MCH	21.7 ± 2.81	20.8 ± 2.55	<0.001
MCHC	31.9 ± 1.98	32.7 ± 2.12	<0.001
RDW	16.6 ± 3.06	15.6 ± 3.03	<0.001
Ferritin	6.75 ± 4.08	79.3 ± 81.2	<0.001
F%	1.00 ± 3.48	1.34 ± 1.64	<0.01
Hb A2	2.32 ± 0.49	5.34 ± 0.95	<0.001
A%	89.0 ± 7.63	86.5 ± 7.16	<0.001

Table 3. step1 binary logistic regression coefficients and odds ratio

Term	Coef	SE Coef	Odds Ratio	P-Value
Constant	19.22	7.04	--------	0.006
RBC	-2.72	1.29	0.0658	0.035
HGB	1.106	0.131	3.0223	0.000
HCT	0.502	0.205	1.6521	0.015
MCV	-0.467	0.107	0.6270	0.000
LN(RDW)	-1.492	0.498	0.2248	0.003

Table 4. Classification Table[a]

			Predicted Dx		
			IDA	TT	Percentage Correct
Step 1	Dx	IDA	726	76	90.4
		TT	116	462	79.9
	Overall Percentage				86.1

a. The cut value is .50

Table 5. Discriminant formula AUC with 95% CI, Youden index, and cut-off value for the diagnosis of Beta-thalassemia trait (N=578) and IDA (N=802)

Discriminant formula	AUC (95% CI)	Youden	βTT Cut–off
Karbala	0.921(0.905 – 0.935)	0.71	> 0.00
England Fraser	0.918(0.901 – 0.932)	0.71	< 2.86
MDHL	0.916(0.899 – 0.931)	0.71	> 1.68
Matos Carvalho	0.909(0.890 – 0.924)	0.69	> 24.2
Hameed	0.907(0.889 – 0.923)	0.68	< 3.14
RBC	0.901(0.883 – 0.917)	0.67	> 5.28
Sirdah	0.895(0.877 – 0.912)	0.66	< 25.4
Das Gupta	0.886(0.866 – 0.903)	0.64	> 1.70
Wongprachum	0.895(0.875 – 0.911)	0.65	< 81.8
Green King	0.881(0.861 – 0.899)	0.64	< 57.8
Rahim Keikhaei	0.869(0.848 – 0.887)	0.59	< 18.2
Sirachainan	0.868(0.847 – 0.887)	0.60	> 13.6
RDW index	0.869(0.849 – 0.887)	0.60	< 198
Mentzer	0.867(0.847 – 0.885)	0.59	< 12.4
Ehsani	0.856(0.834 – 0.875)	0.57	< 11.3
Ricerca	0.840(0.818 – 0.860)	0.54	< 2.88
Hisham	0.837(0.814 – 0.857)	0.53	< 63.7
Srivastava	0.822(0.798 – 0.843)	0.52	< 3.88
Kerman II	0.825(0.801 – 0.846)	0.53	< 8.23
Sehgal	0.825(0.802 – 0.846)	0.52	< 823
Kerman I	0.794(0.769 – 0.816)	0.49	< 262
Nishad	0.698(0.668 – 0.725)	0.35	< 58.9
Huber Herklotz	0.731(0.703 – 0.756)	0.34	< 21.9
Amendolia	0.663(0.633 – 0.691)	0.30	<-2.27
Shine Lal	0.659(0.628 – 0.687)	0.30	< 953
Sargolzaie_Moghaddam	0.656(0.625 – 0.685)	0.25	< 0.53
Bordbar	0.648(0.617 – 0.676)	0.28	> 97.9
D Onofrio	0.613(0.582 – 0.642)	0.18	< 3.14
MCHD	0.618(0.588 – 0.647)	0.18	> 0.32

AUC, area under the curve; CI, Confidence Interval; IDA, Iron Deficiency Anemia.

Table 6. Pairwise comparison of top five ROC curves with Karbala Discriminant formula

	AUC difference	z statistic	P-value
Karbala ~ England Fraser	0.0035	2.13	0.033*
Karbala ~ MDHL	0.0061	2.67	0.007*
Karbala ~ Hameed	0.0140	3.75	0.0002 *
Karbala ~ Matosa Carvalho	0.0128	4.05	0.0001 *
Karbala ~ RBC	0.0199	4.56	0.0001*

Discussion

Different authors have formulated many RBC Indices with contradictory results concerning the accuracy, sensitivity, and specificity [39-41]. The reasons behind these variations are not fully understood. It might be attributable to genetic mutations in specific geographic regions, study sample size, data collection method, selection bias, or analytical techniques. ROC curve analysis predicts the optimal cut-off point and assesses the diagnostic accuracy of many lab tests. A Discriminant formula to be selected as the best test should have the highest accuracy, sensitivity, specificity, area under the curve AUC, and Youden index. This study probed 29 RBC discriminant formulas, including the newly suggested Karbala formula, to select the best that differentiate between β-thalassemia trait and iron deficiency anemia. The first six formulas that yielded the highest accuracy were Karbala, England Fraser, MDHL, Matos Carvalho, Hameed, and RBC index. These formulas also have the highest area under the curve AUC and Youden index. The formulas with the lowest performance were Sargolzaie Moghaddam, Amendolia, Shine Lal, Bordbar, D Onofrio, and MCHD. Sargolzaie and Moghaddam are the first to suggest using logistic regression in predicting an equation suitable for a local region with a homogenous population that may have similar genetic mutation [36]. The logistic regression model is a non-linear transformation of the linear regression, which is similar to the standard normal distribution and constrains the estimated probabilities to lie between 0 and 1[42]. The equation predicted by the binary logistic regression model in This study best fits the data of our center and produced optimal results (accuracy = 0.856, sensitivity = 0.85, specificity = 0.86, AUC = 0.921(0.905 – 0.935) and Youden index = 0.71).

Table 7. Discriminant formulas performance for Beta-thalassemia trait (N=578) and iron-deficiency anemia (N=802)

Discriminant function	Accuracy	Sensitivity	Specificity	PPV	NPV
Karbala	0.856	0.85	0.86	0.81	0.89
MDHL	0.855	0.85	0.86	0.81	0.89
England Fraser	0.85	0.88	0.83	0.79	0.91
Hameed	0.849	0.78	0.90	0.85	0.85
Matos Carvalho	0.845	0.84	0.85	0.8	0.88
RBC	0.839	0.81	0.86	0.8	0.86
Sirdah	0.838	0.78	0.88	0.83	0.84
Wongprachum	0.827	0.81	0.84	0.79	0.86
Green King	0.828	0.77	0.87	0.81	0.84
Das Gupta	0.825	0.75	0.88	0.82	0.83
Sirachainan	0.806	0.76	0.84	0.77	0.83
RDW index	0.798	0.81	0.79	0.74	0.85
Rahim Keikhaei	0.808	0.71	0.88	0.82	0.81
Mentzer	0.799	0.77	0.82	0.75	0.83
Ehsani	0.792	0.74	0.83	0.75	0.82
Ricerca	0.778	0.72	0.82	0.74	0.8
Hisham	0.764	0.77	0.76	0.70	0.82
Kerman I	0.760	0.76	0.76	0.70	0.82
Sehgal	0.760	0.76	0.76	0.70	0.82
Srivastava	0.763	0.70	0.81	0.73	0.79
Kerman II	0.744	0.75	0.74	0.68	0.81
Nishad	0.669	0.71	0.64	0.59	0.75
Huber_Herklotz	0.673	0.65	0.69	0.60	0.73
Bordbar	0.653	0.59	0.70	0.58	0.7
Amendolia	0.641	0.70	0.60	0.56	0.74
Shine Lal	0.641	0.70	0.60	0.56	0.73
Sargolzaie_Moghaddam	0.631	0.61	0.64	0.55	0.69
D Onofrio	0.571	0.67	0.50	0.50	0.68
MCHD	0.571	0.67	0.50	0.50	0.68

PPV, positive predictive value; NPV, negative predictive value.

When Sargolzaie and Moghaddam formula was applied to data from Karbala center for blood diseases it produced inferior results (accuracy= 0.631, sensitivity = 0.61, specificity = 0.64, AUC =0.656 (0.625 – 0.685) and Youden index = 0.25) which are obviously lower than the results calculated by the Sargolzaie and Moghaddam depending on their data (sensitivity = 0.99, specificity = 96.1, AUC = 0.998 (0.995–1.001) (36). The lower performance of their formula when applied to our data compared to their data may be due to the difference in sample size between the two studies or different genetic mutations in different communities.

Conclusion

Variations of β-thalassemia trait in different populations may limit the adoption of various hematological discriminant formulas to differentiate between β-thalassemia trait and iron deficiency anemia. A convenient formula is best tailored for local populations with β-thalassemia trait. Karbala discriminant formula best fits our local community for the reason that it was created by relying on data collected from Karbala population.

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