

## Sensitivity, specificity and efficiency of different discriminative indexes in differentiation of thalassemia trait from iron deficiency anemia

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### ABSTRACT

A complete blood count (CBC), separation of haemoglobin molecules by electrophoresis at pH 8.4 followed by scanning densitometry and ethnic background, all together, are absolutely necessary in the diagnosis of  $\beta$ - thalassemia trait. The aim of this study was to assess the specificity, sensitivity and efficiency of six different discriminative indexes in differentiation of thalassemia trait from iron deficiency anemia and if they alone may replace the haemoglobin electrophoresis. In this study there were analyzed 638 adult and child blood samples by haemoglobin electrophoresis at pH 8.4 on an agarose gel. For all these samples a CBC was initially performed on a fully automated system. 435 patients diagnosed with  $\beta$ - thalassemia minor were further analyzed for thalassemia mutations. An individual evaluation for six discriminative indexes that included the CBC parameters was performed to differentiate thalassemia trait from iron deficiency anemia. The evaluation was performed on 542 samples with a clear diagnosis which included 107 samples with HbA2 within the reference range and 435 samples with the diagnosis of  $\beta$ -thalassemia minor. The Shine and Lal index showed good specificity, sensitivity and efficiency. For 96 patients we could not evaluate these six discriminative indexes, the diagnosis being inconclusive. In conclusion CBC and the discriminative indexes cannot replace the haemoglobin electrophoresis in diagnosing  $\beta$ -thalassemia trait.

**Keywords:**  $\beta$ - thalassemia differential diagnosis, discriminative indexes, haemoglobin electrophoresis, molecular testing

## 1. Introduction

Microcytic anemias are among the most common types of anemia encountered by physicians. The two most common causes of microcytic anemia [1] are iron deficiency and thalassemia minor. The differentiation between thalassemia trait and iron deficiency is an important consideration and requires the use of specialized laboratory procedures, as well as a knowledgeable interpretation of the results. The thalassemias are characterized by a reduction in the amount of the normal globin chain produced [2]. This diminution in globin chain production may result from gene deletion [3] or

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from mutations. The manifestations range from mild anemia with microcytosis (thalassemia trait) to fatal severe anemia (thalassemia major). The 1975 International Committee for Standardization in Hematology expert panel on thalassemias made diagnostic recommendations regarding the laboratory investigation of these conditions. Initial recommended tests include a complete blood count (CBC) followed by separation of haemoglobin molecules by electrophoresis at pH 8.4 and their quantification by scanning densitometry. Red cell indexes are critical to the diagnosis of thalassemias and the key components of the CBC that should be followed are: Hb, RBC (red blood cell), MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin), and RDW (red cell distribution width). MCV ranges in most populations from 80 to 100 fL but a value of less than 72 fL is maximally sensitive and specific for presumptive diagnosis of thalassemia syndromes [4]. The RDW is a measure of variation degree in red cell size. The thalassemias tend to produce a uniform microcytic red cell population without a concomitant increase in RDW, but iron deficiency anemia is characterized by an increase in RDW. The RBC tends to be increased in thalassemia because of microcytic anemia and iron deficiency is associated with a decrease in the RBC number that is proportional to the decreased level in Hb concentration. The Hb concentration typically is decreased in thalassemia. The thalassemia minor conditions produce minimal decreases in the Hb concentration. Different discriminative indexes using the CBC components have been developed with the purpose to reliably differentiate iron deficiency from thalassemia minor [5-8] but none of them have not proved to be 100 percent useful in all clinical settings. In the investigation of microcytosis alongside of CBC it is necessary to obtain some additional data about the patient, such as: the age, the ethnic background, the recent transfusion. In Romania, separation and quantification by scanning densitometry of different molecules of haemoglobin represents the method of choice in diagnosing  $\beta$ -thalassemia that allows us to quantify the HbA2.

## 2. Experimental section

**Patients:** The study was performed on 638 samples collected during March 2008 - March 2010, from the patients with ages between 2-67 years. Demographic data, family history, personal history (including serum iron levels and transfusion antecedents) were noted. Initially all the patients had been seen by the clinicians in the Divisions of Haematology and Pediatrics of Clinical Institute Fundeni and diagnosed, considering the clinical manifestations, with thalassemia: unexplained fatigue, anemia, microcytosis, target cells. **The routine CBC** was performed on a fully automated system Sysmex XE 2100. The analyzer required 1 ml blood collected with Na<sub>2</sub>EDTA and gave a rapid quantification of red cell indexes (Hb, RBC, MCV, MCH, RDW). The reference ranges of the CBC and HbA2 indicated by the manufacturer are presented in table 1.

**Table 1:** Reference ranges of CBC parameters and HbA2

Parameter	Reference range
Hb (g/L)	11.2 – 17.5
RBC count ( $\times 10^6/\mu\text{L}$ )	3.93 – 6.08
MCV (fL)	79 – 94.8
MCH (pg)	25.6 – 32.2
RDW (%)	11.6 – 14.4
HbA2 (%)	$\leq 3.5$

**Electrophoretic separation** was performed with the SEBIA semiautomated using Hydragel Haemoglobin K20 kit, that is designed for separation of the normal haemoglobins (Hb A and A2) and for the detection of the major haemoglobin variants: S or D and C or E by electrophoresis on alkaline agarose gels (pH 8.4) [9]. We used fresh samples collected with Na2EDTA as anticoagulant or samples refrigerated for no more than 5 days at 2 to 8°C. Prior the electrophoresis it was necessary a sample preparation which consisted in two step washing of 500 µL red blood cells with 2.5 mL saline solution followed by a centrifugation at 5000 rpm for 15 minutes. After the plasma was discarded we prepared a hemolysate by using 15 µL packed red cells with 130 µL hemolyzing solution.

For electrophoresis, 10 mL of hemolysate was applied manually to the sample applicator (the applicator is designed for 7 samples). The sample applicator was loaded within 2 minutes. The sample applicator was placed in an applicator carrier which allowed its contact with the gel for 60 seconds. The gel was placed into an electrophoresis chamber and the time of separation was of 15 minutes at 165V. The gel was transferred to the fixative solution for 15 minutes than dried for 10 minutes at 80°C, stained for 5 minutes with amido black, destained with an acidic solution and finally dried for 10 minutes at 80°C. The resulting electrophoregrams were evaluated visually for pattern abnormalities than scanned with the SEBIA densitometer in order to obtain relative concentrations of individual fractions.

**Discriminative index:** In this study we individually evaluated six discriminative indexes which included in their equations some of the CBC parameters to differentiate thalassemia trait from iron deficiency anemia. The six discriminative indexes with their equations and cut offs used in this study are presented in table 2.

**Table 2:** Discriminative used indexes and their equations and cutoffs

Formula	Equation	Cutoff values
Shine and Lal (S & L) Index	$(MCV^2 \times MCH) / 100$	< 1530 TM ; > 1530 IDA
Green and King (G & K) Index	$[(MCV)^2 \times RDW] / (Hb \times 100)$	< 72 TM ; > 72 IDA
Mentzer Index (MI)	MCV / RBC	< 13 TM ; > 13 IDA
RDW Index (RDWI)	MCV X RDW / RBC	< 220 TM ; > 220 IDA
Srivastava Index (SI)	MCH / RBC	< 4.4 TM ; > 4.4 IDA
Ricerca Index (RI)	RDW / RBC	< 3.3 TM ; > 3.3 IDA

### 3. Results section

During this study, there were analyzed 638 samples and for 107 of them the values of HbA2, serum iron and Hb were situated within the reference ranges (Figure 1). The CBC's parameters, MCV >72 fL and MCH >27 pg, did not indicate thalassemia trait. The comparative evaluation of six discriminative factors for these 107 normal patients (Table 3) showed the following results: the Shine and Lal index indicated values > 1530 for all 107 samples, the Mentzel index showed values > 13 for all 107 samples, the Srivastava index showed values > 4.4 for all 107 samples, the Ricerca index showed values > 3.3 for 80 samples and for 27 samples indicated thalassemia trait, the Green and King index showed values < 72 for 46 samples and values >72 for 61 samples, the RDW index showed values < 220 for 22 samples and values >220 for 85 samples.

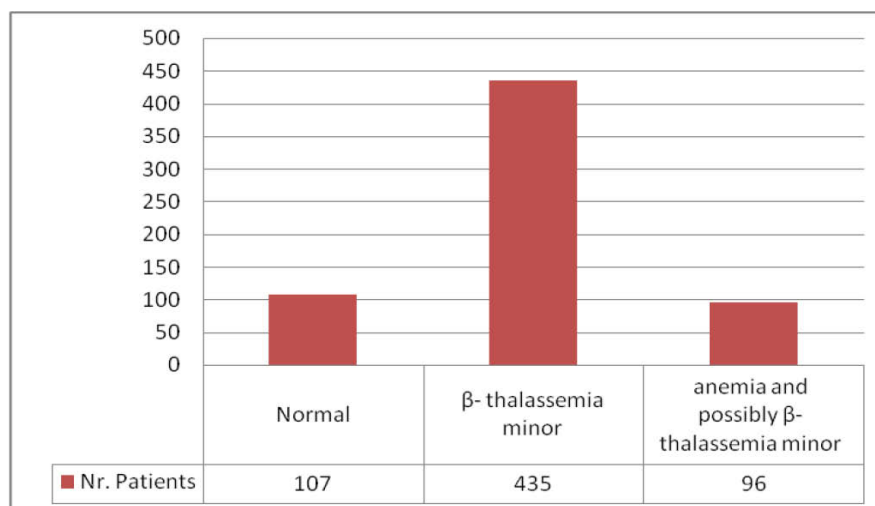


Figure 1. Number of analyzed patients and their diagnosis

Table 3: Range, mean and standard deviation of the CBC parameters and HbA2 for normal patients

Parameter	Range	Mean	Standard Deviation (SD)
Hb	11.6 – 17.5	13.8	1.66
RBC	3.3 – 6.5	4.6	0.62
MCV	79.6 – 107.8	88.7	6.07
MCH	27.2 – 40.9	30.24	2.41
RDW	11.1 – 39.4	13.7	2.37
HbA2	2 – 3.4	2.7	0.4

Three of these indexes (Shine and Lal, Mentzel, Srivastava) were consistent with the results obtained by haemoglobin electrophoresis indicating the absence of thalassemia trait. The other three indexes indicated the presence of thalassemia trait for some patients (RDW- 20.52%, Ricerca-25.2%, Green and King- 42.9%) although the HbA2 levels and the CBC's parameters were within the reference ranges.

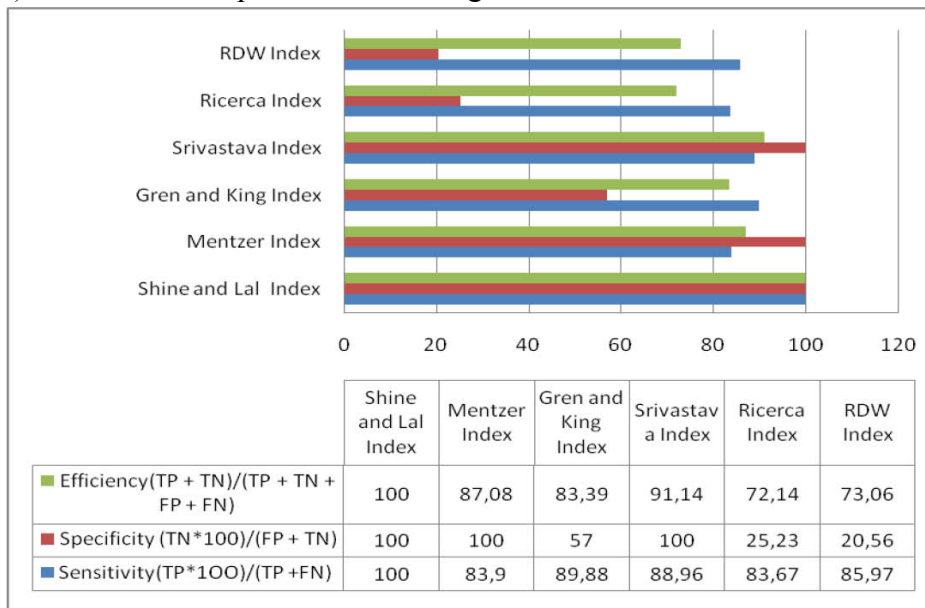
435 patients were diagnosed with  $\beta$ -thalassemia minor (Table 4). The values of HbA2 were situated above the upper limit of the reference range and the CBC parameters also indicated the presence of thalassemia. From those 435 patients, 200 exhibited Hb values < 11.2 g/L and 235 Hb > 11.2 g/L. For all these patients there were performed molecular biology tests which confirmed the diagnosis of thalassemia trait.

Table 4: Range, mean and standard deviation for the CBC parameters and HbA2 of the patients diagnosed with  $\beta$ -thalassemia minor

PARAMETER	RANGE	MEAN	Standard Deviation (SD)
Hb	7.7 – 15	11.1	1.29
RBC	3.5 – 7.42	5.57	0.62
MCV	48.8 -72	63	3.99
MCH	15 – 32.7	20	1.67
RDW	12.3 – 37.2	16.75	3.17
HbA2	3.5 – 7.8	4.67	0.84

We evaluated and compared the six discriminative indexes for these 435 patients with  $\beta$ -thalassemia minor and the results were the following: the Shine and Lal index showed values <1530 for all the patients, the Srivastava identified  $\beta$ -thalassemia for 387 patients and for 48 patients indicated IDA, Green and King indicated thalassemia for 391 patients and for 44 patients indicated

IDA, the RDW index indicated thalassemia for 374 patients and for 61 patients indicated IDA, the Mentzel index indicated thalassemia for 365 patients and for 70 patients indicated IDA, the Ricerca index indicated thalassemia for 364 patients and for 71 patients indicated IDA. The Shine and Lal index 100% confirmed the diagnosis of thalassemia trait but the other five indexes indicated the presence of thalassemia trait for one part of the patients only as follows: Srivastava- 88.9%, Green and King- 89.8%, RDW- 85.9%, Mentzel- 83.9%, Ricerca- 83.6%. For normal and  $\beta$ -thalassemia minor patients together we evaluated the sensitivity, specificity and the efficiency of these six discriminative indexes, based on the following formulas : sensitivity (true positives x100)/(true positives + false negatives), specificity (true negatives x100)/ (true negatives + false positives)], efficiency [(true positives + true negatives) x 100]/ (true positives + true negatives + false positives + false negatives). The results are presented in the figure 2.



**Figure 2.** Sensitivity, specificity and efficiency of the six discriminative indexes for the normal and  $\beta$ - thalassemia patients.

We considered as positive control or negative control the values of the HbA2 obtained by electrophoretic separation and the results obtained by molecular biology tests. The Shine and Lal showed 100% sensitivity, 100% specificity and 100% efficiency in diagnosing thalassemia trait, the Srivastava showed a 100% specificity, 88.96 sensitivity and 91.14% efficiency in diagnosing thalassemia, also the Mentzel index showed a specificity of 100% and a sensitivity of 83.9%, the RDW index showed a specificity of 20.56%.

The results of our study confirm the findings of other publications obtained by studying other populations [4], where Shine and Lal index was found effective in discriminating thalassemic from nonthalassemic microcytosis. Our study also contradicts some other findings [10-13], where the Shine and Lal index was ineffective for the Kuwait population, the Green and King and RDW indexes showed great effectiveness for Palestine population, the RDW index was effective for the Turkey population, in differentiation between thalassemia and IDA.

The inter populations differences in effectiveness of various discriminative indexes in differentiation of thalassemia trait from iron deficiency anemia could be attributed to differences in the mutation spectrum of the thalassemia disease of the different populations. Rund et al. [14] explained that different MCV values are significantly correlated with different  $\beta$ -thalassemia

mutations. Rosatelli et al. [15] mentioned the relationship between the hematological phenotype and the type of mutation in  $\beta$ -thalassaemia minor individuals, which could also explain the inter-populations differences.

For 96 patients haemoglobin electrophoresis could not give an exact answer whereas those patients were thalassaemic or not. For all these patients HbA2 was situated within the reference range but the levels of Hb and serum iron levels were under the reference range, MCV was  $< 72$ fL and MCH was  $< 27$  pg. None of these patients were evaluated with molecular biology tests (Table 5).

**Table 5:** Range, mean and standard deviation for the CBC parameters and HbA2 of those patients thought to be iron deficiency anemia and possibly  $\beta$ - thalassemia minor

Parameter	Range	Mean	Standard Deviation (SD)
Hb	7.1 – 11.2	9.37	1.2
RBC	3.3 – 6.3	4.77	0.668
MCV	51.4 – 72	64.9	3.93
MCH	15 – 24.46	19.7	1.99
RDW	11.8 – 33.2	20	4.5
HbA2	1 – 3.4	2.36	0.61

For these 96 patients thought to be IDA and possibly  $\beta$ - thalassemia minor, we evaluated the six discriminative indexes and the results were the following: the Shine and Lal index showed values  $< 1530$  indicating the presence of thalassemia for all 96 patients, the Srivastava indicated thalassemia for 63 patients, the Green and King index for 34 patients, the Mentzel index for 39 patients, the Ricerca index for 29 patients, the RDW index for 30 patients, while for the rest of the patients all indexes indicated IDA. Because of an uncertain diagnosis of these patients we were not able to evaluate the sensitivity, specificity and efficiency for the six discriminative indexes from this study.

## 4. Conclusions

Although using discriminative indexes might seem an easy way of evaluating patients with microcytosis and differentiating thalassemia from iron deficiency anemia, none of these discriminative indexes cannot replace haemoglobin electrophoresis and molecular biology tests for a correct diagnosis of thalassemia. Also some additional data about the patient, such as: the age, the ethnic background or the recent history of transfusion is a must for a correct evaluation of the patient.

This is the first study trying to characterize the value of different discriminative indexes on Romanian population, but further investigation is needed in order to establish our own formula based on the mutations identified in this particular population.

## 5. References

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