

## Original Research Article

# Use of red cell indices in the diagnosis of beta-thalassemia trait

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## ABSTRACT

**Background:** Hemoglobinopathies are found to be the most common monogenic hereditary disorders. The haematological features of thalassemia trait are similar to that of microcytic hypochromic anemia and differentiation is mainly based on High Performance Liquid Chromatography (HPLC) results. Simple blood count indices have been developed for prediction of possible presence of thalassemia trait. This study aimed to assess the diagnostic role of red cell indices in reporting beta-thalassemia trait.

**Methods:** A total of 124 patients were included in this study. All cases of microcytic hypochromic anemia were included in the study and were classified as positive or negative for beta-thalassemia trait based on the indices analysed. Cases were sent for high performance liquid chromatography for confirmation and statistical analysis was carried out to find the index that best suited the study population.

**Results:** There was a slight male predominance in the study population. Shine and Lal index showed the highest sensitivity of 100%, followed by Red Cell Distribution Width Index (RDWI) and Kandhro index (98.9%). Sirdah index showed the highest specificity of 71.4% followed by Zaghoulou 1 index showing specificity of 67.8%. The low specificity in this study could be attributed to the fact that only a few cases that were flagged negative by the CBC indices were selected.

**Conclusions:** The accuracy of each index in predicting the presence of beta-thalassemia trait varies from population to population. Population based studies are warranted to assess the indices that are most accurate, and thereafter these indices must be included in routine hematology investigations to aid in early detection of beta thalassemia trait.

**Keywords:** Fetal distress, Hypertension in pregnancy, Stillbirths

## INTRODUCTION

Genetic disorders of haemoglobin can be broadly classified into three major categories: structural haemoglobin variants such as Hemoglobin S (HbS), Hemoglobin C (HbC), Hemoglobin D (HbD), and Hemoglobin E (HbE); thalassemias, which are characterized by impaired or reduced synthesis of globin chains and are further classified into  $\alpha$ -,  $\beta$ -,  $\epsilon\delta\beta$ -, and  $\delta\beta$ -thalassemias, with the majority involving either  $\alpha$ - or  $\beta$ -globin chains; and hereditary persistence of fetal

haemoglobin (HPFH), a condition in which significant production of fetal haemoglobin continues into adulthood.<sup>1</sup>

According to the World Health Organization (WHO), approximately 5% of the global population are carriers of a potentially pathological haemoglobin gene, meaning healthy individuals who have inherited a single mutant gene from one parent. Each year, about 300,000 infants worldwide are born with haemoglobin disorders, including thalassemia syndromes (30%) and sickle cell anaemia (70%). The frequency of  $\beta$ -thalassemia carriers ranges from 1% to 17%, and the clinical spectrum varies from

asymptomatic conditions to severe disorders requiring regular blood transfusions and extensive medical care, such as thalassemia major.<sup>2</sup> Recent data from the WHO Hereditary Disease Programme estimate that approximately 269 million individuals worldwide are carriers of haemoglobin disorders. In India, there are an estimated 65,000-67,000 patients with  $\beta$ -thalassemia, with approximately 9,000-10,000 new cases added annually. The carrier rate for the  $\beta$ -thalassemia gene varies geographically, ranging from 1%-3% in southern India to 3%-15% in northern India.<sup>3</sup>

The haematological features of  $\beta$ -thalassemia trait typically include microcytosis and hypochromia, usually accompanied by an increased percentage of hemoglobin A2 (HbA2). The haemoglobin composition is generally 92%-95% HbA, more than 3.8% HbA2, and variable amounts of fetal hemoglobin (HbF) ranging from 0.5% to 4%.<sup>4</sup> In addition to microcytosis and hypochromia, significant anisopoikilocytosis may be observed, with red cells in  $\beta^0$ -thalassemia trait exhibiting lower mean corpuscular volume (MCV) values compared to  $\beta^+$ -thalassemia trait.<sup>5</sup> Recent studies suggest that patients with thalassemia trait may experience symptoms of anaemia, including headache, lethargy, fatigue, and exercise intolerance, despite having normal haemoglobin levels. No significant difference in symptom frequency has been observed between individuals with mildly reduced haemoglobin levels and those with haemoglobin levels within the normal range.<sup>6</sup> Besides anaemia-related symptoms, jaundice, abdominal pain, and hepatosplenomegaly may also occur in symptomatic  $\beta$ -thalassemia trait, being present in approximately 20%–25% of cases. Appropriate screening, early detection, and counselling of at-risk couples remain the most important measures for reducing morbidity and mortality, as missed or incorrect diagnosis can result in homozygous affected offspring.

Typical laboratory findings in  $\beta$ -thalassemia trait include hemoglobin levels ranging from 9-11 g/dL, mean corpuscular hemoglobin (MCH) of 20-22 pg, mean corpuscular volume (MCV) of 50-70 fL, elevated red blood cell counts, normal mean corpuscular hemoglobin concentration (MCHC), and normal or increased red cell distribution width (RDW). Peripheral smear commonly shows microcytosis, hypochromia, anisopoikilocytosis, basophilic stippling, and reticulocytosis. Bone marrow findings include mild to moderate erythroid hyperplasia with rare red cell inclusions and mild ineffective erythropoiesis. Serum hemoglobin electrophoresis typically demonstrates elevated HbA2 levels ranging from 3.5% to 7%, with increased HbF observed in approximately half of the patients.<sup>7,8</sup>

Diagnosis of  $\beta$ -thalassemia is usually established by demonstrating increased HbA2 levels using electrophoresis and/or chromatography. However, in conditions such as coexistent  $\delta$ -thalassemia or severe iron deficiency, HbA2 levels may not be elevated. In  $\delta\beta$ -

thalassemia and hereditary persistence of fetal hemoglobin, HbF is often increased without a diagnostic rise in HbA2.<sup>9</sup> High-performance liquid chromatography (HPLC) is an advanced column chromatography technique commonly used for this purpose. Since 1970, several complete blood count indices have been proposed as simple and inexpensive tools to differentiate  $\beta$ -thalassemia trait from iron deficiency anaemia (IDA). In 1973, Mentzer first described the Mentzer Index for distinguishing  $\beta$ -thalassemia trait from IDA with reasonable reliability.<sup>4</sup> Over the past five decades, numerous studies across different populations have evaluated these formulas, developed new indices, and compared their sensitivity and specificity.<sup>10-12</sup> However, none of the indices has demonstrated 100% sensitivity and specificity across all populations.<sup>13-17</sup> Inter-population variations in discriminating formulas have been attributed to differences in mutation spectra among populations.<sup>10,15</sup>

Data obtained from automated haematology analysers may sometimes be overlooked by clinicians because carriers are often asymptomatic. Since early identification and counselling of carriers is the most effective strategy for thalassemia prevention, discrimination indices such as red blood cell count, red cell distribution width index (RDWI), Mentzer index, Sehgal index, Green and King index, Ricerca index, and Shine and Lal index can be useful for screening  $\beta$ -thalassemia trait. The clinical applicability of incorporating red cell indices from automated analysers into routine evaluation requires further investigation. Therefore, the present study aims to assess the diagnostic role of red cell indices in identifying  $\beta$ -thalassemia trait and to determine the proportion of  $\beta$ -thalassemia trait among individuals with microcytic anaemia screened by complete blood count.

## METHODS

This was a cross-sectional study where anaemic patients diagnosed during the time period of 24 months from January 2019 to January 2021 was studied based on clinical features, and haematological investigations including special investigations like HPLC was performed. All patients presenting to Sri Siddhartha Medical College, Tumkur with microcytic hypochromic anemia and flagged positive for beta thalassemia trait were included in the study. Only a few cases of microcytic hypochromic anemia that were flagged negative for beta thalassemia trait were included in the study and this was a major limitation of the study. Sample size was calculated using the formula,

$$n = z^2(1-a)*p(1-p)/d^2,$$

where; n=sample size, Z=1.96(95% CI), P=prevalence (25%)<sup>7</sup>, d=margin of error (10%).

A total of 124 cases were included in this study. Clinical details including presenting complaints, age, sex, physical examination findings were recorded and basic hematologic

investigation of Complete Blood Count were performed using Sysmex-XP 100 hematology analyser. Blood samples of patients testing positive for red cell indices indicating  $\beta$ -thalassemia trait were sent for HPLC and the results were recorded and analysed. The indices were used for calculation of red cell indices as given in Table 1.

**Table 1: Formulas for calculation of various indices used in flagging beta thalassemia trait.**

| Index                              | Formula                     |
|------------------------------------|-----------------------------|
| Mentzer Index <sup>18</sup>        | MCV/RBC                     |
| Shine and Lal index <sup>18</sup>  | MCV X MCV X MCH/100         |
| Green and King index <sup>18</sup> | MCV X MCV X RDW/ Hb X 100   |
| Ricera index <sup>18</sup>         | RDW/RBC                     |
| RDWI <sup>18</sup>                 | RDW X MCV/ RBC              |
| Sirdah index <sup>18</sup>         | MCV-RBC -3Hb                |
| Ehsani index <sup>18</sup>         | MCV-10 X RBC                |
| Srivastva index <sup>18</sup>      | MCH/ RBC                    |
| HH index <sup>18</sup>             | (MCH X RDW X 0.1/RBC)+ RDW  |
| Bordbar index <sup>18</sup>        | (   80-MCV   X   27-MCH   ) |
| Kandhro index <sup>18</sup>        | (RDW X 5)/ RBC              |
| Keikhaei index <sup>18</sup>       | HbXRDWX100/ (RBC)2 X MCHC   |
| Zaghloul 1 index <sup>18</sup>     | Hb + Hct + RBC              |
| Zaghloul 2 index <sup>18</sup>     | Hb + Hct + RBC – RDW        |
| Kerman 1 index <sup>18</sup>       | MCV X MCH/RBC               |
| Kerman 2 index <sup>18</sup>       | (MCV X MCH/RBC) X 10/MCH    |
| Sehgal index <sup>5</sup>          | MCV X MCV/ RBC              |

The cutoffs taken for the indices used for flagging of beta thalassemia trait are given in Table 2.

**Table 2: Cut off values of red cell indices for flagging beta thalassemia trait.**

| Index used     | Iron deficiency anaemia | Beta thalassemia trait |
|----------------|-------------------------|------------------------|
| Mentzer index  | >14                     | <14                    |
| Sehgal index   | >972                    | <972                   |
| Green and King | >72                     | <72                    |
| Ricera         | >3.3                    | <3.3                   |
| Shine and Lal  | >1530                   | <1530                  |
| RDWI           | >220                    | <220                   |
| Srivastva      | >3.8                    | <3.8                   |
| Ehsani         | >15                     | <15                    |
| Sirdah         | >27                     | <27                    |
| HH             | >21                     | <21                    |
| Kandhro 2      | >16.8                   | <16.8                  |
| Keikaei        | >21                     | <21                    |
| Kerman 1       | >300                    | <300                   |
| Kerman 2       | >85                     | <85                    |
| Zaghloul 1     | <52.5                   | >52.5                  |
| Zaghloul 2     | <37.1                   | >37.1                  |
| Bordbar        | <44.76                  | >44.76                 |

**Inclusion criteria**

All microcytic hypochromic anaemic patients who were suspected cases of  $\beta$ -thalassemia trait based on red cell indices were included in the study.

**Exclusion criteria**

All patients with macrocytic and normocytic anaemia, renal or hepatic derangement were excluded from the study. Children less than 6 months of age were also be excluded from the study.

The collected data were analysed with IBM.SPSS statistics software 23.0 Version. Description of data was done using descriptive statistics frequency analysis for categorical variables and mean & Standard deviation for continuous variables. Difference between the bivariate samples in independent groups was analysed using unpaired sample t-test. The Receiver Operator Characteristic (ROC) curve with sensitivity, specificity, PPV, NPV and Youden's index were used to find the efficacy of the tools. In the above statistical tools the probability value 0.05 is considered as significant level.

**RESULTS**

Total 124 samples that were sent for complete blood count to the Department of Pathology were evaluated and sent for High Performance Liquid Chromatography for confirmation of beta thalassemia trait. Following results were obtained:

There was a slight male predominance in the number of cases studied with 50.8% cases of male gender. The gender distribution of the study population is depicted in Table 3.

**Table 3: The gender distribution of the study population.**

|        | Number of cases | Percentage of cases |
|--------|-----------------|---------------------|
| Male   | 63              | 50.8                |
| Female | 61              | 49.2                |
| Total  | 124             | 100                 |

**Table 4: Presenting complaints of the study population.**

| Presenting complaints            | Number of cases |
|----------------------------------|-----------------|
| Generalised weakness and fatigue | 61              |
| Routine work up                  | 28              |
| Shortness of breath              | 12              |
| Fever                            | 10              |
| Palpitations                     | 7               |
| Loss of appetite                 | 6               |
| Total number of cases            | 124             |

Majority of cases studied presented with generalised weakness and fatigue (49.2%). Few of the cases studied (22.6%) were without any complaints of anaemia and low hemoglobin count was incidentally found during routine blood work up for surgery. The clinical complaints of the study population and the frequency in which each complaints were seen in the study population, is depicted in Table 4.

**Examination findings**

On examination, few cases had pallor of skin (27.4%) and few had yellowish discoloration (10.5%), while majority of cases showed no positive examination findings (53.2%). A minority of cases showed mild hepatomegaly (Table 5).

**Table 5: Clinical examination findings of the study population.**

| Examination findings      | Number of cases |
|---------------------------|-----------------|
| No positive findings      | 66              |
| Pallor                    | 34              |
| Jaundice                  | 13              |
| Hepatomegaly              | 8               |
| Retarded growth (for age) | 3               |
| Total number of cases     | 124             |

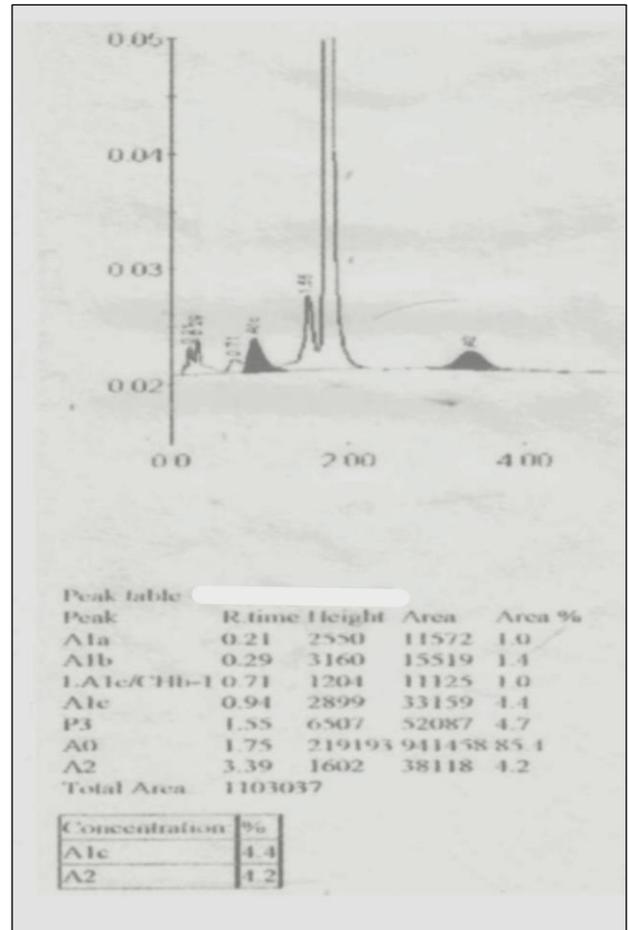
Cases that were flagged positive by the use of CBC indices were sent for high performance liquid chromatography for confirmation. Most of the cases (77.4%) were confirmed to be of beta thalassemia trait (true positive), as seen in Table 6.

**Table 6: Results of the study population sent for HPLC.**

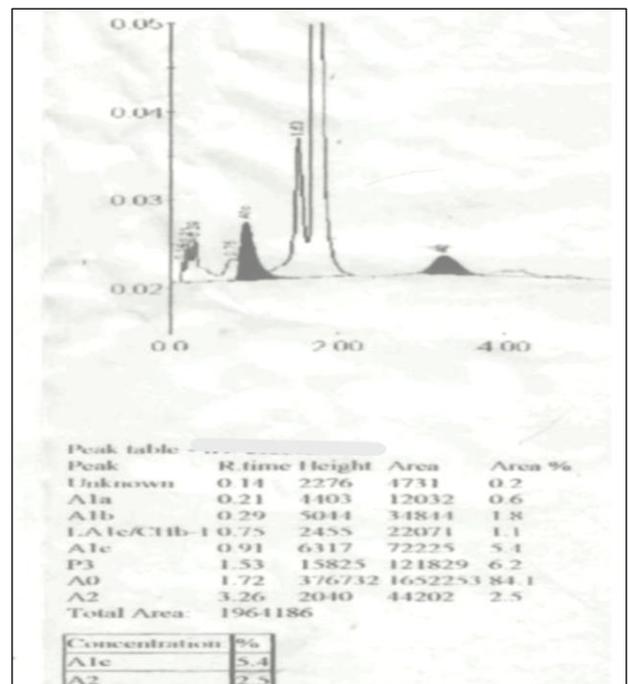
| Result from HPLC                    | Number of cases |
|-------------------------------------|-----------------|
| Positive for beta thalassemia trait | 96              |
| Negative for beta thalassemia trait | 28              |
| Total number of cases               | 124             |

HbA2 peaks above 3.5% were taken as positive for beta thalassemia trait. Figure 1 and 2 shows sample HPLC reports of patients flagged as positive and negative for beta thalassemia trait.

The cases that proved to be positive for beta thalassemia trait by HPLC, showed the following mean values in the RBC indices: Hb showed a mean value of 10.04g/dl with a standard deviation of 0.67. RBC count showed a mean value of 5.51x10<sup>6</sup>/cumm with a standard deviation of 0.54. MCV showed a mean value of 66.1fl with a standard deviation of 4.06. MCH showed a mean value of 18.44pg with a standard deviation of 2.46. Hematocrit showed a mean value of 36.48% with a standard deviation of 4.72. MCHC showed a mean value of 28.04g/dl with a standard deviation of 4.46. RDW showed a mean value of 12.8% with a standard deviation of 0.69.



**Figure 1: HPLC report shows a case positive for beta thalassemia trait.**



**Figure 2: A negative HPLC report, disproving the flagging by CBC indices.**

The cases that were shown to be negative for beta thalassemia trait by HPLC, showed the following range and mean values in the RBC indices: Hb showed a mean value of 10.13g/dl with a standard deviation of 0.59. RBC count showed a mean value of 5.24x10<sup>6</sup>/cumm with a standard deviation of 0.68. MCV showed a mean value of 66.24fl with a standard deviation of 5.74. MCH showed a

mean value of 19.72 pg with a standard deviation of 3.32. Hematocrit showed a mean value of 34.59% with a standard deviation of 4.97. MCHC showed a mean value of 29.88g/dl with a standard deviation of 4.78. RDW showed a mean value of 12.98% with a standard deviation of 0.86. The statistical analysis of the red cell indices are given in Table 7.

**Table 7: Statistical analysis of the accuracy of the red cell indices used in the study.**

| RBC indices                  | Sensitivity | Specificity | PPV   | NPV   | Accuracy | Youden's index | Area under curve | Std error | P value |
|------------------------------|-------------|-------------|-------|-------|----------|----------------|------------------|-----------|---------|
| <b>Mentzer index</b>         | 95.83       | 21.43       | 80.7  | 60.0  | 58.63    | 17.26          | 0.586            | 0.066     | 0.166   |
| <b>Sehgal index</b>          | 94.79       | 25.00       | 81.25 | 58.33 | 59.90    | 19.79          | 0.599            | 0.066     | 0.112   |
| <b>Green and King index</b>  | 93.75       | 14.29       | 78.95 | 40.0  | 54.02    | 8.04           | 0.540            | 0.064     | 0.519   |
| <b>Ricera Index</b>          | 97.92       | 10.71       | 78.99 | 60.0  | 54.32    | 8.63           | 0.543            | 0.065     | 0.488   |
| <b>Shine &amp; Lal index</b> | 100.00      | 3.57        | 78.05 | 100.0 | 51.79    | 3.57           | 0.518            | 0.063     | 0.774   |
| <b>RDWI</b>                  | 98.96       | 7.14        | 78.51 | 66.67 | 53.05    | 6.10           | 0.531            | 0.064     | 0.624   |
| <b>Srivastava Index</b>      | 85.42       | 14.29       | 77.36 | 22.22 | 49.85    | -0.30          | 0.499            | 0.062     | 0.981   |
| <b>Ehsani index</b>          | 76.04       | 42.86       | 82.02 | 34.29 | 59.45    | 18.90          | 0.594            | 0.063     | 0.129   |
| <b>Sirdah index</b>          | 23.96       | 71.43       | 74.19 | 21.51 | 47.69    | -4.61          | 0.477            | 0.063     | 0.711   |
| <b>Bordbar index</b>         | 96.88       | 14.29       | 79.49 | 57.14 | 55.58    | 11.16          | 0.556            | 0.065     | 0.370   |
| <b>HH index</b>              | 98.96       | 7.14        | 78.51 | 66.67 | 53.05    | 6.10           | 0.531            | 0.064     | 0.624   |
| <b>Kandhro 2 index</b>       | 98.96       | 10.71       | 79.17 | 75.00 | 54.84    | 9.67           | 0.548            | 0.065     | 0.437   |
| <b>Kerman 1 index</b>        | 91.67       | 14.29       | 78.57 | 33.33 | 52.98    | 5.95           | 0.530            | 0.064     | 0.633   |
| <b>Kerman 2 index</b>        | 73.96       | 42.86       | 81.61 | 32.43 | 58.41    | 16.82          | 0.584            | 0.063     | 0.177   |
| <b>Zaghloul 1 index</b>      | 54.17       | 67.86       | 85.25 | 30.16 | 61.01    | 22.02          | 0.610            | 0.060     | 0.077   |
| <b>Zaghloul 2 index</b>      | 63.54       | 42.86       | 79.22 | 25.53 | 53.20    | 6.40           | 0.532            | 0.063     | 0.607   |

Shine and Lal index showed the highest sensitivity and negative predictive value of 100% while Zaghloul 1 index showed the highest specificity of 67.86%, accuracy of 61.01% and positive predictive value of 85.25%.

## DISCUSSION

In this study, Shine and Lal index showed the highest sensitivity of 100%, followed by RDWI and Kandhro index (98.9%).

Sirdah index showed the highest specificity of 71.4% followed by Zaghloul 1 index showing specificity of 67.8%. The low specificity of the indices in this study could be attributed to the fact that only a limited number of cases flagged negative by the CBC indices were selected. The confirmatory tests done for these patients would not have been of any benefit to the patients.

In this study, Zaghloul 1 index showed the highest positive predictive value of 85.2% followed by Ehsani index (82%). Shine and Lal index showed the highest negative predictive value of 100% followed by Kandhro index (75%).

The study conducted by Al Fadhli et al in Kuwait showed Ricera index and Mentzer index to have the highest sensitivity (85.7%) while Shine and Lal index showed the highest specificity (97.8%).<sup>19</sup> The study conducted by George Ntaios in Europe showed Green and King index to have the highest sensitivity of 75%, whereas the study conducted by Rathod et al in the same year showed highest sensitivity for Shine and Lal index (99%) followed by Ricera index (92.9%).<sup>4</sup> Highest specificity was seen in Mentzer index and Srivastava index(100%).<sup>20</sup>

The study conducted by Diplani et al in Amritsar showed Mentzer index to have the highest sensitivity (89%) and specificity of 87.9%. Positive predictive value of Mentzer index was 83.2%.<sup>21</sup> Study conducted by Niazi et al in the same year in Pakistan showed Ricera index to have the highest sensitivity (92%) while Shine and Lal had the highest specificity (100%) and positive predictive value (100%). Ricera index had the highest negative predictive value of 82%.<sup>22</sup>

The study conducted by Shen et al in China in the year 2010 showed Shine and Lal index to have the highest sensitivity of 99.2%, HH index showed specificity of 95.4% and highest predictive value of 100%. Sirdah index showed the highest negative predictive values of 93.5%.<sup>23</sup> Study done by Eloisa et al in Spain in the year

2011 showed 100% sensitivity for Shine and Lal index while Green and King index showed highest specificity of 95.7%.<sup>24</sup>

A study done by Sehgal et al in Maharashtra in the year 2015 showed Shine and Lal to have the highest sensitivity (97%) while Mentzer index had the highest specificity (92%).<sup>5</sup> The study done by Sirdah et al in Palestine in the year 2018 showed Sirdah index to have the highest sensitivity of 84.3% while Shine and Lal index showed the highest specificity of 94.1% and positive predictive value of 99%. Srivastava index was found to have the highest negative predictive value of 87%.<sup>18</sup>

Study done by Jahangiri et al in Iran in the year 2019 showed the Shine and Lal index to have the highest sensitivity of 100% and Sirdah index to have the highest specificity of 88.65%.<sup>25</sup>

Thus, by comparing all the results of the studies done and the present study, it is evident that the reliability of the indices varies depending on the population on which it is used. Nevertheless, it can be concluded without doubt that once the indices for the population are worked out, the indices are very reliable to be used as the initial flagging of beta thalassemia trait. All CBC analyzers should have the indices programmed into them to avoid unnecessary iron therapy (and subsequent iron overload) and also to facilitate genetic counselling.

This study has certain limitations. First and foremost, the hospital-based study population may not accurately represent the general population. Second, only a small number of cases that were flagged as negative for  $\beta$ -thalassemia trait by red cell indices were included in the assessment, which likely contributed to the low specificity observed for several indices. Additionally, iron studies were not performed in the study population, which may have resulted in misclassification of iron-deficiency anemia. Iron deficiency can also alter red cell indices and may also influence HPLC patterns, thereby potentially affecting the interpretation of results. Finally, the relatively small sample size may have affected the precision of sensitivity and specificity estimates. Larger, population-based studies including iron profile assessment are required to validate these findings.

## CONCLUSION

The differentiation between IDA and  $\beta$ TT is important because anaemia will not improve in  $\beta$ TT if it is misdiagnosed as IDA. Also, iron therapy prescribed by the physician might result in toxicity. Therefore, it is important to flag the population with possible beta thalassemia trait. However, it is difficult to obtain accurate prevalence within different populations. This is due to limitations in diagnostic testing, as well as the fact that many studies have focused on small, biased hospital populations. This emphasizes the need of including the indices in routine hematology examinations after a

population based study is done for analyzing the accuracy of the indices in the specific population.

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