

## Unresolved laboratory issues of the heterozygous state of $\beta$ -thalassemia: a literature review

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## **TITLE PAGE**

### **Unresolved laboratory issues of the heterozygous state of $\beta$ -thalassemia: a literature review**

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***Running head:*** Laboratory issues of beta-thalassemia trait

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## **Abstract**

Although considered a mild clinical condition, many laboratory issues of the carrier state of beta-thalassaemia remain unresolved. Accurate laboratory screening of beta-thalassaemia traits is crucial for preventing the birth of a beta-thalassaemia major child. Identification of carriers in the laboratory is affected by factors that influence red cell indices and HbA2 quantification. Silent mutations and co-inheriting genetic and non-genetic factors affect red cell indices which decreases the effectiveness of the conventional approach. Similarly, the type of beta mutation, co-inheriting genetic and non-genetic factors, and technical aspects, including the analytical method used and variations in the HbA2 cutoff values, affect the HbA2 results leading to further confusion. However, the combination of MCV, MCH and haemoglobin analysis increases the diagnostic accuracy. Diagnostic problems arising from non-genetic factors can be eliminated by carefully screening the patient's clinical history. Still, issues due to certain genetic factors, such as Krüppel-like factor 1 gene mutations and alpha triplication remain unresolved. Each laboratory should determine the population-specific reference ranges and be wary of machine-related variations of HbA2 levels, the prevalence of silent mutations in the community.

**Keywords:** Beta-thalassaemia trait, Beta-thalassaemia minor, Heterozygous state of beta-thalassaemia, Diagnostic problems, Laboratory issues, HbA2 Cutoff.

## **Introduction**

Beta-thalassaemia is one of the commonest hereditary blood disorders.<sup>1</sup> It is characterized by the reduction of ( $\beta^+$ ) or absence of ( $\beta^0$ ) synthesis of the beta globin chains of the haemoglobin (Hb) tetramer. More than 200 different mutations of beta globin genes are currently recognized.<sup>1</sup> Most of the mutations are single nucleotide substitutions, deletions, or insertions of oligonucleotides leading to frameshifts. Rarely beta-thalassaemia results from gene deletions. Clinical severity could be affected by the mutation type.<sup>2</sup>

Based on clinical and haematological severity, there are three main forms of beta-thalassaemia syndromes, *i.e.*, the beta-thalassaemia carrier (trait) state, thalassaemia intermedia (non-transfusion dependent thalassaemia), and thalassaemia major (transfusion dependent thalassaemia). The current review would focus on beta-thalassaemia carrier state (BTT).

It has been estimated that about 1.5% of the global population (80 to 90 million people) are carriers of beta-thalassaemia.<sup>3</sup> Beta-thalassaemia traits have a 25% risk of having children affected with thalassaemia major if their partner too is a carrier. World Health Organization (WHO) emphasizes the importance of incorporating carrier screening in basic health services in countries with a high incidence of thalassaemia.<sup>4</sup>

Haematology laboratories play a vital role in carrier identification. Screening thalassaemia traits using blood analysis has two methodological approaches;

1. Primary screening followed by a secondary screening
2. Complete screening

In the former approach, only subjects with reduced mean corpuscular volume (MCV) and/or mean corpuscular haemoglobin (MCH) will be assessed for haemoglobin patterns and HbA<sub>2</sub> levels. In contrast, haemoglobin analysis is done for all subjects from the outset in the latter.<sup>5</sup> The first approach has notable disadvantages over its limited advantages, *i.e.*, low cost. In the presence of mild/silent mutations, beta promoter mutations, alpha thalassaemia and HbD and/or beta variants; beta-thalassaemia carriers may show normal/marginal red cell indices.<sup>6</sup> In addition, megaloblastic anaemia and hereditary persistence of fetal haemoglobin (HPFH) too can mask the microcytosis of beta-thalassaemia carriers.<sup>7,8</sup> Other than these clinical conditions, technical problems such as sample storage can affect MCV results. Thus the first approach will misdiagnose a proportion of beta-thalassaemia carriers. The second approach is also not foolproof. High-Performance Liquid Chromatography (HPLC), the conventional method, and Capillary Electrophoresis (CE) are two techniques used for quantifying HbA<sub>2</sub>. When the same sample is analyzed using both techniques, machine-related variations in HbA<sub>2</sub> have been reported.<sup>9,10</sup> Factors such as  $\delta$ -haemoglobinopathies,  $\delta\beta^0$ -thalassaemia,  $\alpha$ -thalassaemia, triple-alpha, haemoglobin variants, iron deficiency anaemia (IDA), hyperthyroidism, megaloblastic anaemia, HPFH, antiretroviral drugs, and Krüppel-like factor 1 (*KLF1*) gene mutations can affect the HbA<sub>2</sub> levels.<sup>8,11-12</sup> Labeling a person as BTT based on HbA<sub>2</sub> level without considering the above factors may be precarious. An HbA<sub>2</sub> cutoff of 3.5% is generally used for the diagnosis of BTT,<sup>13</sup> though different reference ranges for HbA<sub>2</sub> are used in some countries.<sup>14</sup> HbA<sub>2</sub> values between the upper limit of the normal range and the cutoff value are considered borderline. The diagnosis of borderline samples is usually made only by doing confirmatory genetic tests such as PCR and beta gene sequencing. The current review aims to discuss these unresolved problems in BTT condition pertaining to the laboratory aspects.

## **Materials and Methods**

### **Search strategy**

We searched databases of MEDLINE via Pub Med, Google Scholar and Taylor & Francis for research studies published in English for the past 20 years (October 2002 to September 2022) using the following keywords in many combinations: Beta-thalassaemia trait, Beta-thalassaemia minor, Heterozygous state of beta-thalassaemia, Screening carriers, Diagnostic problems, Laboratory issues, HbA2, Borderline, Cutoff, Techniques, Iron deficiency.

### **Inclusion criteria**

Prospective, descriptive and retrospective studies, including laboratory issues of the beta-thalassaemia carrier state in different countries of the world (India, China, Italy, Thailand, Iran, Saudi Arabia, Turkey, Egypt, Malaysia, US, UK, Bangladesh, Pakistan, Canada, Netherland, UAE, Portugal, Iraq, Bahrain, Singapore and Spain) were included in the present review.

### **Exclusion criteria**

Studies including beta-thalassaemia traits that did not describe problems in laboratory diagnosis were excluded. In addition, case reports, abstracts, reviews, unpublished studies and duplicates of previously included studies were excluded from the present review.

### **Data Extraction**

Two researchers (ST and UPJ) independently reviewed all abstracts of journal articles gathered by web search to identify articles that required full-text review. All selected articles were discussed with a third independent reviewer (AP). Data on study design, objectives, methodology, and results of the selected articles were extracted and methodically reviewed.

## **Results**

Through the investigation strategy, we identified 810 citations, from which 68 articles were selected for qualitative synthesis (Figure 1). The study designs excluding case studies, abstracts and reviews, included prospective studies, retrospective studies and short communication. Out of 68 articles, 14 (20.6%) originated from India, 10 (14.7%) from Thailand, 9 (13.2%) from Italy, 8 (11.8%) from China, 6 (8.8%) from Iran, 2 (2.9%) each from Malaysia, Saudi Arabia, Pakistan, Netherland and UK, 1 (1.5%) each from Sri Lanka, Bangladesh, Bahrain, Singapore, Egypt, UAE, Portugal, Iraq, Canada, U.S., Turkey and Spain.

Of 68 studies, 7 studies (10.3%) have analyzed the effectiveness of the conventional approaches of beta-thalassaemia carrier screening. There were 60 studies (77.9%) focused on factors affecting RBC indices (37 studies) and/or HbA2 value (43 studies). Only 1 study (1.5%) assessed the conventional cutoff values used in BTT diagnosis.

### **1. Effectiveness of the conventional approaches**

Out of 7 studies that analyzed the effectiveness of conventional haematological-analysis-based approaches of beta-thalassaemia carrier screening, 57.1% (n=4) compared the effectiveness of MCV and MCH values in diagnosing BTT based on the first approach.<sup>17-20</sup> Sensitivity, specificity, positive predictive values, negative predictive values and false negatives in different combinations have been used for comparisons. Of 4 studies, 3 concluded that MCH is more appropriate than MCV,<sup>17,18,20</sup> while one concluded that MCV is more appropriate than MCH.<sup>19</sup> Out of 7 studies, 14.3% (n=1) showed that using Hb analysis as the first test is unreliable in detecting beta-thalassaemia traits.<sup>21</sup> About 28.6% (n=2) compared the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy among the conventional two approaches for thalassaemia carrier detection.<sup>22,23</sup> According to both studies, the combination of MCV, MCH and Hb analysis resulted in high sensitivity, specificity and diagnostic accuracy.

### **2. Factors affecting MCV and MCH among beta-thalassaemia carriers**

Of 37 studies regarding factors affecting RBC indices, 67.6% (n=25) identified silent or mild beta-thalassaemia carriers with normal or marginal MCV and/or MCH. Of 25 studies, 6 (24%) originated from Italy,<sup>6,24-27</sup> 5 (20%) from India,<sup>14,29-32</sup> 4 (16%) from China,<sup>33-36</sup> 2 (8%) each from Thailand,<sup>37,38</sup> Iran<sup>39,40</sup> and Pakistan,<sup>41,42</sup> 1 (4%) each from Turkey,<sup>43</sup> Bahrain,<sup>44</sup> Malaysia<sup>45</sup> and Spain<sup>46</sup>. Silent or mild beta-thalassaemia mutations reported from different countries are listed in the table 1.

Out of 37 studies, 13.5% (n=5) analyzed the effect of the coinheritance of alpha and beta-thalassaemia on MCV and MCH.<sup>36,37,47-49</sup> SEA type was the most common alpha thalassaemia type that coexisted with beta-thalassaemia in three studies from Thailand, China and Singapore while 3.7 kb deletion was the most common type in the other two studies from Thailand and Iran. Of 5 studies, 4 (80%) reported a significant increase in MCV and MCH values among heterozygotes for alpha and beta-thalassaemia compared to the simple beta-thalassaemia carriers.<sup>36,37,48,49</sup> The same 4 studies revealed that this increase in MCV and

MCH values is highest when one or two alpha globin gene deletions coexisted with beta-thalassaemia than in other alpha gene arrangements. However, in all 4 studies, mean values of MCV and MCH in heterozygotes for alpha and beta-thalassaemia were less than 80 fl and 27 pg. Out of 37 studies, 2.7% (n=1) showed the effect of fetal haemoglobin in HPFH on MCV and MCH of BTT.<sup>8</sup> The study showed significant increase on MCH and MCV values, accounting for 29% and 30% of their respective variances. This effect was highest in individuals who have inherited two copies of the HPFH quantitative trait loci (QTL), least in those without HPFH, and intermediate in subjects with only one copy of the QTL. However, mean values of MCV and MCH were less than 80 fl and 27 pg even in individuals who have inherited two copies of the HPFH QTL.

We observed some non-genetic factors affecting MCV and MCH in the present review. Out of 37 studies, 2.7% (n=1) focused on normalized red cell parameters of BTT in patients with megaloblastic anaemia.<sup>7</sup> Treatment of Vitamin B<sub>12</sub> and folic acid reduced the mean MCV of patients from 85.28±12.49 to 74.45±9.74. According to 13.5% studies (n=5), antiretroviral therapy (ART) causes an increase in MCV and MCH among HIV-infected patients.<sup>50-54</sup> Pornprasert *et al.* (2009) revealed that at 6 and 12 months after the ART therapy, the mean MCV of beta-thalassaemia patients shifted from microcytic level (<80 fL) to normocytic level (80–100 fL).<sup>52</sup> Mean MCH increased to normal levels (27–31 pg).

### **3. Factors affecting HbA2**

Of 43 studies focusing on factors affecting HbA2 levels, 34.9% (n=15) reported the type of beta-thalassaemia mutation itself as one of the factors associated with reduced HbA2 level among beta-thalassaemia carriers. Four studies (26.7%) from Thailand,<sup>38,55,56,57</sup> 3 (20%) from Italy,<sup>6,11,58</sup> 2 (13.3%) from China,<sup>35,36</sup> 2 (13.3%) from India,<sup>14,32</sup> 1 (6.7%) each from Iran,<sup>40</sup> Malaysia,<sup>59</sup> Saudi Arabia,<sup>60</sup> and Portugal<sup>61</sup> reported different types of beta-thalassaemia mutations resulting in borderline or normal HbA2 levels in beta-thalassaemia carriers.

Out of 43 studies, 39.5% (n=17) investigated the effect of alpha thalassaemia on HbA2 level. Of 17 studies, 2 (11.8%) compared pure alpha-thalassaemia traits and normal individuals and both reported reduced HbA2 levels in alpha-thalassaemia traits.<sup>62,63</sup> One study reported that the reduction in HbA2 expression depends on the number of alpha-globin gene defects.<sup>62</sup> Co-inheritance of beta thalassaemia with Hb Constant Spring was associated with lower HbA2 expression when compared to other types of alpha thalassaemia.<sup>62</sup> Out of 17 studies, 47.1%

(n=8) compared HbA2 levels between beta-thalassaemia traits and beta-thalassaemia traits co-inherited with alpha-thalassaemia.<sup>36-38,47-49,57,62</sup> Although 4 studies (50%) observed a reduction in HbA2 level when alpha thalassaemia coexists,<sup>36,37,49,57</sup> only two studies (25%) reported a possible effect of it on BTT diagnosis.<sup>49,57</sup> Of these two, one showed that HbA2<4.0% can happen when Hb H disease (alpha-thalassaemia intermedia) coexists with the BTT.<sup>57</sup> Surprisingly, out of 17 studies, 47.1% (n=8) reported a marginal increase in HbA2 levels among alpha-thalassaemia traits without beta-thalassaemia heterozygosis.<sup>6,11,35,40,56,57,59,60</sup> None of the studies could explain the underlying mechanism.

Of the studies that investigated factors affecting HbA2, 13.9% (n=6) from 4 different countries, *i.e.* Italy,<sup>6,11,58</sup> India,<sup>64</sup> Thailand,<sup>62</sup> and Portugal<sup>61</sup> identified that coinheritation of delta-haemoglobinopathy decrease the HbA2 level in beta-thalassaemia carriers resulting in misdiagnosis. About 11.6% (n=5) evaluated the effect of alpha gene triplication on the HbA2 level. Out of 5, 4 reported the presence of  $\alpha\alpha\alpha$  condition in healthy individuals with borderline increased HbA2 levels but without a beta globin mutation.<sup>6,11,35,36</sup> Only 1 study said alpha gene triplication does not affect haematological parameters.<sup>65</sup> Similarly, out of 43 studies, only 2.3% (n=1) found that co-inherited HPFH in BTT reduced HbA2 levels.<sup>8</sup> The effect increased with the number of copies of the HPFH allele.

Similarly, 18.6% (n=8) eligible studies commented on the effect of *KLF1* gene mutations. *KLF1* or Erythroid Kruppel-Like Factor is a zinc-finger transcription factor that has different functions in erythropoiesis including modifying chromatin architecture, regulating beta-like globin gene switching, and activating or repressing gene transcription.<sup>66</sup> Of 8 studies on the effect of *KLF1* gene mutations, 7 (87.5%) observed a high prevalence of *KLF1* gene defects among individuals with borderline increased HbA2 levels without an *HBB* gene mutation.<sup>12,28,35,36,56,67,68</sup> They concluded a possible effect of *KLF1* gene mutations on increased HbA2 levels among normal individuals. Only 1 study (12.5%), which was a cross-sectional analysis in Saudi Arabia, reported that *KLF1* gene mutations are not only associated with borderline high HbA2 results but also normal (HbA2< 3%) and High (HbA2>4.3%) results.<sup>60</sup> Out of 7 studies which determined the effect of *KLF1* gene mutations on HbA2 level, the studies of Perseu *et al.* (2011), Hariharan *et al.* (2019) and Liu *et al.* (2014) revealed 0.8%, 1.1% and 0.5% increase in HbA2 level among individuals with *KLF1* gene mutations when compared to the group without *KLF1* gene mutations.<sup>12,67,68</sup> Interestingly, Liu *et al.* (2014) had shown a high prevalence of *KLF1* genes in beta-thalassaemia endemic regions in China and an amelioration of the severity of beta-thalassaemia by *KLF1* gene



changes.<sup>68</sup> We were able to determine the commonest *KLF1* mutations in different countries. G176Afsx179 was the most common *KLF1* mutation in China and Thailand, while p ser. 270 and -148 (G<A) were common in Italy and India, respectively.

In the present study, we analyzed the effect of non-genetic factors on HbA2 level. Out of 43 studies, 20.9% (n=9) investigated the association between iron deficiency anaemia (IDA) and HbA2 levels. Of them, 4 studies (44.4%) determined the effect of IDA on HbA2 results of normal (non-thalassaemic) individuals.<sup>58,62,69,70</sup> All concluded that IDA causes a significant reduction in HbA2 levels in normal people. Eight studies (88.9%) compared the results of HbA2 among beta-thalassaemia traits with and without IDA. Out of eight, five (62.5%) showed significantly higher HbA2 values for beta-thalassaemia traits without IDA than in those with IDA.<sup>32,63, 69,71,72</sup> In the other three no difference was observed.<sup>58,70,73</sup> Of 5 studies that observed higher values, 3 (60%) suggested possible effects on beta-thalassaemia diagnosis,<sup>32,63,69</sup> while others showed no effect due to the steadying of all HbA2 values above the cutoff.<sup>71,72</sup> Of studies that suggested possible effects, one study proposed a dose effect of severity of iron deficiency on HbA2 level of beta-thalassaemia traits.<sup>63</sup>

Out of 43 studies, 4.6% (n=2) reported the effect of megaloblastic anaemia in elevated HbA2 levels among normal individuals without beta-thalassaemia.<sup>7,70</sup> One of the above studies reported a change of mean HbA2 of the study group from 4.56% to 3.81% with vitamin B<sub>12</sub> and folic acid treatments, whereas 93.75% of subjects diagnosed as BTT (HbA2>3.5%) before treatment became normal (HbA2<3.5%) after the treatment.<sup>7</sup> Among 43 studies that discussed factors affecting HbA2 levels, 2.3% (n=1) indicated the effect of thyroid hormones on the production of HbA2.<sup>74</sup> According to the results, HbA2 was significantly higher in hyperthyroid (mean; 2.77%) patients than in the controls (2.39%). However, this increase in HbA2 didn't pass the cutoff level of HbA2, giving less chance of misdiagnosing BTT.

Five studies analyzed the effect of antiretroviral therapy (ART) on HbA2 results.<sup>50-54</sup> All of them showed an increase in HbA2 levels among HIV-infected patients treated with ART leading to a misdiagnosis of BTT. Of 5 studies, 3 (60%) confirmed that zidovudine increased HbA2 levels.<sup>50,53,54</sup> Bhagat *et al.* (2015) evaluated the effect of 3 different drugs and concluded that both zidovudine and stavudine increase HbA2 levels, while tenofovir had no effect.<sup>50</sup>

In the present review, we observed technical issues due to the variations in analytical methods used to determine HbA2 level in beta-thalassaemia screening. Out of 43 studies, 6.9% (n=3) compared HbA2 results by different analytical systems in the presence of Hb variants.<sup>9,10,75</sup> All 3 studies determined falsely increased HbA2 in HPLC reports of patients with Hb E and Hb S variants. However, CE method clearly separated Hb E and HbA2. One study reported the variant Hb Lepore, which co-elutes with HbA2 in the HPLC method causing problems in diagnosis.<sup>75</sup> Similarly, when HPLC technique is used, falsely reduced HbA2 had been experienced in the presence of Hb D Punjab in two studies.<sup>9,75</sup> According to 2 studies HbA2 peak was included in Hb C peak when the CE method was used.<sup>9,10</sup> Interestingly, all three studies reported variations in HbA2 values between HPLC and CE methods when comparing HbA2 measurements in samples within the normal range. Two studies reported higher HbA2 values by Bio-Rad variant II HPLC method (mean, 2.95% and 2.67%, respectively) than Sebia CE method (mean, 2.49% and 2.51%, respectively).<sup>9,75</sup> On the contrary, one study reported HbA2 levels by the Sebia CE method (mean, 2.8%) was higher than that of the Primus HPLC method (mean, 2.3%).<sup>10</sup>

#### **4. Problems with HbA2 cutoff values**

In the present review, we analyzed the variations in cutoff values of HbA2 used in beta-thalassaemia screening. Out of 68 studies, 54.4% (n=37) articles (excluding studies based on borderline HbA2 values) clearly mentioned the HbA2 cutoff value used in diagnosing beta-thalassaemia carriers. Of 37 articles, the majority (48.6%; n=18) used 3.5% as the cutoff value, while 32.4% (n=12) of studies used 4.0% as the cutoff. Eighteen studies that used 3.5% as the cutoff include 5 from China,<sup>20,23,34,36,72</sup> 5 from Iran,<sup>17,18,39,49,69</sup> 2 from Thailand,<sup>48,55</sup> 1 each from India,<sup>53</sup> Bangladesh,<sup>22</sup> Saudi Arabia,<sup>21</sup> Portugal,<sup>61</sup> UAE<sup>63</sup> and UK<sup>54</sup>. Similarly, 12 studies that used 4.0% as the cutoff include 4 from India,<sup>7,19,29,50</sup> 4 from Thailand,<sup>37,51,52,57</sup> 1 each from Malaysia,<sup>45</sup> Bahrain,<sup>44</sup> Italy<sup>26</sup> and Netherland<sup>75</sup>. 3.9% was the cutoff for 3 studies from India (n=2)<sup>70,73</sup> and Italy (n=1)<sup>12</sup>. Two studies from Italy<sup>28</sup> and Turkey<sup>43</sup> used 3.8% as the cutoff. 3.4%, 3.6% and 3.7% were used as the cutoff values by 3 separate studies from Italy,<sup>58</sup> Thailand<sup>38</sup> and India,<sup>30</sup> respectively.

Abdel-Messih *et al.* (2015) determined the effectiveness of the 2 most common cutoff values used in BTT screening.<sup>76</sup> The results revealed the sensitivity (100.0% and 97.4%), specificity (70.0% and 72.7%), positive predictive value (75.0% and 92.6%), negative predictive value

(100.0% and 88.8%) and accuracy (70.0% and 92.0%) to identify beta-thalassaemia carriers at 3.5% and 4.0% cutoff values, respectively.

There were 16 (23.5%) studies which analyzed borderline HbA2 results in BTT diagnosis. Nine different borderline ranges were identified among these studies (Table 2).

## **Discussion**

The diagnosis of beta-thalassaemia using red cell indices as the primary screening or using HPLC or CE as the first test both seem to have deficiencies in their own right. In the first approach, MCV and MCH are crucial haematological parameters to rule out possible beta-thalassaemia traits. Several studies reported MCH is more appropriate than MCV.<sup>17,18,20</sup> Most of these studies generate sensitivity figures based on their own cutoff values for MCV and MCH. MCH is considered to be more stable in ambient temperature while MCV increases with time after being stored for several hours before testing.<sup>77</sup> This stability of MCH may be the reason for its higher sensitivity. Some clinicians use only MCV to exclude beta-thalassaemia in the primary screening.<sup>38</sup> In such cases, falsely high MCV results due to stored samples may give misleading results. Therefore, it is important to consider not one but both MCV and MCH results in screening for beta-thalassaemia. Reduction in any parameter should be considered as having diagnostic importance.

One of the main disadvantages of using red cell indices for screening includes the possibility of missing silent beta-thalassaemia carriers, as they may have normal red cell indices. Silent beta-thalassaemia mutations exist not only in codons but also in the promoter region and/or/cap region of the HBB gene.<sup>26,30,43,78</sup> Screening for thalassaemia based on red cell indices will also result in missing individuals with Hb D trait and individuals with triple alpha genes.<sup>79</sup> Although phenotypically not important and globally rare, the coinheritance of the above conditions with beta-thalassaemia mutations leads to severe phenotype in the offspring.

In the present review, we analyzed the multitude of factors that may affect the HbA2 levels; thus the diagnosis of (Table 2). The problems may arise in two circumstances, *i.e.*, decreased HbA2 levels in beta-thalassaemia traits or deceptively elevated HbA2 levels in non-carriers. The type of beta-thalassaemia mutation is the main reason for reduced or borderline HbA2 levels among carriers.<sup>32</sup> Silent or mild beta-thalassaemia mutations show reduced/borderline

HbA2 levels along with normal or slightly reduced RBC indices. Most of the time, homozygotes for classic and silent beta-thalassaemia mutations have thalassaemia intermedia phenotype.<sup>78,80</sup> Unfortunately, silent carriers will be missed until the birth of a child with  $\beta$  thalassaemia intermedia.

Coinheritance of alpha thalassaemia with beta-thalassaemia results in decreased HbA2 levels due to the unavailability of free alpha chain pool to partner with  $\delta$ -globin chains.<sup>2</sup> This reduction seems insufficient to cause problems in BTT diagnosis<sup>38,62</sup> unless mild or silent beta-thalassaemia coexists. Sathakar *et al.* (2020) showed HbA2 less than 4.0% only in Hb H and Hb H-CS diseases.<sup>57</sup> Since both these conditions are apparent in HPLC patterns, beta-thalassaemia traits will not be missed. Surprisingly, borderline increased HbA2 levels also have been identified among individuals with alpha thalassaemia but without beta-thalassaemia mutations.<sup>35,57,60</sup> The exact reason for that is still not discovered, while some researchers consider regulation factors or mutations in regulatory genes as causes.<sup>35,40</sup>

Coinheritance of delta-haemoglobinopathy<sup>62</sup> and HPFH<sup>8</sup> cause reduced HbA2 levels in beta-thalassaemia carriers. The synthesis of insufficient amounts of  $\delta$ -globin monomers results in lower HbA2 levels in  $\delta$  haemoglobinopathy. On the other hand, co-inheritance of heterocellular HPFH leads to a primary increase in  $\gamma$ -chain synthesis resulting in high HbF levels.<sup>8</sup> The reason for decreased HbA2 level is not clear.

The effect of alpha-triplication and *KLF1* gene mutations on increased HbA2 levels is contradictory. Alpha-triplications are considered uncommon because de novo crossover events generating triplications are rare.<sup>65</sup> *KLF1* gene has a regulatory effect on erythropoiesis.<sup>81</sup> Perseu *et al.* (2013) first identified a delay in the transcriptional switch from the *HBD* to the *HBB* gene by *KLF1* mutations.<sup>12</sup> Majority of the studies regarding alpha-triplication and *KLF1* gene mutations in the current review reported possible effects on increased HbA2 levels. Most of these studies have specifically selected cohorts with borderline HbA2 results. Because of the marginally increased HbA2 levels and absence of beta-thalassaemia mutations, possible effects are suspected. Among eligible studies, there were a few cross-sectional studies, including in the general population, but they revealed no association between increased HbA2 level and alpha-triplication/*KLF1* mutations.<sup>60,65</sup> Therefore, larger scale of studies, including the general population, is necessary to gather more comprehensive and informative data.

IDA is the major cause of anaemia in the world.<sup>82</sup> Coexistence of IDA may decrease the HbA2 level in beta-thalassaemia traits. Intracellular lack of iron reduces  $\alpha$ -globin chain

synthesis than non- $\alpha$  globin chains. In beta-thalassaemia,  $\beta$  globin chain synthesis is also limited. Therefore, beta chains compete more effectively for  $\alpha$ -globin chains than  $\delta$ -globin chains, resulting in reduced levels of HbA2.<sup>83</sup> It is widely believed that IDA cannot reduce HbA2 levels of beta-thalassaemia traits below the cutoff values. Then, the effect of IDA on beta-thalassaemia diagnosis should be minimal unless in cases of severe IDA or silent/mild beta-thalassaemia mutations.

Megaloblastic anaemia, ART therapy and hyperthyroidism are other non-genetic factors which increase HbA2 levels in beta-thalassaemia traits. Both megaloblastic anaemia and long-term ART therapy (particularly, zidovudine and stavudine) affect red cell synthesis. Nuclear maturation of red cell precursors is delayed in megaloblastic anaemia, while antiretroviral medications inhibit nucleic acid synthesis.<sup>52</sup> Then, more Hb synthesis occurs in immature erythroid precursors. Since the synthesis of  $\delta$  chains is relatively greater in these cells, HbA2 level is increased.<sup>83</sup> Besides, thyroid hormones are considered to specifically increase the expression of  $\delta$  globin resulting in high HbA2 levels in hyperthyroidism.<sup>74</sup> No literature was found which demonstrated an increased HbA2 level above the cutoff in hyperthyroidism. Nevertheless a careful medical history of person is well warranted prior to beta-thalassaemia screening.

There are machine related variations in HbA2 results of normal individuals or beta-thalassaemia traits. When compared, Bio-Rad variant II HPLC method and Sebia CE method, consistently high results have been obtained from HPLC method,<sup>9,75</sup> although there is good correlation between the methods. When using a single cutoff value to diagnose beta-thalassaemia carriers, this will be a major problem especially in borderline HbA2 results. Therefore, when issuing a diagnosis of BTT in borderline values, machine-related variation of the HbA2 level should be borne in mind. The main reason for this unresolved problem is the unavailability of a standardization of HbA2 results between methods. Unavailability of a reference method and international reference HbA2 calibrators make this problem more complex.<sup>9</sup>

Also, in the present review, we identified marked differences in the HbA2 cut off values, (3.0-4.0%) used for diagnosis of BTT. Although it is recommended to define population or country specific cutoff values, sometimes, 2 or 3 different cutoffs have been used within a single country. Similarly, different authors have defined borderline ranges arbitrarily. In the present review, the percentages of beta-thalassaemia heterozygotes in each borderline group

ranged from 1.9%- 83.3%, which is very broad (Table 3). The reason may be the differences in inclusion/exclusion criteria of the study group and variations in analytical methods used to identify beta-thalassaemia mutations. However, it is noteworthy that all these beta-thalassaemia traits would be missed if  $HbA2 \geq 4.0\%$  was used as the cutoff. We, believe a cutoff with a higher sensitivity than specificity is suitable for screening to prevent missing beta-thalassaemia traits. If problems arise in accurate diagnosis of beta-thalassaemia heterozygosis due to the borderline results, the parental studies may provide prompt solutions. If not further genetic or molecular tests are necessary.

## **Conclusions and Recommendations**

Many laboratory issues of the heterozygous state of beta-thalassaemia remain unresolved. During the screening of beta-thalassaemia, both RBC indices and HbA2 level should be taken into consideration. Population specific reference ranges and machine related variations of HbA2 level should be determined within the laboratory. Borderline HbA2 levels should be further investigated for factors affecting HbA2 levels. Careful screening of patient's clinical history can identify most non-genetic factors that affect HbA2 screening. IDA and alpha thalassaemia are unlikely to affect the BTT diagnosis unless in individuals with silent  $\beta$  mutations. Based on the prevalence of silent mutations and other co-inheriting genetic factors, diagnostic approach of BTT should be modified for each population. For instance, screening the partners of patients with  $\beta$  thalassaemia heterozygosity for common silent beta mutations, HbE, Hb D, etc. is possible. However, effects of some genetic factors, such as *KLF1* gene mutations and alpha triplication on HbA2 level are still controversial, and further investigations are needed. Moreover, there may be other unidentified factors such as environmental factors, lifestyle related factors, regulation factors or mutations in regulatory genes affecting HbA2 level. Further studies should be expanded to identify those factors including large number of participants.

### **Practice points;**

1. Results of MCV, MCH and Hb analysis should be considered together prior to issuing a report on beta-thalassaemia trait. Changes in any parameter should be considered as having diagnostic importance.
2. Non-genetic factors such as IDA, (B12/Folate deficiency) megaloblastic anemia, ART therapy and hyperthyroidism are known to interfere with red cell indices and HbA2

levels. Thus questionnaires/forms filled by the health care workers prior to thalassaemia screening should include questions related to them.

3. The diagnostic approach should be modified based on the prevalence of any silent beta thalassaemia mutations or other genetic factors that affect HbA2 specific to the community.
4. Each laboratory should make decisions on beta thalassaemia carrier status based on the population-specific reference ranges and machine-related variations of HbA2 levels.

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**TABLES**

Country	Silent/mild mutations*
Italy	CAP+1570 (T>C), $\beta$ -101C $\rightarrow$ G, $\beta$ -101, $\beta$ +45 (G>C), $\beta$ -101(C $\rightarrow$ T), $\beta$ -92 (C $\rightarrow$ T), $\beta$ -54 (G $\rightarrow$ A), Poly A
India	Capsite +1 (A $\rightarrow$ C), CD 16 (-C), -88 (C $\rightarrow$ T), Poly A (T>C), IVS-1-5 (G>C)
Malaysia	codon 17 (A>T), IVS-1-5 (G>C)
Pakistan	CAP+1 (A>C)
Bahrain	-71 (C>T) -101 (C>T),
Turkey	HBB:c.*+108 A>G and HBB:c.*+132 C>T
Iran	-101 (C $\rightarrow$ T) , IVS II 844 (C $\rightarrow$ G), IVS I 128 (T>G)
China	-31 (A>C), -50 (G>A), -73 (A>T), -90(C>T), -88 (C>T)
Spain	-27 (A>T), -28 (A>G), -101 (C>T), -29 (A>G), -86 (C>A), -88 (C>T)
Thailand	NT-28 (A>G), NT-31 (A>G), NT-87 (C>A), NT-50 (G>A), CAP+1 (A > C), Codon 126 (T > G)

\*MCV/ mean MCV &gt; 70 fl

**Table 1.** Silent/mild beta-thalassaemia mutations causing normal or marginal MCV

	HPLC	CE
<b>Falsely increased HbA2</b>	<i>KLF1</i> gene mutation*	
	Megaloblastic anaemia*	
	Hyperthyroidism*	
	Antiretroviral therapy*	
	Hb E	
	Hb S	-
	Hb Lepore	
<b>Falsely decreased HbA2</b>	Alpha-thalassaemia*	
	Delta-haemoglobinopathy*	
	Hereditary persistence of fetal haemoglobin*	
	Iron deficiency anaemia*	
	Hb D Punjab	Hb C

\* These factors affect HbA2 results of both HPLC and CE methods

**Table 2.** Factors affecting HbA2 results of HPLC and capillary electrophoresis methods



Borderline Range (HbA2%)	Study	% Beta-thalassaemia heterozygotes	Remarks
3.0-3.9	Rosnah <i>et al.</i> , 2017	30.8% (36/117)	10 (27.0%) had HbA2 level of 3.0%.
	Colaco <i>et al.</i> , 2022	72.6% (149/205)	20 of 149 [14.0%] of all beta-thalassaemia traits would be missed if MCV < 80 fL and HbA2 ≥ 3.5% were used as cutoff.
3.0-4.0	Rangan <i>et al.</i> , 2011	32% (8/25)	All had HbA2 levels ranging from 3.5%–3.9%
3.1-3.9	Borgio <i>et al.</i> , 2017	83.3% (60/72)	
	Giambona <i>et al.</i> , 2008	14.8% (61/410)	<b>Exclusion criteria:</b> Iron deficiency anemia & Hb variants 20, out of 61 beta-thalassaemia carriers had MCV>80fl.
	Chaweephisal <i>et al.</i> , 2018	5.6% (6/106)	<b>Exclusion criteria:</b> MCV>80fl
	Moradi <i>et al.</i> , 2022	36.4% (159/436)	<b>Exclusion criteria:</b> MCV>80fl MCH>27pg
3.3-3.7	Mosca <i>et al.</i> , 2008	16.2% (38/234)	Majority with MCV>80
3.3-3.9	Hariharan <i>et al.</i> , 2019	Not mentioned	
3.3-4.0	Lou <i>et al.</i> , 2014	2.4% (4/165)	
3.3-4.1	Perseu <i>et al.</i> , 2011	Not mentioned	
	Paglietti <i>et al.</i> , 2015	18.9 (7/37)	
3.5-3.9	Jiang <i>et al.</i> , 2018	17.7% (11/62)	
	Satthakarn <i>et al.</i> , 2020	5.33% (16/300)	
3.5-4.0	Rungsee <i>et al.</i> , 2016	1.9% (3/158)	Tested only for beta <sup>0</sup> mutations
	Srivorakun <i>et al.</i> , 2020	10.9% (22/202)	

**Table 3.** Nine different borderline ranges for HbA2 in diagnosing beta-thalassaemia traits by 16 studies included in the present review

## **FIGURES**

**Figure 1. Flow chart of article selection for the systematic review**

824 records identified through  
selected database searching

12 records identified through  
other sources

810 records after removing duplicates

688 records removed  
after screening

122 full-text articles assessed for eligibility

54 full-text articles  
excluded with reasons

68 articles included in the review