REVIEW ARTICLE

Unresolved laboratory issues of the heterozygous state of β-thalassemia: a literature review

Shyamali Thilakarathne,[1,2] Udayanga P. Jayaweera[3] and Anuja Premawardhena[4]

[1]Faculty of Graduate Studies, University of Kelaniya, Dalugama; [2]Department of Medical Laboratory Science, Faculty of Allied Health Sciences, University of Peradeniya, Peradeniya; [3]Divisional Hospital, Medawala-Haarispatthuwa and [4]Department of Medicine, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka

Correspondence: S. Thilakarathne
shyamali5thilakarathne@gmail.com
shyamali.thilakarathne@ahs.pdn.ac.lk

Received: February 15, 2023.
Accepted: May 19, 2023.
Early view: June 1, 2023.

https://doi.org/10.3324/haematol.2022.282667

Abstract

Although considered a mild clinical condition, many laboratory issues of the carrier state of β-thalassemia remain unresolved. Accurate laboratory screening of β-thalassemia traits is crucial for preventing the birth of a β-thalassemia 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 β mutation, co-inheriting genetic and non-genetic factors, and technical aspects, including the analytical method used and variations in the HbA2 cut-off values, affect the HbA2 results, leading to further confusion. However, the combination of mean corpuscular volume, mean corpuscular hemoglobin, and hemoglobin analysis increases the diagnostic accuracy. Diagnostic problems arising from non-genetic factors can be eliminated by carefully screening the patient's clinical history. However, issues due to certain genetic factors, such as Krüppel-like factor 1 gene mutations and α triplication still 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.

Introduction

β-thalassemia is one of the most common hereditary blood disorders.[1] It is characterized by the reduction of (β^+) or absence of (β^0) synthesis of the β globin chains of the hemoglobin (Hb) tetramer. More than 200 different mutations of β globin genes are currently recognized.[1] Most of the mutations are single nucleotide substitutions, deletions, or insertions of oligonucleotides leading to frameshifts. More rarely, β-thalassemia results from gene deletions. Clinical severity could be affected by the mutation type.[2]

Based on clinical and hematologic severity, there are three main forms of β-thalassemia syndromes, i.e., the β-thalassemia carrier (trait) state, thalassemia intermedia (non-transfusion-dependent thalassemia), and thalassemia major (transfusion-dependent thalassemia). The current review will focus on β-thalassemia carrier state (BTT).

It has been estimated that about 1.5% of the global population (80-90 million people) are carriers of β-thalassemia.[3] β-thalassemia traits have a 25% risk of having children affected with thalassemia major if their partner is also a carrier. The World Health Organization (WHO) emphasizes the importance of incorporating carrier screening in basic health services in countries with a high incidence of thalassemia.[4]

Hematology laboratories play a vital role in carrier identification. Screening thalassemia 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 hemoglobin (MCH) will be assessed for hemoglobin patterns and HbA2 levels. In contrast, hemoglobin analysis is carried out for all subjects from the outset in the latter.[5] The first approach has notable disadvantages over its limited advantages, which include low cost. In the presence of mild/silent mutations, β-promoter mutations, α thalassemia and HbD and/or β variants, β-thalassemia carriers may show normal/marginal red cell indices.[6] In addition, also megaloblastic anemia and hereditary persistence of fetal hemoglobin (HPFH) can mask the microcytosis of β-thalassemia carriers.[7,8] Other than these clinical conditions, technical problems such as sample storage can affect

MCV results. Thus the first approach will misdiagnose a proportion of β-thalassemia carriers. The second approach is also not foolproof. The conventional method, high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) are two techniques used for quantifying HbA2. When the same sample is analyzed using both techniques, machine-related variations in HbA2 have been reported.[9,10] Factors such as δ-hemoglobinopathies, $\delta\beta^0$-thalassemia, α-thalassemia, triple-α, hemoglobin variants, iron deficiency anemia (IDA), hyperthyroidism, megaloblastic anemia, HPFH, antiretroviral drugs, and Krüppel-like factor 1 (*KLF1*) gene mutations can affect the HbA2 levels.[8,11,12] Labeling a person as BTT based on HbA2 level without considering the above factors may not be reliable. An HbA2 cut-off of 3.5% is generally used for the diagnosis of BTT,[13] though different reference ranges for HbA2 are used in some countries.[14] HbA2 values between the upper limit of the normal range and the cut-off value are considered borderline. The diagnosis of borderline samples is usually made only by doing confirmatory genetic tests such as polymerase chain reaction and β-gene sequencing.

The current review aims to discuss these unresolved problems in the BTT state with reference to the laboratory aspects.

Methods

Search strategy

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

Inclusion criteria

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

Exclusion criteria

Studies including β-thalassemia 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 also excluded from the present review.

Data extraction

Two researchers (ST and UPJ) independently reviewed all abstracts of journal articles gathered by the 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 communications. Out of the 68 articles selected, 14 (20.6%) originated from India, ten (14.7%) from Thailand, nine (13.2%) from Italy, eight (11.8%) from China, six (8.8%) from Iran, two (2.9%) each from Malaysia, Saudi Arabia, Pakistan, the Netherlands, and the UK, and one (1.5%) each from Sri Lanka, Bangladesh, Bahrain, Singapore, Egypt, the UAE, Portugal, Iraq, Canada, the US, Turkey, and Spain.

Of these 68 studies, seven (10.3%) analyzed the effectiveness of the conventional approaches of β-thalassemia carrier screening. There were 60 studies (77.9%) focused on factors affecting red cell indices (37 studies) and/or HbA2 value (43 studies). Only one study (1.5%) assessed the conventional cut-off values used in BTT diagnosis.

Effectiveness of the conventional approaches

Out of seven studies that analyzed the effectiveness of conventional hematologic-analysis-based approaches of β-thalassemia carrier screening, 57.1% (n=4) compared the effectiveness of MCV and MCH values in diagnosing BTT based on the first approach.[15-18] Sensitivity, specificity, positive predictive values, negative predictive values, and false negatives in different combinations have been used for comparisons. Of four studies, three concluded that MCH is more appropriate than MCV,[15,16,18] while one concluded that MCV is more appropriate than MCH.[17] Out of seven studies, 14.3% (n=1) showed that using Hb analysis as the first test is unreliable in detecting β-thalassemia traits.[19] About 28.6% (n=2) compared the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy among the conventional two approaches for thalassemia carrier detection.[20,21] According to both studies, the combination of MCV, MCH and Hb analysis resulted in high sensitivity, specificity, and diagnostic accuracy.

Factors affecting mean corpuscular volume and mean corpuscular hemoglobin among β-thalassemia carriers

Of 37 studies regarding factors affecting RBC indices,

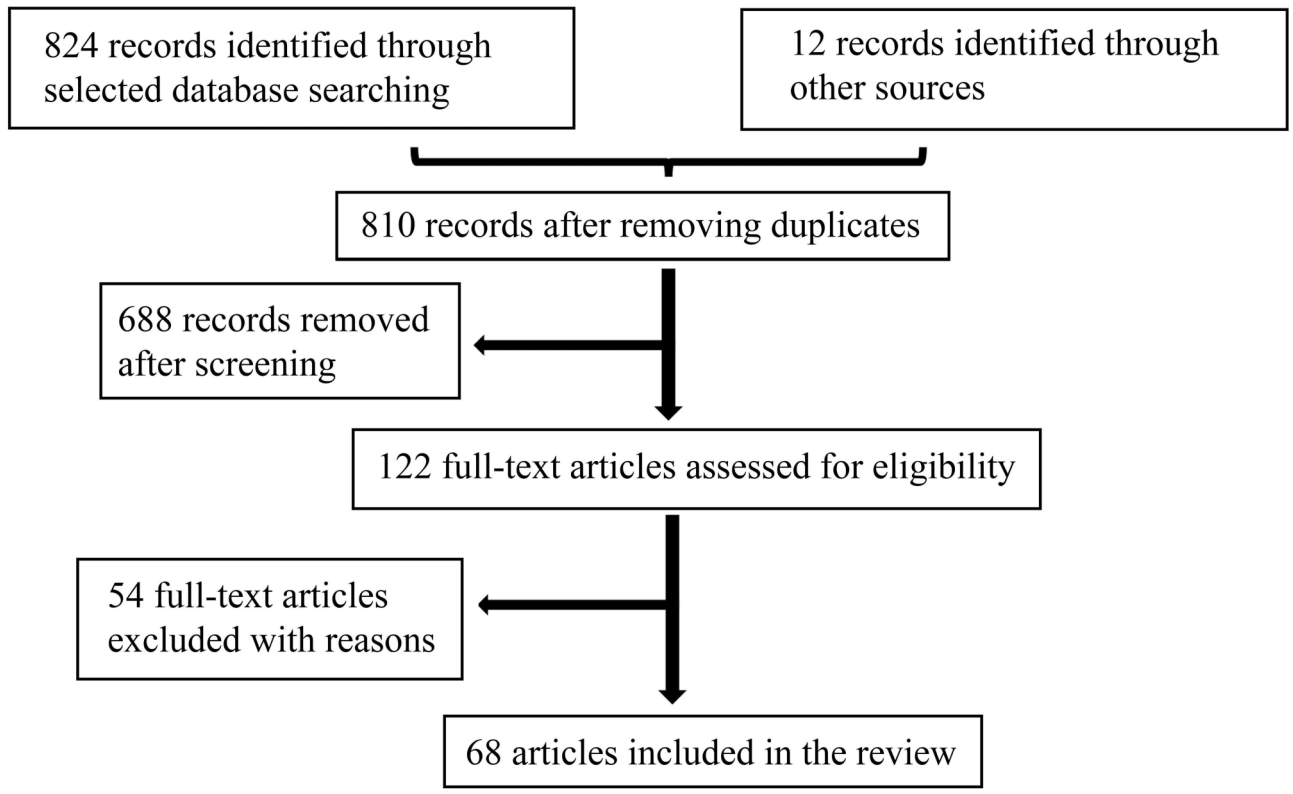

Figure 1. Flow chart of article selection for the systematic review.

67.6% (n=25) identified silent or mild β-thalassemia carriers with normal or marginal MCV and/or MCH. Of 25 studies, 6 (24%) originated from Italy,[6,22-26] 5 (20%) from India,[14,27-30] 4 (16%) from China,[31-34] 2 (8%) each from Thailand,[35,36] Iran[37,38] and Pakistan,[39,40] one (4%) each from Turkey,[41] Bahrain,[42] Malaysia,[43] and Spain.[44] Silent or mild β-thalassemia mutations reported from different countries are listed in Table 1.

Out of 37 studies, 13.5% (n=5) analyzed the effect of the co-inheritance of α- and β-thalassemia on MCV and MCH.[34,35,45-47] SEA type was the most common α-thalassemia type that co-existed with β-thalassemia 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 five studies, four (80%) reported a significant increase in MCV and MCH values among heterozygotes for α- and β-thalassemia compared to the simple β-thalassemia carriers.[34,35,46,47] The same four studies revealed that this increase in MCV and MCH values is higher when one or two α-globin gene deletions co-existed with β-thalassemia than in other α-gene arrangements. However, in all four studies, mean values of MCV and MCH in heterozygotes for α- and β-thalassemia were less than 80 fL and 27 pg. Out of 37 studies, 2.7% (n=1) showed the effect of fetal Hb in HPFH on MCV and MCH of BTT.[8] The study showed a significant increase in 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.

In the present review, we observed some non-genetic factors affecting MCV and MCH. Out of 37 studies, 2.7% (n=1) focused on normalized red cell parameters of BTT in patients with megaloblastic anemia.[7] Treatment with vitamin B_{12} and folic acid reduced the mean MCV of patients from 85.28±12.49 to 74.45±9.74. According to 13.5% of studies (n=5), antiretroviral therapy (ART) causes an increase in MCV and MCH among HIV-infected patients.[48-52] Pornprasert *et al.*[50] revealed that at 6 and 12 months after the ART therapy, the mean MCV of β-thalassemia patients shifted from microcytic level (<80 fL) to normocytic level (80-100 fL). Mean MCH increased to normal levels (27-31 pg).

Factors affecting HbA2

Of 43 studies focusing on factors affecting HbA2 levels, 34.9% (n=15) reported the type of β-thalassemia mutation itself as one of the factors associated with reduced HbA2 level among β-thalassemia carriers. Four studies (26.7%) from Thailand,[36,53-55] three (20%) from Italy,[6,11,56] two (13.3%) from China,[33,34] two (13.3%) from India,[14,30] and one (6.7%) each from Iran,[38] Malaysia,[57] Saudi Arabia,[58] and Portugal[59] reported different types of β-thalassemia mutations resulting in borderline or normal HbA2 levels in β-thalassemia carriers.

Out of 43 studies, 39.5% (n=17) investigated the effect of α-thalassemia on HbA2 level. Of 17 studies, two (11.8%) compared pure α-thalassemia traits and normal individuals, and both reported reduced HbA2 levels in α-thalassemia traits.[60,61] One study reported that the reduction in HbA2 expression depends on the number of α-globin gene defects.[60] Co-inheritance of β-thalassemia with Hb Constant Spring was associated with lower HbA2 expression when compared to other types of α-thalassemia.[60] Out of

Table 1. Silent/mild β-thalassemia mutations causing normal or marginal mean corpuscular volume.

Country	Silent/mild mutations*
Italy	CAP+1570 (T>C), β -101C →G, β-101, β +45 (G>C), β -101 (C →T), β -92 (C →T), β -54 (G →A), Poly A
India	Capsite +1 (A→C), CD 16 (-C), -88 (C →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 → T) , IVS II 844 (C → 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)

*Mean corpuscular volume (MCV) / mean MCV >70 fL.

17 studies, 47.1% (n=8) compared HbA2 levels between β-thalassemia traits and β-thalassemia traits co-inherited with α-thalassemia.[34-36,45-47,55,60] Although four studies (50%) observed a reduction in HbA2 level when α-thalassemia co-exists,[34,35,47,55] only two studies (25%) reported a possible effect of this on BTT diagnosis.[47,55] Of these two, one showed that HbA2<4.0% can occur when HbH disease (α-thalassemia intermedia) co-exists with the BTT.[55] Surprisingly, out of 17 studies, 47.1% (n=8) reported a marginal increase in HbA2 levels among α-thalassemia traits without β-thalassemia heterozygosis.[6,11,33,38,54,55,57,58] None of the studies could explain the underlying mechanism.

Of the studies that investigated factors affecting HbA2, 13.9% (n=6) from four different countries (Italy,[6,11,56] India,[62] Thailand,[60] and Portugal[59]) identified that co-inheritance of δ-hemoglobinopathy decreases the HbA2 level in β-thalassemia carriers resulting in misdiagnosis. About 11.6% (n=5) evaluated the effect of α-gene triplication on the HbA2 level. Out of these five studies, four reported the presence of an ααα condition in healthy individuals with borderline increased HbA2 levels but without a β-globin mutation.[6,11,33,34] Only one study stated that α-gene triplication does not affect hematologic parameters.[63] Similarly, out of 43 studies, only 2.3% (n=1) found that co-inherited HPFH in BTT reduced HbA2 levels.[8] 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 β-like globin gene switching, and activating or repressing gene transcription.[64] Of eight studies on the effect of *KLF1* gene mutations, seven (87.5%) observed a high prevalence of *KLF1* gene defects among individuals with borderline increased HbA2 levels without an HBB gene mutation.[12,26,33,34,54,65,66] They concluded a possible effect of *KLF1* gene mutations on increased HbA2 levels among normal individuals. Only one 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.[58] Out of seven studies which determined the effect of *KLF1* gene mutations on HbA2 level, the studies of Perseu *et al.*,[12] Hariharan *et al.*,[65] and Liu *et al.*[66] revealed 0.8%, 1.1%, and 0.5% increases, respectively, in HbA2 level among individuals with *KLF1* gene mutations when compared to the group without *KLF1* gene mutations. Interestingly, Liu *et al.*[66] had shown a high prevalence of *KLF1* genes in β-thalassemia endemic regions in China and an amelioration of the severity of β-thalassemia by *KLF1* gene changes.[66] We were able to determine the most common *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 the most 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 anemia (IDA) and HbA2 levels. Of these nine studies, four (44.4%) determined the effect of IDA on HbA2 results of normal (non-thalassemic) individuals.[56,60,67,68] All concluded that IDA causes a significant reduction in HbA2 levels in normal individuals. Eight studies (88.9%) compared the results of HbA2 among β-thalassemia traits with and without IDA. Out of these eight studies, five (62.5%) showed significantly higher HbA2 values for β-thalassemia traits without IDA than in those with IDA;[30,61,67,69,70] in the other three, no difference was observed.[56,68,71] Of five studies that observed higher values, three (60%) suggested possible effects on β-thalassemia diagnosis,[30,61,67] while others showed no effect

due to the steadying of all HbA2 values above the cut-off.[69,70] Among those studies that suggested possible effects, one proposed a dose effect of severity of iron deficiency on HbA2 level of β-thalassemia traits.[61]

Out of 43 studies, 4.6% (n=2) reported the effect of megaloblastic anemia in elevated HbA2 levels among normal individuals without β-thalassemia.[7,68] One of the above studies reported a change of mean HbA2 of the study group from 4.56% to 3.81% with vitamin B_{12} 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.[7] Among 43 studies that discussed factors affecting HbA2 levels, 2.3% (n=1) indicated the effect of thyroid hormones on the production of HbA2.[72] 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 did not pass the cut-off level of HbA2, meaning there was less chance of misdiagnosing BTT.

Five studies analyzed the effect of antiretroviral therapy (ART) on HbA2 results.[48-52] All of them showed an increase in HbA2 levels among HIV-infected patients treated with ART leading to a misdiagnosis of BTT. Of five studies, three (60%) confirmed that zidovudine increased HbA2 levels.[48,51,52] Bhagat *et al.*[48] evaluated the effect of three different drugs, and concluded that both zidovudine and stavudine increase HbA2 levels, while tenofovir had no effect.

In the present review, we observed technical issues due to the variations in analytical methods used to determine HbA2 level in β-thalassemia screening. Out of 43 studies, 6.9% (n=3) compared HbA2 results by different analytical systems in the presence of Hb variants.[9,10,73] All three studies determined falsely increased HbA2 in HPLC reports of patients with HbE and HbS variants. However, the CE method clearly separated HbE and HbA2. One study reported the variant Hb Lepore, which co-elutes with HbA2 in the HPLC method causing problems in diagnosis.[73] Similarly, when the HPLC technique is used, falsely reduced HbA2 had been observed in the presence of HbD Punjab in two studies.[9,73] According to two studies, HbA2 peak was included in HbC peak when the CE method was used.[9,10] 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 the Bio-Rad variant II HPLC method (mean: 2.95% and 2.67%, respectively) than the Sebia CE method (mean: 2.49% and 2.51%, respectively).[9,73] 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%).[10]

Problems with HbA2 cut-off values

In the present review, we analyzed the variations in cut-off values of HbA2 used in β-thalassemia screening. Out of 68 studies, 54.4% (n=37) articles (excluding studies based on borderline HbA2 values) clearly mentioned the HbA2 cut-off value used in diagnosing β-thalassemia carriers. Of 37 articles, the majority (48.6%; n=18) used 3.5% as the cut-off value, while 32.4% (n=12) of studies used 4.0% as the cut-off. Eighteen studies that used 3.5% as the cut-off include five from China,[18,21,32,34,70] five from Iran,[15,16,37,47,67] two from Thailand,[46,53] one each from India,[51] Bangladesh,[20] Saudi Arabia,[19] Portugal,[59] the UAE,[61] and the UK.[52] Similarly, 12 studies that used 4.0% as the cut-off include four from India,[7,17,27,48] four from Thailand,[35,49,51,55] and one each from Malaysia,[43] Bahrain,[42] Italy,[24] and the Netherlands.[73] A 3.9% cut-off was used for three studies from India (n=2)[68,71] and Italy (n=1).[12] Two studies from Italy[26] and Turkey[41] used 3.8% as the cut-off while 3.4%, 3.6%, and 3.7% were used as the cut-off values by three separate studies from Italy,[56] Thailand,[36] and India,[28] respectively.

Abdel-Messih *et al.*[74] determined the effectiveness of the two most common cut-off values used in BTT screening. 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%) in identifying β-thalassemia carriers at 3.5% and 4.0% cut-off 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 β-thalassemia using red cell indices as the primary screening or using HPLC or CE as the first test each seem to have their own deficiencies. In the first approach, MCV and MCH are crucial hematologic parameters to rule out possible β-thalassemia traits. Several studies reported that MCH is more appropriate than MCV.[15,16,18] Most of these studies generate sensitivity figures based on their own cut-off 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;[75] this stability of MCH may be the reason for its higher sensitivity. Some clinicians use only MCV to exclude β-thalassemia in the primary screening.[36] In such cases, falsely high MCV values 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 β-thalassemia. 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 β-thalassemia carriers, as red cell indices may be normal in these individuals. Silent β-thalassemia mutations exist not only in codons, but also in the promoter region and/or the

cap region of the HBB gene.[24,28,41,76] Screening for thalassemia based on red cell indices will also result in missing individuals with HbD trait and individuals with triple α genes.[77] Although phenotypically not important, and globally rare, the co-inheritance of the above conditions with β-thalassemia mutations leads to severe phenotype in the offspring.

In the present review, we analyzed the multitude of factors that may affect HbA2 levels, and thus the diagnosis of β-thalassemia (Table 2). The problems may arise in two circumstances: decreased HbA2 levels in β-thalassemia traits, or deceptively elevated HbA2 levels in non-carriers. The type of β-thalassemia mutation is the main reason for reduced or borderline HbA2 levels among carriers.[30] Silent or mild β-thalassemia mutations show reduced/borderline HbA2 levels along with normal or slightly reduced RBC indices. Most of the time, homozygotes for classic and silent β-thalassemia mutations have thalassemia intermedia phenotype.[76,78] Unfortunately, silent carriers will be missed until the birth of a child with β-thalassemia intermedia.

Co-inheritance of α-thalassemia with β-thalassemia results in decreased HbA2 levels due to the unavailability of a free α-chain pool to partner with δ-globin chains.[2] This reduction seems insufficient to cause problems in BTT diagnosis[36,60] unless mild or silent β-thalassemia also coexists. Satthakar *et al.*[55] showed HbA2 less than 4.0% only in HbH and HbH-CS diseases; since both these conditions are apparent in HPLC patterns, β-thalassemia traits will not be missed. Surprisingly, borderline increases in HbA2 levels have also been identified among individuals with α-thalassemia but without β-thalassemia mutations.[33,55,58] The exact reason for that has still not been discovered, while some researchers consider regulation factors or mutations in regulatory genes to be causes.[33,38]

Co-inheritance of δ-hemoglobinopathy[60] and HPFH[8] cause reduced HbA2 levels in β-thalassemia carriers. The synthesis of insufficient amounts of δ-globin monomers results in lower HbA2 levels in δ-hemoglobinopathy. On the other hand, co-inheritance of heterocellular HPFH leads to a primary increase in γ-chain synthesis resulting in high HbF levels.[8] However, the reason for decreased HbA2 level is not clear.

The effect of α-triplication and *KLF1* gene mutations on increased HbA2 levels is contradictory. α-triplications are considered uncommon because *de novo* crossover events generating triplications are rare.[63] The *KLF1* gene has a regulatory effect on erythropoiesis.[79] Perseu *et al.*[12] first identified a delay in the transcriptional switch from the *HBD* to the *HBB* gene by *KLF1* mutations. The majority of the studies regarding α-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 β-thalassemia mutations, possible effects have been hypothesized. Among eligible studies, there were a few of a cross-sectional nature, with some studies including members of the general population, but they revealed no association between increased HbA2 level and α-triplication/*KLF1* mutations.[58,63] Therefore, larger scale studies that include the general population are needed in order to gather more comprehensive and informative data.

Table 2. Factors affecting HbA2 results of high-performance liquid chromatography and capillary electrophoresis methods.

	HPLC	CE
Falsely increased HbA2	*KLF1* gene mutation* Megaloblastic anemia* Hyperthyroidism* Antiretroviral therapy*	
	HbE HbS Hb Lepore	-
Falsely decreased HbA2	α-thalassemia* δ-hemoglobinopathy* Hereditary persistence of fetal Hb* Iron deficiency anemia*	
	HbD Punjab	HbC

*These factors affect HbA2 results of both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) methods. Hb: hemoglobin.

IDA is the major cause of anemia in the world.[80] Co-existence of IDA may decrease the HbA2 level in β-thalassemia traits. Intracellular lack of iron reduces α-globin chain synthesis more than non-α-globin chains. In β-thalassemia, β-globin chain synthesis is also limited. Therefore, β chains compete more effectively for α-globin chains than δ-globin chains, resulting in reduced levels of HbA2.[81] It is widely believed that IDA cannot reduce HbA2 levels of β-thalassemia traits below the cut-off values. If this is the case, the effect of IDA on β-thalassemia diagnosis would be minimal, except in cases of severe IDA or silent/mild β-thalassemia mutations.

Megaloblastic anemia, ART therapy, and hyperthyroidism are other non-genetic factors which increase HbA2 levels in β-thalassemia traits. Both megaloblastic anemia and long-term ART therapy (in particular, zidovudine and stavudine) affect red cell synthesis. Nuclear maturation of red cell precursors is delayed in megaloblastic anemia, while antiretroviral medications inhibit nucleic acid synthesis.[50] So, more Hb synthesis occurs in immature erythroid precursors. Since the synthesis of δ chains is relatively greater in these cells, HbA2 level is increased.[81] Besides this, thyroid hormones are considered to specifically increase the expression of δ globin resulting in high HbA2 levels in hyperthyroidism.[72] No literature was found which demonstrated an increased HbA2 level above the cut-off in hyperthyroidism. Nevertheless, a careful medical history

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

Borderline range (HbA2%)	Study	% β-thalassemia heterozygotes	Remarks
3.0-3.9	Bahar *et al.*[57]	30.8 (36/117)	10 (27.0%) had HbA2 level of 3.0%
	Colaco *et al.*[30]	72.6 (149/205)	20 of 149 [14.0%] of all β-thalassemia traits would be missed if MCV < 80 fL and HbA2≥3.5% were used as cut-off
3.0-4.0	Rangan *et al.*[14]	32 (8/25)	All had HbA2 levels ranging from 3.5% to 3.9%
3.1-3.9	Borgio *et al.*[58]	83.3 (60/72)	-
	Giambona *et al.*[6]	14.8 (61/410)	Exclusion criteria: iron deficiency anemia and Hb variants 20 out of 61 β-thalassemia carriers had MCV>80fL
	Chaweephisal *et al.*[36]	5.6 (6/106)	Exclusion criteria: MCV>80fL
	Moradi *et al.*[38]	36.4 (159/436)	Exclusion criteria: MCV>80fL MCH>27pg
3.3-3.7	Mosca *et al.*[11]	16.2 (38/234)	Majority with MCV>80fl
3.3-3.9	Hariharan *et al.*[65]	NM	-
3.3-4.0	Lou *et al.*[33]	2.4 (4/165)	-
3.3-4.1	Perseu *et al.*[12]	NM	-
	Paglietti *et al.*[26]	18.9 (7/37)	-
3.5-3.9	Jiang *et al.*[34]	17.7 (11/62)	-
	Satthakarn *et al.*[55]	5.33 (16/300)	-
3.5-4.0	Rungsee *et al.*[53]	1.9 (3/158)	Tested only for β^0 mutations
	Srivorakun *et al.*[54]	10.9 (22/202)	-

MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; Hb: hemoglobin; NM: not mentioned.

of the individual is warranted prior to β-thalassemia screening.

There are machine-related variations in HbA2 results of normal individuals or β-thalassemia traits. A comparison of the Bio-Rad variant II HPLC and Sebia CE methods showed consistently high results have been obtained from the HPLC method,[9,73] although there is good correlation between the two. When using a single cut-off value to diagnose β-thalassemia carriers, this will be a major problem, especially in borderline HbA2 results. Therefore, when issuing a diagnosis of BTT based on borderline values, machine-related variation of the HbA2 level should be borne in mind. The main reason for this unresolved problem is the non-availability of standardized HbA2 results between methods. Non-availability of a reference method and international reference HbA2 calibrators makes this problem more complex.[9]

In addition, 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 cut-off values, sometimes 2 or 3 different cut-offs have been used within a single country. Similarly, different authors have defined borderline ranges arbitrarily. In the present review, the percentages of β-thalassemia heterozygotes in each borderline group ranged from 1.9% to 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 the analytical methods used to identify β-thalassemia mutations. However, it is noteworthy that all these β-thalassemia traits would be missed if HbA2≥4.0% was used as the cut-off. We believe a cut-off with a higher sensitivity than specificity is suitable for screening to prevent missing β-thalassemia traits. If problems arise in the accurate diagnosis of β-thalassemia 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 β-thalassemia remain unresolved. During the screening of β-thalassemia, both RBC indices and HbA2 level should be taken into consideration. Population-specific reference ranges and machine-related variations in HbA2 level should be determined within the laboratory. Borderline HbA2 levels should be further investigated for factors affecting HbA2 levels. Careful screening of the patient's clinical history can identify most non-genetic factors that affect HbA2 screening. IDA and α-thalassemia are unlikely to affect the BTT diagnosis unless in individuals with silent β mutations. Based on the prevalence of silent mutations

and other co-inheriting genetic factors, the diagnostic approach of BTT should be modified for each population. For example, screening the partners of patients with β-thalassemia heterozygosity for common silent β mutations, HbE, HbD, etc. is possible. However, effects of some genetic factors, such as *KLF1* gene mutations and α-triplication on HbA2 level are still controversial, and further investigations are needed. Moreover, there may be other as yet unidentified factors, such as the environment, lifestyle, regulation factors or mutations in regulatory genes affecting HbA2 level. Further studies should aim to identify these factors and should include large numbers of participants.

Disclosures

No conflicts of interest to disclose.

Contributions

ST and UPJ wrote the first version of the manuscript. AP revised the manuscript critically for important intellectual content. All authors read, revised, and approved the final manuscript.

References

1. Weatherall DJ. Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat Rev Genet. 2001;2(4):245-255.
2. Thein SL. Genetic insights into the clinical diversity of beta-thalassaemia. Br J Haematol. 2004;124(3):264-274.
3. Colah R, Gorakshakar A, Nadkarni A. Global burden, distribution and prevention of β-thalassaemias and hemoglobin E disorders. Expert Rev Hematol. 2010;3(1):103-117.
4. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ. 2008;86(6):480-487.
5. Angastiniotis M, Eleftheriou A, Galanello R, et al. Prevention of thalassaemias and other haemoglobin disorders: Volume 1: principles [Internet]. Old J, editor. 2nd ed. Nicosia (Cyprus): Thalassaemia International Federation; 2013.
6. Giambona A, Passarello C, Renda D, Maggio A. The significance of the hemoglobin A2 value in screening for hemoglobinopathies. Clin Biochem. 2009;42(18):1786-1796.
7. Sahoo S, Sahu N, Das P, Senapati U. Effect of megaloblastic anemia on hemoglobin A2 and diagnosis of β thalassaemia trait. Indian J Pathol Microbiol. 2023;66(2):327-331.
8. Garner C, Dew T, Sherwood R, Rees D, Thein S. Heterocellular hereditary persistence of fetal haemoglobin affects the haematological parameters of beta-thalassaemia trait. Br J Haematol. 2003;123:353-358.
9. Higgins TN, Khajuria A, Mack M. Quantification of HbA(2) in patients with and without beta-thalassaemia and in the presence of HbS, HbC, HbE, and HbD Punjab hemoglobin variants: comparison of two systems. Am J Clin Pathol. 2009;131(3):357-362.
10. Keren DF, Hedstrom D, Gulbranson R, Ou C-N, Bak R. Comparison of Sebia Capillarys capillary electrophoresis with the Primus high-pressure liquid chromatography in the evaluation of hemoglobinopathies. Am J Clin Pathol. 2008;130(5):824-831.
11. Mosca A, Paleari R, Ivaldi G, Galanello R, Giordano PC. The role of haemoglobin A2 testing in the diagnosis of thalassaemias and related haemoglobinopathies. J Clin Pathol. 2009;62(1):13-17.
12. Perseu L, Satta S, Moi P, et al. KLF1 gene mutations cause borderline HbA(2). Blood. 2011;118(16):4454-4458.
13. Ryan K, Bain BJ, Worthington D, et al. Significant haemoglobinopathies: guidelines for screening and diagnosis. Br J Haematol, 2010;149(1):35-49.
14. Rangan A, Sharma P, Dadu T, Saxena R, Verma IC, Bhargava M. β-thalassaemia mutations in subjects with borderline HbA2 values: a pilot study in North India. Clin Chem Lab Med. 2011;49(12):2069-2072.
15. Tari K, Alikhani S, Abbaszadehdibavar M, Kianinodeh F, Karami F, Atashi A. Evaluation of the sensitivity and specificity of MCH and MCV for screening of beta-thalassaemia minor. Int J Biomed Pub Health. 2018;1(4):184-186.
16. Karimi M, Rasekhi AR. Efficiency of premarital screening of beta-thalassaemia trait using MCH rather than MCV in the population of Fars Province, Iran. Haematologia. 2002;32(2):129-133.
17. Rathod DA, Kaur A, Patel V, et al. Usefulness of cell counter-based parameters and formulas in detection of beta-thalassaemia trait in areas of high prevalence. Am J Clin Pathol. 2007;128(4):585-589.
18. Liao C, Xie X-M, Zhong H-Z, Zhou J-Y, Li D-Z. Proposed screening criteria for β-thalassaemia trait during early pregnancy in Southern China. Hemoglobin. 2009;33(6):528-533.
19. Al-Amodi AM, Ghanem NZ, Aldakeel SA, et al. Hemoglobin A2 (HbA2) has a measure of unreliability in diagnosing β-thalassaemia trait (β-TT). Curr Med Res Opin. 2018;34(5):945-951.
20. Noor FA, Sultana N, Bhuyan GS, et al. Nationwide carrier detection and molecular characterization of β-thalassaemia and hemoglobin E variants in Bangladeshi population. Orphanet J Rare Dis. 2020;15(1):15.
21. Zhao J, Li J, Lai Q, Yu Y. Combined use of gap-PCR and next-generation sequencing improves thalassaemia carrier screening among premarital adults in China. J Clin Pathol. 2020;73(8):488-492.
22. Dell'Edera D, Epifania AA, Malvasi A, et al. Incidence of β-thalassaemia carrier on 1495 couples in preconceptional period. J Matern Fetal Neonatal Med. 2013;26(5):445-448.
23. Moi P, Faà V, Marini MG, et al. A novel silent beta-thalassaemia mutation in the distal CACCC box affects the binding and responsiveness to EKLF. Br J Haematol. 2004;126(6):881-884.
24. Vinciguerra M, Passarello C, Cassarà F, et al. Co-heredity of silent CAP + 1570 T>C (HBB:c*96T>C) defect and severe β-thal mutation: a cause of mild β-thalassaemia intermedia. Int J Lab Hematol. 2016;38(1):17-26.
25. De Angioletti M, Lacerra G, Sabato V, Carestia C. Beta+45 G C: a novel silent beta-thalassaemia mutation, the first in the Kozak sequence. Br J Haematol. 2004;124(2):224-231.
26. Paglietti M E, Satta S, Sollaino MC, et al. The problem of borderline hemoglobin A2 levels in the screening for β-thalassaemia carriers in Sardinia. Acta Haematol. 2016;135(4):193-199.
27. Colah RB, Surve R, Sawant P, et al. HPLC studies in hemoglobinopathies. Indian J Pediatr. 2007;74(7):657-662.

28. Garewal G, Das R, Awasthi A, Ahluwalia J, Marwaha RK. The clinical significance of the spectrum of interactions of CAP+1 (A→C), a silent β-globin gene mutation, with other β-thalassaemia mutations and globin gene modifiers in north Indians. Eur J Haematol. 2007;79(5):417-421.
29. Italia K, Sawant P, Surve R, et al. Variable haematological and clinical presentation of β-thalassaemia carriers and homozygotes with the Poly A (T→C) mutation in the Indian population. Eur J Haematol. 2012;89(2):160-164.
30. Colaco S, Colah R, Nadkarni A. Significance of borderline HbA2 levels in β thalassaemia carrier screening. Sci Rep. 2022;12(1):5414.
31. Chen X-W, Mo Q-H, Li Q, Zeng R, Xu X-M. A novel mutation of -73(A-->T) in the CCAAT box of the beta-globin gene identified in a patient with the mild beta-thalassaemia intermedia. Ann Hematol. 2007;86(9):653-657.
32. Zhao Y, Jiang F, Li D-Z. Hematological characteristics of β-globin gene mutation -50 (G>A) (HBB: C.-100G>A) carriers in mainland China. Hemoglobin. 2020;44(4):240-243.
33. Lou J-W, Li D-Z, Zhang Y, et al. Delineation of the molecular basis of borderline hemoglobin A2 in Chinese individuals. Blood Cells Mol Dis. 2014;53(4):261-264.
34. Jiang F, Chen G-L, Li J, Zhou J-Y, Liao C, Li D-Z. Analysis of the genotypes in a Chinese population with increased HbA2 and low hematological indices. Hemoglobin. 2018;42(3):154-158.
35. Yamsri S, Singha K, Prajantasen T, et al. A large cohort of β+-thalassaemia in Thailand: molecular, hematological and diagnostic considerations. Blood Cells Mol Dis. 2015;54(2):164-169.
36. Chaweephisal P, Phusua A, Fanhchaksai K, Sirichotiyakul S, Charoenkwan P. Borderline hemoglobin A2 levels in northern Thai population: HBB genotypes and effects of coinherited alpha-thalassaemia. Blood Cells Mol Dis. 2019;74:13-17.
37. Nezhad FH, Nezhad KH, Choghakabodi PM, Keikhaei B. Prevalence and genetic analysis of α- and β-thalassaemia and sickle cell anemia in Southwest Iran. J Epidemiol Glob Health. 2018;8(3-4):189-195.
38. Moradi K, Alibakhshi R, Shafieenia S, Azimi A. Problem of borderline hemoglobin A2 levels in an Iranian population with a high prevalence of α- and β-thalassaemia carriers. Egypt J Med Hum Genet. 2022;23(1):61.
39. Khattak SAK, Ahmed S, Anwar J, Ali N, Shaikh KH. Prevalence of various mutations in beta-thalassaemia and its association with haematological parameters. J Pak Med Assoc. 2012;62(1):40-43.
40. Karim M, Moinuddin M, Babar S. Cap +1 mutation; an unsuspected cause of beta-thalassaemia transmission in Pakistan. Turk J Hematol. 2009;26:167-170.
41. Bilgen T, Clark OA, Ozturk Z, Yesilipek MA, Keser I. Two novel mutations in the 3' untranslated region of the beta-globin gene that are associated with the mild phenotype of beta-thalassaemia. Int J Lab Hematol. 2013;35(1):26-30.
42. Al Moamen NJ, Mahdi F, Salman E, et al. Silent β-thalassaemia mutations at -101 (C>T) and -71 (C>T) and their coinheritance with the sickle cell mutation in Bahrain. Hemoglobin. 2013;37(4):369-377.
43. Abdullah UYH, Ibrahim HM, Mahmud NB, et al. Genotype-phenotype correlation of β-thalassaemia in Malaysian population: toward effective genetic counseling. Hemoglobin. 2020;44(3):184-189.
44. Ropero P, Erquiaga S, Arrizabalaga B, et al. Phenotype of mutations in the promoter region of the β-globin gene. J Clin Pathol. 2017;70(10):874-878.
45. Law HY, Chee MKL, Tan GP, Ng ISL. The simultaneous presence of alpha- and beta-thalassaemia alleles: a pitfall of thalassaemia screening. Community Genet. 2003;6(1):14-21.
46. Viprakasit V, Limwongse C, Sukpanichnant S, et al. Problems in determining thalassaemia carrier status in a program for prevention and control of severe thalassaemia syndromes: a lesson from Thailand. Clin Chem Lab Med. 2013;51(8):1605-1614.
47. Saleh-Gohari N, Bami MK, Nikbakht R, Karimi-Maleh H. Effects of α-thalassaemia mutations on the haematological parameters of β-thalassaemia carriers. J Clin Pathol. 2015;68(7):562-566.
48. Bhagat P, Sachdeva RK, Sharma P, et al. Effect of antiretroviral therapy on hemoglobin A2 values can have implications in antenatal beta-thalassaemia screening programs. Infect Dis (Lond). 2016;48(2):122-126.
49. Pornprasert S, Leechanachai P, Klinbuayaem V, et al. Effect of haematological alterations on thalassaemia investigation in HIV-1-infected Thai patients receiving antiretroviral therapy. HIV Med. 2008;9(8):660-666.
50. Pornprasert S, Sonboon P, Kiatwattanacharoen S, et al. Evolution of hematological parameters in HIV-1-infected patients with and without thalassaemia carriages during highly active antiretroviral therapy. HIV Clin Trials. 2009;10(2):88-93.
51. Nigam JS, Bharti JN, Kumar D, Sharma A. A comparison of hemoglobin A2 levels in untreated and treated groups of HIV patients on ART including zidovudine. Patholog Res Int. 2013;828214.
52. Wilkinson MJ, Bain BJ, Phelan L, Benzie A. Increased haemoglobin A2 percentage in HIV infection: disease or treatment. AIDS (Lond). 2007;21(9):1207-1208.
53. Rungsee P, Kongthai K, Pornprasert S. Detection of the common South-East Asian β0-thalassaemia mutations in samples with borderline HbA2 levels. Clin Chem Lab Med. 2017;55(1):e17-e20.
54. Srivorakun H, Thawinan W, Fucharoen G, Sanchaisuriya K, Fucharoen S. Thalassaemia and erythroid transcription factor KLF1 mutations associated with borderline hemoglobin A2 in the Thai population. Arch Med Sci. 2020;18:112-120.
55. Satthakarn S, Panyasai S, Pornprasert S. Molecular characterization of β- and α-globin gene mutations in individuals with borderline HbA2 levels. Hemoglobin. 2020;44(5):349-353.
56. Passarello C, Giambona A, Cannata M, Vinciguerra M, Renda D, Maggio A. Iron deficiency does not compromise the diagnosis of high HbA2 β thalassaemia trait. Haematologica. 2012;97(3):472-473.
57. Bahar R, Hassan MN, Marini R, Shafini MY, Abdullah WZ. The diagnosis of beta-thalassaemia with borderline HbA2 level among Kelantan population. J Blood Disord Transfus. 2017;08:396.
58. Borgio JF, AbdulAzeez S, Al-Muslami AM, et al. KLF1 gene and borderline hemoglobin A2 in Saudi population. Arch Med Sci. 2018;14(1):230-236.
59. Morgado A, Picanço I, Gomes S, et al. Mutational spectrum of delta-globin gene in the Portuguese population. Eur J Haematol. 2007;79:422-428.
60. Singha K, Sanchaisuriya K, Fucharoen G, Fucharoen S. Genetic and non-genetic factors affecting hemoglobin A2 expression in a large cohort of Thai individuals: implication for population screening for thalassaemia. Am J Transl Res. 2021;13(10):11632-11642.
61. Denic S, Agarwal MM, Al-Dabbagh B, et al. Hemoglobin A2 lowered by iron deficiency and α-thalassaemia: should screening recommendation for β-thalassaemia change? Int Sch Res Notices. 2013:e858294.
62. Colaco S, Trivedi A, Colah RB, Ghosh K, Nadkarni AH. Masking of a β-thalassaemia determinant by a novel δ-globin gene defect [HbA2-Saurashtra or δ100(G2)Pro→Ser; HBD: C.301C>T] in Cis.

Hemoglobin. 2014;38(1):24-27.
63. Giordano PC, Bakker-Verwij M, Harteveld CL. Frequency of alpha-globin gene triplications and their interaction with beta-thalassaemia mutations. Hemoglobin. 2009;33(2):124-131.
64. Gallagher PG. HbA2: at the borderline of the KLF. Blood. 2011;118(16):4301-4302.
65. Hariharan P, Colah R, Ghosh K, Nadkarni A. Differential role of Kruppel like factor 1 (KLF1) gene in red blood cell disorders. Genomics. 2019;111(6):1771-1776.
66. Liu D, Zhang X, Yu L, et al. KLF1 mutations are relatively more common in a thalassaemia endemic region and ameliorate the severity of β-thalassaemia. Blood. 2014;124(5):803-811.
67. Keramati MR, Maybodi N. The effect of iron deficiency anemia (IDA) on the HbA2 level and comparison of hematologic values between IDA and thalassaemia minor. Int J Hematol Oncol. 2006;17:151-156.
68. Rao S, Kar R, Gupta SK, Chopra A, Saxena R. Spectrum of haemoglobinopathies diagnosed by cation exchange-HPLC & modulating effects of nutritional deficiency anaemias from north India. Indian J Med Res. 2010;132(5):513-519.
69. Verma S, Gupta R, Kudesia M, Mathur A, Krishan G, Singh S. Coexisting iron deficiency anemia and beta-thalassaemia trait: effect of iron therapy on red cell parameters and hemoglobin subtypes. ISRN Hematol. 2014;293216.
70. Verhovsek M, So C-C, O'Shea T, et al. Is HbA2 level a reliable diagnostic measurement for β-thalassaemia trait in people with iron deficiency? Am J Hematol. 2012;87(1):114-116.
71. Sharma P, Das R, Trehan A, et al. Impact of iron deficiency on hemoglobin A2% in obligate β-thalassaemia heterozygotes. Int J Lab Hematol. 2015;37(1):105-111.
72. Jaafar FH, Al-Tameemi W, Ali HH. The influence of thyroid hormones on hemoglobin A2 and F expression. New Iraqi J Med. 2009;5:78-81.
73. Van Delft P, Lenters E, Bakker-Verweij M, et al. Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations. Int J Lab Hematol. 2009;31(5):484-495.
74. Abdel-Messih IY, Youssef SR, Mokhtar GM, et al. Clinical to molecular screening paradigm for β-thalassaemia carriers. Hemoglobin. 2015;39(4):240-246.
75. Rogers M, Phelan L, Bain B. Screening criteria for beta-thalassaemia trait in pregnant women. J Clin Pathol. 1995;48(11):1054-1056.
76. Aslan D. "Silent" β-thalassaemia mutation (promoter nt-101 C > T) with increased hemoglobin A2. Turk J Pediatr. 2016;58(3):305-308.
77. Giordano PC. Strategies for basic laboratory diagnostics of the hemoglobinopathies in multi-ethnic societies: interpretation of results and pitfalls. Int J Lab Hematol. 2013;35(5):465-479.
78. Bain BJ. Haemoglobinopathy diagnosis: algorithms, lessons and pitfalls. Blood Rev. 2011;25(5):205-213.
79. Tallack MR, Whitington T, Yuen WS, et al. A global role for KLF1 in erythropoiesis revealed by ChIP-seq in primary erythroid cells. Genome Res. 2010;20(8):1052-1063.
80. Miller JL. Iron deficiency anemia: a common and curable disease. Cold Spring Harb Perspect Med. 2013;3(7):a011866.
81. Steinberg M H, Adams J G. Hemoglobin A2: Origin, evolution, and aftermath. Blood. 1991;78(9):2165-2177.