

# The Impact of Repeated Hb A<sub>2</sub> Measurements on $\beta$ -Thalassemia Trait Diagnosis

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## Abstract

**Introduction:** Hemoglobin A<sub>2</sub> (Hb A<sub>2</sub>) is a minor component of the hemoglobin present in normal adult red blood cells, accounting for 1.5-3.5% of the total hemoglobin in healthy individuals. The Hb A<sub>2</sub> level is the most significant parameter to identify  $\beta$ -thalassemia carriers. We aimed to study the effect of repeated measurements of Hb A<sub>2</sub> levels on  $\beta$ -thalassemia trait diagnosis.

**Methods:** Repeated HPLC tests were conducted for 107 patient samples. Results were categorized into variant interval ranges (VIRs); each interval has 0.25% deviation from the repeated test. The following six VIRs were created: 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50%. HPLC results were interpreted in correlation with red blood cell indices, and serum ferritin. Serum ferritin was available for 76 patients from medical records.

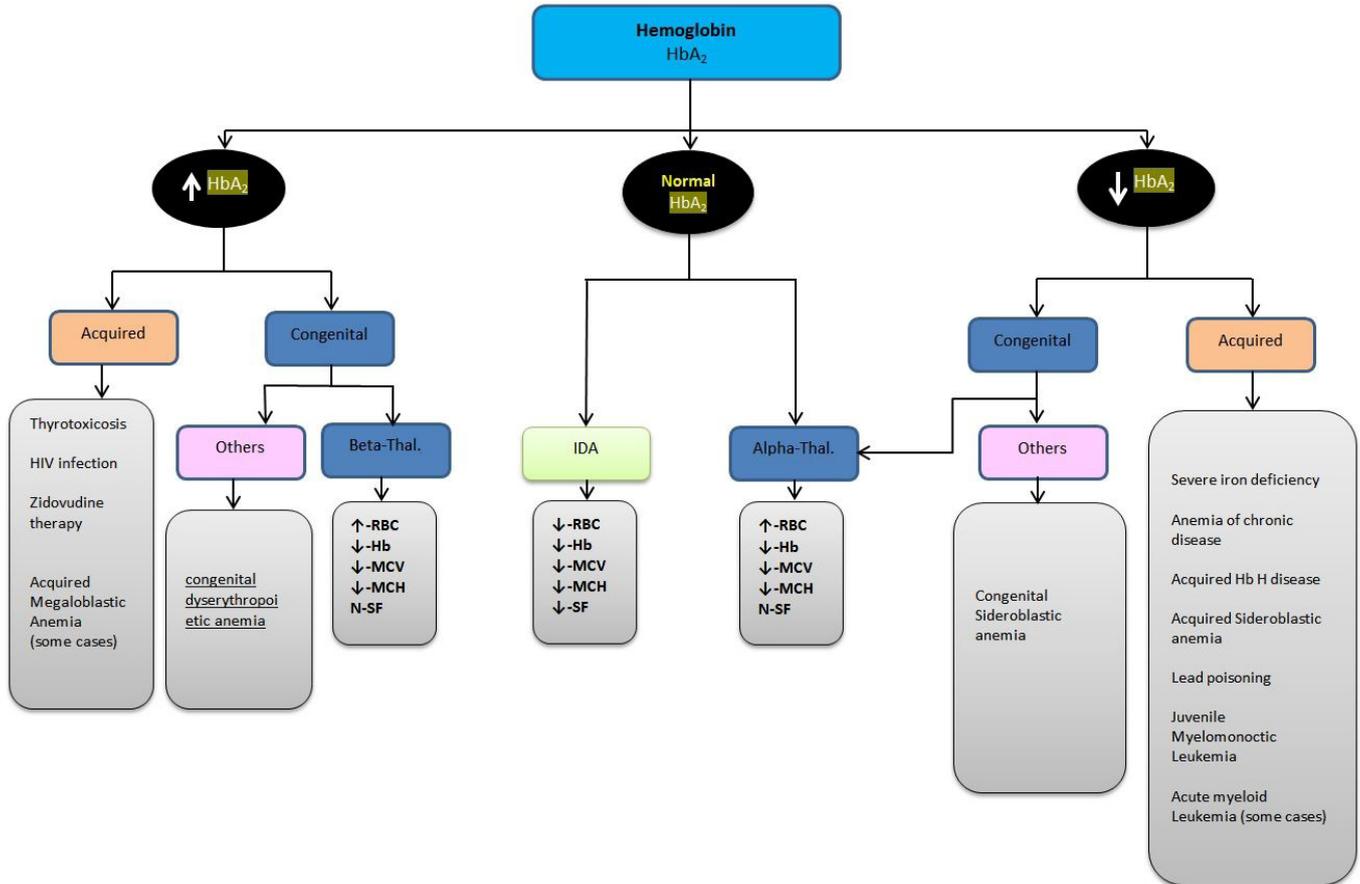
**Results:** All samples showed different degrees of variation between repeated test results. The variations ranged from 0.25% VIR in 51 (47%) of the repeated test results and 1.5% VIR in 7 (7%).

**Conclusion:** The normal range Hb A<sub>2</sub> levels are very narrow window. Repeated HPLC results for samples showed degrees of variation that could alter  $\beta$ -thalassemia trait diagnosis. Alert technical work and careful clinical interpretation are mandatory to avoid wrong diagnosis related to Hb A<sub>2</sub> level in  $\beta$ -thalassemia trait and other related hemoglobin disorders. This comparative study on repeated HPLC for Hb A<sub>2</sub> levels has great importance to avoid either false negative or false positive results.

**Keywords:** Hemoglobinopathy; Standardization; HPLC; Thalassemia minor; Quality control

## Introduction

Hemoglobin A<sub>2</sub> (Hb A<sub>2</sub>) level is the most significant parameter in the identification of  $\beta$ -thalassemia carriers. A small percentage of hemoglobin A<sub>2</sub> is present in normal adult red blood cells (RBC), accounting for about 1.5-3.5% of the total Hb in healthy individuals [1]. Several research studies have confirmed typical increase of Hb A<sub>2</sub> in  $\beta$  thalassemia carriers, thus providing evidence for the use of Hb A<sub>2</sub> as a diagnostic tool for such condition [2]. From that time on,  $\beta$  thalassemia carriers have been diagnosed by their Hb A<sub>2</sub> levels, which are usually above normal average. Other evidence, collected through the United Kingdom National External Quality Assessment Service (NEQAS) survey showed that some High Performance Liquid Chromatography (HPLC) methods are not well aligned with those of other systems, with a positive bias of up to 0.5% in Hb A<sub>2</sub> results [3]. Iron deficiency could interfere with the diagnosis of  $\beta$ -thalassemia trait [4]. In patients with the  $\beta$ -thalassemia trait, iron level could be elevated and iron-depleted  $\beta$ -thalassemia carriers are found to have no significant reduction in Hb A<sub>2</sub> level, although other studies reported low Hb A<sub>2</sub> in iron deficiency anemia [5,6]. Moreover, reduced Hb A<sub>2</sub> levels can be detected in the presence of  $\alpha$ -thalassemia mutations, probably due to preferential binding of the  $\alpha$ -chains with the  $\beta$  rather than with their  $\delta$  counterparts. This is clearly seen in hemoglobin H disease (Hb H), where Hb A<sub>2</sub> can drop to less than 1% [7]. Hb A<sub>2</sub> levels could also be affected by congenital or acquired factors (Figure 1). The Hb A<sub>2</sub> variations detected by repeated HPLC tests in this study could potentially influence the diagnosis of the  $\beta$ -thalassemia trait and another hemoglobinopathies. Therefore, this study highlights the impact of such variations on Hb A<sub>2</sub> level quality control determined by HPLC testing.



β-Thal = β -Thalassemia; α-Thal = αThalassemia; IDA = Iron deficiency anemia; RBC = Red blood count; Hb = Hemoglobin; MCV = Mean corpuscular volume; MCH = Mean corpuscular Hemoglobin; SF = Serum ferritin; n = normal

**Figure 1:** Causes of different HbA<sub>2</sub> levels with the hematology laboratory results comparison between β-Thal, α-Thal, IDA

## Method

A retrospective study was conducted to assess the variation between repeated HPLC results for the same sample. In our medical records, 107 patients were found to have repeated HPLC tests between 2010 and 2015 without known the reasons why the test was repeated, most likely it were repeated without checking the availability of the results in the records. HPLC analysis was performed using the Waters Corporation machine model 2487 Dual λ Absorbance Detector. From available records, samples that were repeat tested by HPLC and had high Hb A<sub>2</sub> levels were included in this study. Samples with another hemoglobinopathy such as S, D, G, E, thalassemia major, or thalassemia intermedia were excluded. HPLC results were interpreted in correlation with red blood cell indices and serum ferritin levels. All the tested samples performed on same machine throughout this period. Each sample was repeated only one time but not on the same day. The variation interval ranges were vary between few days and several months. Serum ferritin was available in our data for 76 patients but it was not done at the same time as HPLC. It was included to give a useful available relevant data.

## Results

Repeated HPLC measurements revealed variations in Hb A<sub>2</sub> levels (Table 1). These variations were categorised into variation interval ranges (VIRs); each VIR spanned 0.25%. Six VIRs were created 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50%. Variations were detected in all the 107 samples had repeated HPLC tests. Analysis of the results of the 107 repeat tested samples revealed that 51 (47%) samples were in the 0.25% VIR; 28 (26%) samples were in the 0.5% VIR; 14 (13%) samples were in the 0.75% VIR; 5 (5%) samples were in the 1.00% VIR; 2 (2%) samples were in the 1.25% VIR; and 7 (7%) samples were in the 1.5% VIR. Serum ferritin level were available for 76 patients. Of these, 63 patients had normal serum ferritin levels; 2 patients had high serum ferritin; and 11 patients had low serum ferritin. After repeated testing, Hb A<sub>2</sub> were corrected from normal level to high level for 2 samples according to high RBC, high Hb, low MCV and low MCH from normal level to high level for 2 samples (Table 2). The Hb A<sub>2</sub> VIRs for all 107 samples were correlated to their RBC indices, Hb levels, and serum ferritin levels according to the hematology reference values based on age and gender (Table 3).

HbA <sub>2</sub> Variations intervals	Count	percentage	Hb A Mean ± SD (96 - 98)	HbA <sub>2</sub> Mean ± SD (1.5 - 3.5)	Hb F Mean ± SD <1	RBC (4.5 - 5.5) 10 <sup>12</sup> /L	Hb (130 - 170) g/L	MCV (83 - 101) fL	MCH (27 - 32) pg
0.25	51	47%	92.86 ± 2.56	5.19 ± 0.67	3.30 ± 2.46	5.62 ± 0.59	110 ± 12.2	62.2 ± 4.00	19.6 ± 1.41
0.50	28	26%	93.52 ± 2.48	5.12 ± 0.75	2.52 ± 3.00	5.59 ± 0.83	107 ± 13.1	60.9 ± 4.47	19.3 ± 1.66
0.75	14	13%	93.24 ± 2.53	5.29 ± 0.62	2.58 ± 2.82	5.43 ± 0.90	106 ± 16.7	62.8 ± 4.04	19.6 ± 1.45
1.00	5	5%	92.70 ± 3.49	5.11 ± 0.62	3.63 ± 3.73	5.29 ± 0.83	104 ± 12.5	63.6 ± 5.06	19.8 ± 1.72
1.25	2	2%	90.54 ± 2.67	5.43 ± 0.54	5.38 ± 2.37	5.47 ± 0.43	100 ± 3.77	56.3 ± 2.28	18.5 ± 1.12
1.50	7	7%	94.31 ± 2.13	4.53 ± 1.11	2.41 ± 1.58	5.14 ± 0.83	106 ± 15.7	64.8 ± 5.41	20.7 ± 1.85

Table 1: Hb A<sub>2</sub> variation intervals range

No	Gender	HbA <sub>2</sub> variations	Repeated	Hb A (96 - 98%)	HbA <sub>2</sub> (1.5 - 3.5%)	Hb F <1%	RBC (4.5 - 5.5) 10 <sup>12</sup> /L	Hb (130 - 170) g/L	MCV (83 - 101) fL	MCH (27 - 32) pg	Ferritin (23 - 336) ng/mL
1	M	2.64	1 <sup>st</sup>	97.07	2.7	<1	5.32	102	60	19	16.3
			2 <sup>nd</sup>	94.10	5.4	<1	5.29	99	58	19	37.6
2	M	2.27	1 <sup>st</sup>	98.00	2.4	-	5.74	125	68	22	84.9
			2 <sup>nd</sup>	90.73	4.7	4.60	4.99	105	66	21	

Table 2: Hb A<sub>2</sub> level before and after repetition in correlation to RBC indices and serum ferritin level

HbA <sub>2</sub> AIR	Gender	Age	N	Hb A (96 - 98)	HbA <sub>2</sub> (1.5 - 3.5)	Hb F <1	RBC (4.5 - 5.5) 10 <sup>12</sup> /L	Hb (130 - 170) g/L	MCV (83 - 101) fL	MCH (27 - 32) pg	Ferritin (23 - 336) ng/mL
0.25	F	1 - 16	11	91.86 ± 2.65	5.17 ± 0.49	3.87 ± 2.63	5.59 ± 0.50	106 ± 10.0	60.5 ± 2.52	19.0 ± 1.00	22 ± 16
		18 - 82	17	93.08 ± 2.29	5.26 ± 0.76	2.77 ± 1.68	5.33 ± 0.50	107 ± 10.0	63.6 ± 4.38	20.2 ± 1.29	29 ± 23
0.25	M	1 - 16	9	91.31 ± 2.88	5.54 ± 0.55	4.33 ± 3.42	5.78 ± 0.39	106 ± 9.0	59.2 ± 2.35	18.3 ± 1.02	44 ± 24
		19 - 77	14	94.06 ± 2.14	4.87 ± 0.57	3.33 ± 2.75	5.96 ± 0.67	120 ± 14.0	63.7 ± 3.59	20.2 ± 1.23	129 ± 145
0.50	F	3 - 14	8	92.23 ± 2.53	5.54 ± 0.86	3.13 ± 2.66	5.69 ± 0.58	104 ± 9.5	58.5 ± 2.16	18.3 ± 1.00	48 ± 46
		18 - 69	12	94.59 ± 1.07	4.79 ± 0.50	1.26 ± 1.00	5.26 ± 0.70	105 ± 13.0	63.2 ± 5.12	20.2 ± 1.93	122 ± 236
0.50	M	1 - 15	6	93.15 ± 3.34	5.23 ± 0.69	3.70 ± 4.94	5.85 ± 1.14	111 ± 15.5	60.4 ± 4.32	19.2 ± 1.33	43 ± 4
		63	2	94.69 ± 0.65	4.62 ± 0.17	1.06 ± 0.60	6.11 ± 0.34	117 ± 8.3	62.5 ± 1.12	19.2 ± 0.43	103 ± 53
0.75	F	13 - 16	3	94.21 ± 0.59	5.28 ± 0.56	0.69 ± 0.28	5.56 ± 0.89	106 ± 19.7	60.6 ± 3.77	19.0 ± 1.41	16 ± 12
		22 - 45	6	92.39 ± 3.26	5.39 ± 0.49	4.41 ± 3.74	4.75 ± 0.55	98 ± 12.5	66.0 ± 3.44	20.6 ± 1.44	769 ± 893
0.75	M	4 - 14	3	92.26 ± 2.25	5.51 ± 0.73	3.25 ± 0.67	5.90 ± 0.34	111 ± 7.1	60.2 ± 2.27	19.0 ± 1.0	31
		39 - 58	2	94.90 ± 0.74	4.81 ± 0.59	0.46 ± 0.09	6.33 ± 0.80	121 ± 15.3	62.0 ± 1.67	19.2 ± 1.0	99 ± 23
1.00	F	10 - 16	2	93.81 ± 1.73	5.20 ± 0.59	3.87 ± 0.00	5.62 ± 0.20	105 ± 2.9	60.5 ± 1.12	18.7 ± 0.43	12
		22 - 30	2	94.54 ± 1.07	5.07 ± 0.70	0.52 ± 0.37	4.65 ± 0.99	101 ± 19.3	69.2 ± 2.86	21.7 ± 0.83	458 ± 317
1.00	M	1	1	86.83 ± 2.97	5.00 ± 0.44	8.18 ± 2.53	5.91 ± 0.15	106 ± 2.0	58.5 ± 0.50	18.0 ± 0.00	13
1.25	M	1 - 6	2	90.54 ± 2.67	5.43 ± 0.54	5.38 ± 2.37	5.47 ± 0.43	100 ± 3.77	56.2 ± 2.28	18.5 ± 1.12	32 ± 3.3
1.50	F	23 - 56	3	94.54 ± 1.67	4.58 ± 1.08	2.16 ± 1.50	4.57 ± 0.70	95 ± 11.0	66.0 ± 6.0	21.0 ± 2.00	235 ± 24
		1	1	95.59 ± 1.49	4.06 ± 1.32	<1	5.31 ± 0.02	101 ± 1.50	59.0 ± 1.0	19.0 ± 0.00	38
1.50	M	49 - 57	3	93.63 ± 2.54	4.59 ± 1.05	2.56 ± 1.60	5.82 ± 0.50	121 ± 11.0	65.0 ± 4.0	21.0 ± 2.00	100 ± 15

Table 3: Hb A<sub>2</sub> variations in correlation with RBC indices, hemoglobin and serum ferritin according to hematology reference values in different age and gender

## Discussion

An elevated Hb A<sub>2</sub> determined by the HPLC test, normal or high serum ferritin, microcytic hypochromic features, high RBC count, and low to normal total hemoglobin, are parameters to determine β-thalassemia trait diagnosis. Hb A<sub>2</sub> plays an integral role in identifying the carrier status of a subject. The range for considering Hb A<sub>2</sub> levels as normal is very narrow; therefore, a slight

increase or decrease in the Hb A<sub>2</sub> level could change the diagnosis from being considered positive for the  $\beta$ -thalassemia trait to normal and vice versa. The repeated HPLC tests show variation differences according to VIRs which could change the diagnosis (Figure 2).

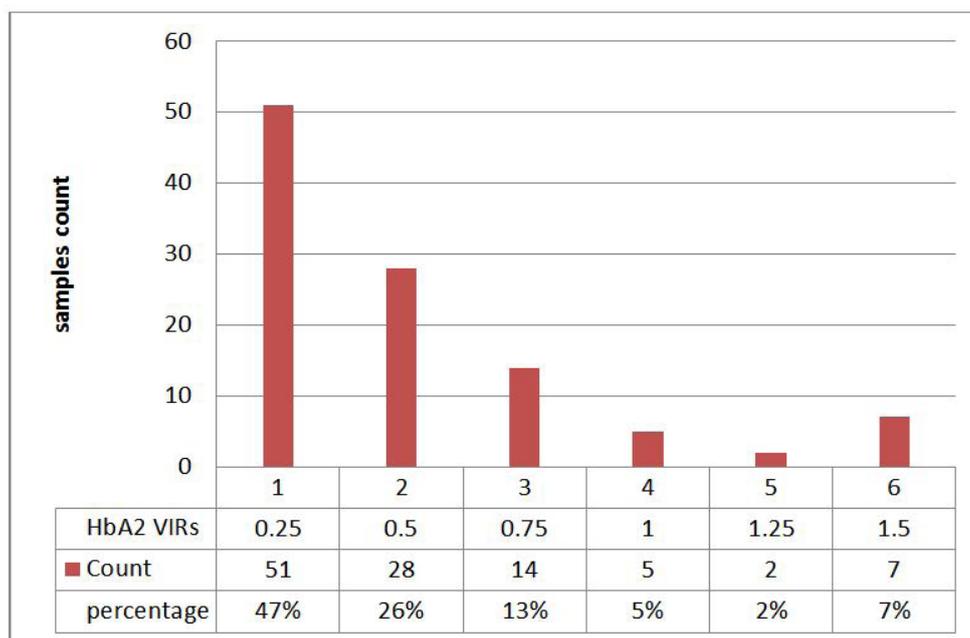


Figure 2: Number of the samples in each variant interval range

For two patients, the first HPLC results showed normal Hb A<sub>2</sub>, which was in contradiction with the other parameters. However, when the test was repeated, the Hb A<sub>2</sub> became higher than normal, which was consistent with  $\beta$ -thalassemia trait (Table 2). These results highlight the importance of correlating the Hb A<sub>2</sub> level with other parameters before assigning a final diagnosis. The VIRs for Hb A<sub>2</sub> levels were compared with RBC indices after sorting the samples based on age and gender, which confirmed the correlation between Hb A<sub>2</sub> levels and RBC indices (Table 3).

Serum ferritin was available in our records for 76 patients but it was not done at the same time as HPLC. As expected, serum ferritin level was within the normal reference range in 63 patients. The serum ferritin level was high (520–824  $\mu\text{g/L}$ ) in only 2 patients. Depletion of iron stores was found in 11 patients, Four of these patients were males, with serum ferritin ranged between 10.8 and 17.1  $\mu\text{g/L}$ ; and 7 were females with a serum ferritin between 3.5 and 10.6  $\mu\text{g/L}$ . Interestingly, all patients with low serum ferritin levels, had higher than normal Hb A<sub>2</sub> levels when the HPLC was repeated, and the Hb A<sub>2</sub> stayed greater than normal. Therefore, the diagnosis of  $\beta$ -thalassemia trait was not altered. This study showed no effect to the low serum ferritin level on Hb A<sub>2</sub> level because with low serum ferritin the RBC count usually drop below the normal range, but the RBC count was high, in consistent to  $\beta$ -thalassemia trait rather than iron deficiency anemia. It is important finding although 11 samples with low serum ferritin and high Hb A<sub>2</sub> and high RBC count is not large enough number to make conclusion about iron deficiency and its effect on the HbA<sub>2</sub> level a further study needed for this significant results. This is an important finding because some other studies showed that low serum ferritin was thought to reduce Hb A<sub>2</sub> level to normal or below normal in patients with  $\beta$ -thalassemia trait [8,9]. Serum ferritin level in patients with the  $\beta$ -thalassemia trait could be high [10]; our results revealed 2 patients with elevated serum ferritin levels without receiving blood transfusion. It is important to be aware of any interference that may falsely alter Hb A<sub>2</sub> levels, leading to false positive (high) or false negative (low) Hb A<sub>2</sub> level result, which may result in inappropriate diagnosis or management [11]. For example, as reported in the literature false negative Hb A<sub>2</sub> could be caused by an iron deficiency or concurrent inheritance of the  $\alpha$ -thalassemia trait [6]. Therefore, the quantification of Hb A<sub>2</sub> levels has to be with high accuracy [12]. Many factors such as the lack of awareness among working technologists and clinical pathologists, or inadequate facilities, may result in imprecise outcomes and interpretations [13]. Another important finding in this study is that the repeated Hb A<sub>2</sub> test results of 7 patients (7%) showed a difference of 1.5% from their first test results, whereas Hb A<sub>2</sub> variation is only, up to 0.5% has been reported in literature [14]. This relatively large difference between the initial and repeated results of Hb A<sub>2</sub> levels which may affect result in the interpretation, if the diagnosis is made solely based on HPLC results only. Equipment calibration, regular maintenance, test validation and the laboratory quality control are necessary to ensure high and accurate performance. Equivocal borderline high Hb A<sub>2</sub> results warrant repeat testing using other modalities and/or genetic tests of the globin genes. There are a few cases of symptomatic refractory microcytic hypochromic anemia with borderline, normal, or reduced Hb A<sub>2</sub> levels. These cases should be investigated for the presence of  $\alpha$ -thalassemia or its co-inheritance with  $\beta$ -thalassemia gene [15], or silent  $\beta$ -thalassemia trait [16]. This study need to be repeated as a prospective study to confirm the findings with molecular biology techniques which couldn't be done in this retrospective study.

## Conclusion

The normal range for Hb A<sub>2</sub> is very narrow. Small changes in Hb A<sub>2</sub> levels can result in incorrect diagnosis. In this study the low serum ferritin did not decrease the Hb A<sub>2</sub> to a normal level. Proper machine maintenance, standardized reagents, adequate training of HPLC testing for technologists and interpretive aspects from pathologists and physicians are crucial to make accurate diagnosis. HPLC result interpretation must be done in correlation with family study, RBC parameters, serum ferritin levels, and blood film morphology. High degree of suspicion helps to identify possible false positive or false negative results. Equivocal or inconclusive Hb A<sub>2</sub> results should be investigated further by other methods such as DNA analysis.

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