

HbA₂ measurements in β -thalassemia and in other conditions

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Abstract

Quite a few papers have been written on the significance of elevated hemoglobin (Hb) A₂ as a parameter for the diagnosis of β -thalassemia trait, on the cutoff values to be used in diagnostics and on the significance and effects of factors reducing or elevating the expression of HbA₂ and last but not least on the need for reliable measurement methods and precise calibrations with accurate standards. However, little has been published on the causes that elevate or reduce the HbA₂ levels in β - and α -thalassemia and in other conditions. For a better understanding of the value of a precise measurement of this parameter we summarize and elucidate in this review the direct and indirect mechanisms that cause the variations in HbA₂ expression and that influence the value of this parameter in particular conditions. We conclude by explaining the advantages and disadvantages of trusting on a precise measurement in the complete diagnostic contest.

Introduction

To understand the significance of the expression of the normal hemoglobin (Hb) fractions measured in the lysates in pre and postnatal life in normal and abnormal conditions, one must be first aware of the structure of the proteins and of the function of the genes coding for these proteins. Hemoglobins are tetrameric molecules consisting of four subunits, two α -like and two β -like globin chains and four iron atoms placed in four porphyrine rings (the hemes) which are imbedded in the globin chains.

The globin genes are clustered and located on different chromosomes. The β cluster is located on chromosome 11, contains one non-active pseudo gene ($\psi\beta$) one embryonic (ϵ), the two fetal genes ($G\gamma$ and $A\gamma$) and two postnatal genes, the *low expression* δ and the *full expression* β

gene. The δ gene is a kind of pseudo gene, practically silent at birth that, in normal conditions, reaches 2.5-3.5% expression within one year after birth. Therefore, the expression rate between the two β and the two δ genes (one from maternal and one from paternal origin) is roughly 97:3 and this amount of β and δ chains and some traces (\approx 0.5%) of γ chains, is the only counterpart available for binding to the α chains expressed by four α genes in postnatal life.

The α -like chains are produced by the a cluster, which is located on chromosome 16 and contains 3 non-active pseudo genes, one embryonic (ζ) gene and two α genes ($\alpha 1$ and $\alpha 2$), which are active during embryonic, fetal and postnatal life. After a brief embryonic phase in which only the ζ and the ϵ genes are active coding for the first embryonic tetramer ($\zeta 2/\epsilon 2$ Hb Gower 1), the four α genes, two per chromosome 16, contribute to the formation of the second embryonic hemoglobin Gower 2 ($\alpha 2/\epsilon 2$), the fetal hemoglobin HbF ($\alpha 2/\gamma 2$), and both postnatal HbA₂ ($\alpha 2/\delta 2$) and HbA ($\alpha 2/\beta 2$). Noteworthy, while four α genes (two from the maternal and two from the paternal chromosome 16) are contributing to the formation of HbA and HbA₂ only two β genes and two $\beta\delta$ genes are providing the non- α counterpart to form these post-natal tetramers (Figure 1).

Mechanisms causing the hemoglobin A₂ levels to rise in β -thalassemia carriers and in other conditions

Loss of expression of one β gene

In the carrier of β thalassemia (heterozygous) one of the two β globin genes is either not or just barely expressed. This means that to match for the 100% expression of the four normally expressed $\beta\alpha$ genes only half of the β genes expression is available. Consequently, the normal expression rate between the four β -like genes will change from two β versus two δ into one β versus two δ and thus from 97:3 to 48:3, increasing the proportion of δ chains versus β chains from 3 to 6% resulting into a comparably elevated HbA₂ (Figure 2 and Table 1).

This basic mechanism explains the HbA₂ elevation and eventually also why, in some mild β -thalassemia mutations with only a partial reduction in β expression, the elevated levels of HbA₂ may become less evident. However, this is not always the case and the simple calculation of the β : δ rate does not explain why the elevation of HbA₂ may go from 4% or less to 8% or more in different but equally severe β^0 thalassemia defects, or be even variable in carriers of the same defect. Then we must consider a number of modulating factors and first of all the measurement of artifacts.

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Measuring artifacts

All dedicated devices or manual kits for HbA₂ estimation are subjected to different degrees of imprecision and to measuring artifact.¹ Factors such as co-elutions, integration modes, calibrations, different columns, different buffers, different temperatures, different sample quality and concentrations have to be considered. These and other factors contribute to an inevitable variety of artifact that makes the use of *normal cutoff* intervals very risky when the HbA₂ value alone is used to confirm or exclude a possible β -thalassemia trait. Moreover, normal intervals established using a *local normal population* consisting of blood donors or perhaps undiagnosed α thalassemia carriers (very common in Black) may down scale the *normal range* by 0.5% or more as shown during measurements of extended specific cohorts.²

In addition, dedicated devices are loading very small volumes of samples that may be fresh or not, may be homogeneous or partially sedimented or particularly hypochromic [low mean corpuscular hemoglobin (MCH)] resulting in less Hb loaded and smaller HbA₂ peaks and less precise integrations.³ Having summarized all these variables the most reliable measurement of Hb fractions remains the alkaline electrophoresis separation of a fresh sample on cellulose acetate strips, the elution in equal volume of Drabkin solution of the unstained hemoglobin bands excised from the cellulose, followed by colorimetric measurement at 418 nm. Although still ideal for measuring the precise HbA₂ value of standard stock solutions, such a laborious method, is not practical in the modern everyday routine diagnostics that requires well-calibrated measurements on dedicated high performance liquid chromatography (HPLC) and/or capillary electrophoresis (CE), the best options in the modern laboratory² (summarized in Table 1).

More interfering factors

Because of the loss of expression of one of the two β genes one would expect that the 14 g/dL Hb level measured on average in the normal individual should always drop by half in the β -thalassemia carrier. Fortunately this is not the case and most healthy β -thalassemia carrier present with Hb values that are barely subnormal (≈ 12 g/dL) due to a number of compensating factors. Conversely, some carriers may present with more severe hematological pictures due to insufficient compensation, particular semi dominant mutations, hemolysis, inefficient proteolysis⁴ or additional α genes (triplications)^{5,6} that may aggravate the carrier state to a thalassemia intermedia. All these factors may also influence the HbA₂ levels and the first factor to be considered is the compensation mechanism triggered by the chronic anemia and tissue hypoxia that increases the red blood cell (RBC) production in the carrier.

Compensatory erythrocytosis

With sufficient folic acid intake β -thal carriers may present with elevated red cell counts up to 6, 7, 8 or more RBCx10¹²/L. Red cells will stay smaller with a lower Hb content than normal (low mean corpuscular volume and MCH) but because of the higher number of red cells the Hb level will increase while the packed cell volume values will remain within normal range. The HbA in these cells derives from the expression of the normal allele that could be enhanced in one case more than in another producing also more δ chains. In addition, the quantity of Hb loaded on the dedicated device will also be higher in well compensated carriers adding to the HbA₂ variability as mentioned above under artifacts.

Balanced gene expression, hemolysis and proteolysis

Due to chronic anemia and tissue hypoxia the expression of the β gene on the normal allele and of the δ and γ genes on both alleles will become enhanced producing more β , δ and γ chains if genetically predisposed. This will partially restore the α /non- α balance within the cell, with some cells more balanced than others. Those cells with the best balance (and the highest δ gene expression) and with the best proteolysis eliminating the free α chains and the unused β polypeptides left from the β -thalassemia mutation, will stay longer in circulation increasing the average value of HbA₂ measured in the lysates.

Coexisting iron deficiency

Iron deficiency is rarely seen in β thalassemia because carriers absorb more iron through the gut than normal and present with elevated ferritin levels and eventually also due to histories of iron supplementation. Nonetheless, it has been shown that in iron deficiency the HbA₂ level is usually at the lower edge of the normal distribution, possibly due to a preferential imbedding of the heme into the β rather than the δ chains as it has been shown for the $\beta^A\beta^S$ chains.⁷

Cases in which iron deficiency has reduced the elevated HbA₂ levels of the typical *high HbA₂ β -thalassemia carriers* to normal, compromising a diagnosis as postulated in the past⁸ have never been observed by these authors and neither by colleagues that have examined iron depleted β -thalassemia carriers in malnutrition areas.⁹ On the other hand, one cannot exclude that atypical β -thalassemia carriers with near normal or only

slightly elevated HbA₂ levels could be missed if, in the presence of iron deficiency, their HbA₂ levels would marginally drop and herewith falling within the normal range.

More than an iron problem this is an atypical β -thalassemia problem and in all these *grey zone* cases the conscientious clinical chemist should never trust on the cut of value and should always carefully examine the complete hematological picture and look at iron status, blood counts and erythromorphology and should ask for DNA analysis in case of doubt (summarized in Table 1).

Coexisting δ thalassemia, δ variants and large deletions

Although not considered relevant because of the insignificant expression and absence of pathology, δ gene mutations causing δ -thalassemia or δ Hb variants are important not only as a model but mainly because of their interference with the measurement of HbA₂. This is particularly important if δ defects occur in association with β -thalassemia and especially because carriers could be misdiagnosed during basic screening and couples at risk seeking prevention could be wrongly counseled. Delta gene mutations have been often described solely and in association with β thalassemia^{10,11} showing that also in these cases complete blood count, erythromorphology and DNA analysis are essential for a conscientious diagnosis.

HbA₂ variants are relatively common and generally easy to spot not only because they reduce by half the HbA₂ value but also because δ variants usually show a second HbA₂ fraction of almost equal expression, either preceding or following the normal HbA₂ that can be confirmed by

Table 1. Summary of common conditions increasing, decreasing or influencing hemoglobin A₂ levels and measurements.

Causes	Increase	Decrease
High A ₂ β -thalassemia	Diagnostic level >4%	
Atypical β -thalassemia	Grey zone (3-4%) (DNA)	
High A ₂ β -thalassemia + α -thal	Diagnostic level >4% (DNA)	
α -thalassemia from ($-\alpha/\alpha\alpha$) to ($-/-\alpha$)		Marginal to significant (DNA)
α -variant (generates a second HbA ₂)		$\approx 25\%$ + second small HbA ₂ (DNA)
Unstable β variants	Marginal to significant (DNA)	
Stable β variants (HbS, C, E, D, ...)	Measurement obsolete	Measurement obsolete
δ -variants		$\approx 50\%$ + second $\approx 50\%$ HbA ₂
δ -thalassemia		$\approx 50\%$
$A\gamma\delta\beta$ -deletions		$\approx 50\%$ + high HbF
$G\gamma A\gamma\delta\beta$ -deletions		$\approx 50\%$ no high HbF
Iron depletion		Marginal
Iron depletion + α -thal		Marginal to significant (DNA)
Iron depletion + high A ₂ β -thal	Diagnostic parameter >4%	
Iron depletion + atypical β -thal	Grey zone (3-4%) (DNA)	
Other diseases	HIV and reference 15	
Sample related artifacts hemolysis, low Hb		Marginal to significant
Device related artifacts	Overlapping, integration modes, calibration, column/buffer match	Integration modes, low Hb, calibration, column/buffer match

Hb, hemoglobin.

DNA sequencing.¹⁰ However, while the normal A_2 is reduced, the second HbA_2 may become invisible because of low expression or because it migrates or elutes under the HbA fraction if the amino acid substitution has caused the loss of one positive charge gaining a negative one. The opposite charge change, could delay the elution of the second HbA_2 peak that could end in the washing cycle of the dedicated instrument and mimic a δ -thalassemia condition. The δ -thalassemia mutations, also reducing the HbA_2 level by half in the heterozygous, will be suspected when, in normocytic normochromic conditions an HbA_2 level is measured below 2.0%. Then, in absence of severe iron depletion or artifacts, direct sequencing of the delta genes will usually reveal a point mutation defect in heterozygous form or, in total absence of HbA_2 , in homozygous or hemizygous form (deletion in TRANS).¹² More risky are those cases of combined β -thalassemia and δ -thalassemia traits when the elevated HbA_2 of the β -thalassemia carrier can be reduced to normal levels by the δ -thalassemia defect.¹¹ Finally, large deletions involving the locus control region and/or one or both the γ genes will present with microcytosis and normal or reduced HbA_2 levels with or without the elevation of HbF that characterize the classic normal HbA_2 $\delta\beta$ thalassemia deletions¹² (summarized in Table 1).

Coexisting α thalassemia

Combinations of β and α thalassemia are not that uncommon and usually present with the same elevated HbA_2 levels expected for that specific β -defect. This can be explained by the more or less restored balance between free α and β chains, whereas no preferential binding occurs (summarized in Table 1).

Hemoglobin A_2 in β -thalassemia major

Estimating HbA_2 in β -thalassemia major is first of all a methodical problem and is not an important diagnostic parameter. One should keep in mind that the switch from fetal to postnatal genes expression, normally completed within one year, is delayed in β -thalassemia major and that, while the expression of the only active postnatal (δ) genes is retarded, the expression of the fetal γ genes is prolonged. This will result into absence or severe deficiency of HbA and high HbF expression that will however not be sufficient to prevent a severe anemic condition. Then around 6-7 months of age the patient will have to be urgently treated with a first blood transfusion and with more transfusions to follow. In this situation the HbA_2 values measured after transfusion will be those of the blood donors. In some cases, when patients have

been diagnosed before becoming transfusion dependent or when diligent hematologists have measured the Hb fractions prior the first transfusion, it has been observed that patients homozygous or compound heterozygous for β -thalassemia mutations associated with elevated HbA_2 , also present with an elevated HbA_2 value similar to the one measured in the heterozygous or higher. Conversely, cases that manage to stay transfusion free for a longer time because of a particularly high γ gene expression and a reasonably balanced erythropoiesis, may present with an HbA_2 level which is either normal or even reduced.¹³ This could be explained by a consistent erythropoietic stress which accelerates the maturation process of the red cell precursors, shifting the gene expression to the active fetal genes,¹⁴ not reaching the inactive β and the still active δ genes which will fail to be enhanced.

Hemoglobin A_2 in α -thalassemia conditions

A reduction in a gene expression will progressively reduce the expression of HbA_2 below the normal average probably because of preferential binding to the more abundant β chains. The reduction will be marginal in the mild α thalassemia forms and therefore not always measurable in a reliable way mainly due to the inevitable technical variability and sample conditions (read above in measuring artifact).

As previously reported, values below 2.5 or 2% are however measured in homozygous $\alpha\alpha$ ($-\alpha/\alpha$) or heterozygous α^0 ($-\alpha/\alpha$) conditions, reaching levels around 1.5% in HbH disease ($-\alpha/\alpha$)¹⁵ (summarized in Table 1).

Hemoglobin A_2 in the presence of hemoglobin variants

Over and underestimations, useless measurements and risks

Dedicated devices may overestimate or underestimate the HbA_2 levels in the presence of Hb variants. Classic artifacts are for instance measured on HPLC in the presence of the common HbS or the less common HbD^{Punjab} respectively.³ In the first case HbA_2 overlaps with glycosylated HbS , in the second underestimation possibly due to the integration method is constantly observed. This is not a diagnostic problem because the measurement of HbA_2 in carriers of β variants is obsolete. The presence of HbS or HbD together with HbA implies (in non transfused patients) that both β genes (we have only two of them; Figure 1) are expressed and that consequently there is no need to measure HbA_2

to establish the presence of a non-expressed β thalassemia gene. However, in homozygous or hemizygous conditions an overestimated HbA_2 may wrongly suggest HbS/β thal in one case or a false homozygosis HbD/D in the other, leading to wrong diagnosis and mistaken risk prediction¹⁶ (summarized in Table 1).

Increased hemoglobin A_2 in presence of thalassemic or unstable hemoglobin variants

Higher or borderline values of HbA_2 can also be measured in the presence of structural defects of the β globin. Unstable structural defects or variants with a thalassemic phenotype may induce an elevation of the HbA_2 fraction by different mechanisms. For the thalassemic variants at low expression, like for instance HbE , the basic mechanism will be similar to that of β -thalassemia (Figure 1). For unstable variant the mechanism will be more complex. Intracellular precipitation and hemolysis eliminating the unstable β/α tetramers and allowing those red cells with higher δ expression to survive longer will play a role while the modified polypeptide chains will be less suitable for the formation of dimers and tetramers producing a post translational thalassemic effect. Carriers of Hb Köln, Hb Zürich, Hb Bushwick, Hb Hope may present with moderately increase of HbA_2 levels as it is in general the case in over 50% of the unstable variants reported in the literature.¹³ These conditions are usually suspected because of their often atypical semi-dominant character similar to a *thalassemia intermedia* but they cannot be diagnosed without molecular analysis especially if the abnormal hemoglobin is not detectable on HPLC or CE (summarized in Table 1).

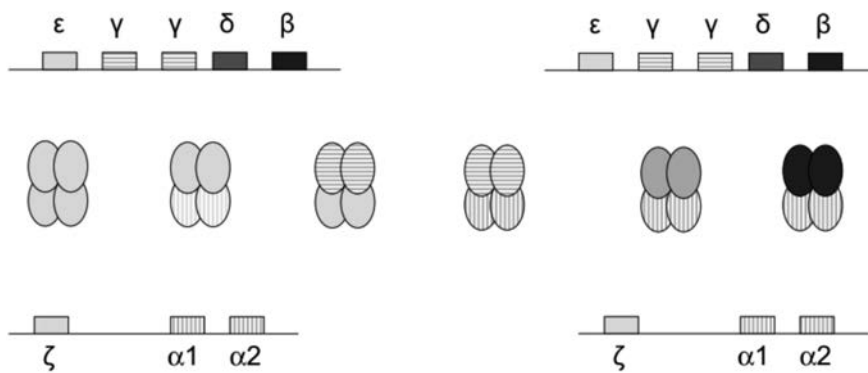
Elevated hemoglobin A_2 occasionally measured in other diseases

Elevated HbA_2 levels have been measured in absence of β -thalassemia in isolated cases of pseudoxanthoma elasticum, megaloblastic anemia, hypertrophic osteoarthropathy and hyperthyroidism. Although apparently reliable these measurement are difficult to be explained by the specific diseases but for the elevated HbA_2 measured in HIV patients treated with thymidine derivate, possibly influencing the expression of the δ gene¹⁵ (summarized in Table 1).

Take home message

The complexity of the mechanisms influencing the HbA_2 levels tells us that confirming or excluding a β -thalassemia carrier state by measuring the HbA_2 levels only and by trusting on the presumed precision of our HPLC or CE can be very risky. One may be reasonably sure when an HbA_2 level of $>4\%$ is measured but should not forget that atypical β -thalassemia carriers with HbA_2 levels between 3 and 4% are not that uncommon. The HbA_2 value as a diagnostic

The β clusters on the two parental chromosomes 11



The α clusters on the two parental chromosomes 16

Figure 1. Globin genes coding for embryonic, fetal and postnatal hemoglobin (Hb) tetramers. From left to right: Hb Gower1, Hb Gower2, Hb Portland, HbF, HbA₂ and HbA.

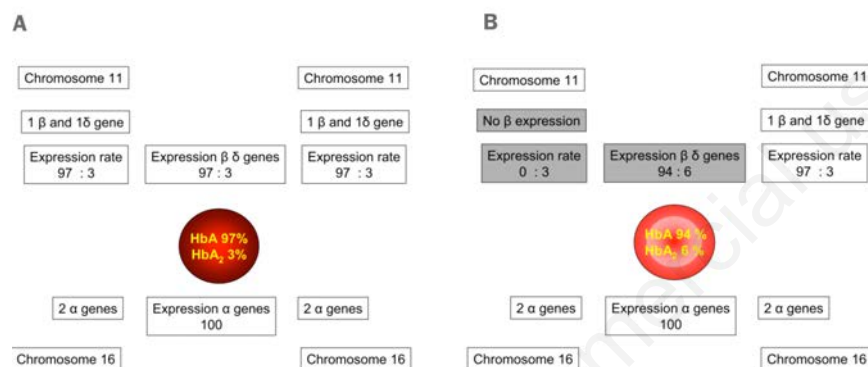


Figure 2. A) The normal rate HbA:HbA₂ in the normal red cell, derived from the normal expression of the β and δ genes from both chromosomes 11. B) Elevated HbA₂ value in the β thalassemic red cell, derived from the expression of a single β gene (β thalassemia minor) from only one chromosome 11 and 2 δ genes from both chromosomes 11.

parameter should always be considered together with the complete blood count, iron status and RBC morphology and in case of doubt a reference lab should be asked for DNA analysis, especially in case of risk assessment.¹⁷

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