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The role of haemoglobin A_2 testing in the diagnosis of thalassaemias and related haemoglobinopathies

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ABSTRACT

The increase in haemoglobin (Hb)A_2 level is the most significant parameter in the identification of β thalassaemia carriers. However, in some cases the level of HbA_2 is not typically elevated and some difficulties may arise in making the diagnosis. For these reasons the quantification of HbA_2 has to be performed with great accuracy and the results must be interpreted together with other haematological and biochemical evidence. The present document includes comments on the need for accuracy and standardisation, and on the interpretation of the HbA_2 value, reviewing the most crucial aspects related to this test. A practical flow-chart is presented to summarise the significance of HbA_2 estimation in different thalassaemia syndromes and related haemoglobinopathies.

Haemoglobin (Hb)A ($\alpha_2\beta_2$) is the main haemoglobin component in postnatal life, accounting for more than 96% of total Hb, followed by HbA_2 ($\alpha_2\delta_2$) representing 2.5–3.5% of the total, and HbF ($\alpha_2\gamma_2$) constituting less than 1%. While α globin chains are the same for the three different haemoglobins (A, A_2 and F), δ chains are specific for HbA_2, and differ from the similar β chains by only 10 residues between positions 22 and 115, resulting in a higher isoelectric point with respect to HbA.

HbA_2 is barely detectable at birth, while the β gene is already active at the eighth week of gestation; normal newborns present with 20–30% HbA, which gradually increases while γ gene expression is reduced (switch HbF→HbA). The adult expression of postbirth haemoglobins is reached after the first year of life.

First reported in the 1950s using electrophoretic techniques,[1] HbA_2 was soon afterwards observed to be elevated in parents of children affected by Cooley anaemia.[2] Several authors later confirmed the typical increase of HbA_2 in β thalassaemia carriers, thus providing the evidence for the use of HbA_2 for the diagnosis of such a condition.[3]

From that time on, β thalassaemia carriers have been diagnosed by their HbA_2 levels, which are usually above 4%. However, a notable proportion of carriers with values between 3.5 and 4%, or even with values in the normal range, have been found.[4]

Therefore, accurate measurement of HbA_2 is needed. We summarise the latest information available with regard to this, and make recommendations to help in the avoidance of pitfalls and in understanding the significance of this laboratory test.

METHODOLOGICAL ASPECTS

Almost all quantitative methods for HbA_2 are based on the separation of this Hb from other Hb fractions by electrophoresis or chromatography. Separation by cellulose acetate electrophoresis followed by elution and colorimetry has been recommended,[5] but this technique does not integrate readily into modern laboratory practice. Scanning densitometry must be avoided due to overestimation.[6] Ion exchange methods from the late 1960s have been adapted to manual and automated systems and are still used today; some years ago the International Committee for Standardization in Haematology recommended ion exchange microcolumn chromatography as a reference method for HbA_2 quantification.[6]

Currently, high-performance liquid chromatography (HPLC) on dedicated commercial apparatus is the method of choice in most laboratories,[7 8] often with direct loading from the primary tube. Capillary electrophoresis[9] is becoming a valid alternative, while proposed immunochemical methods[10 11] are not yet sufficiently validated.

Unfortunately, only few data are available on the degree of concordance of the various methods used. A recent investigation, performed using HPLC methods, revealed a between-laboratory variability between 6.0 and 9.6% (expressed as coefficient of variation, %).[12] Other evidence, collected through a NEQAS survey (Barbara De La Salle, personal communication), showed that some HPLC methods are not well aligned to those of other systems, with a positive bias of up to +0.5 %. These data indicate that there is a need for a better alignment between different procedures. Therefore the International Federation of Clinical Chemistry has recently launched a programme to develop a reference system for HbA_2 and to prepare secondary reference materials that could be used, mostly at the manufacturer level, to standardise all routine tests. Work is in progress on this.[13]

Pre-analytical variables

Case-related variables

Several pre-analytical factors may influence the HbA_2 levels in blood, and the most relevant are reported in table 1.

Increased levels have been reported in the absence of β thalassaemia trait in a few significant cases of hyperthyroidism (values over 4%),[14] in HIV-infected patients treated with antiretroviral therapy, [15 16] by Piero Giordano (personal communication), and in the presence of megaloblastic anaemia.[17]

In the presence of HbS, HbA_2 measured by HPLC is often increased, because of co-eluted glycated,[18] or otherwise modified HbS.[19] However, in many HbS carriers the exact quantification of HbA_2 is not crucial, since the expression of both β^A

and β^S genes excludes the presence of a β thalassaemia allele. However, in microdrepanocytosis (HbS/β thalassaemia) an elevated HbA_2 can be significant if microcytosis is also present, depending on β chain expression (β^0 or β^+), and the microcytosis is not caused by α thalassaemia, which can be very frequent in populations at risk of HbS. Finally, a slight increase in HbA_2 in the presence of triplicate α genes has also been reported in some cases, although recent evidence does not seem to confirm this observation (Piero Giordano, personal communication).

Decreased HbA_2 levels can be detected in iron depletion,[20] possibly due to the preferential binding of β to α chains, rather than δ chains, or to an inhibition of low iron levels on δ globin synthesis. Some authors have suggested that iron deficiency could interfere with the diagnosis of β thalassaemia trait.[21] Generally, in patients with β thalassaemia trait, iron is elevated and, even when iron-depleted carriers are found, no significant reduction of HbA_2 is detected.[22] However, in other cases (patients from the Indian subcontinent) who are iron depleted or frankly iron deficient, HbA_2 has sometimes been observed to be reduced to the normal range (Barbara J Bain, personal communication). Finally, reduced HbA_2 levels can be detected in the presence of α thalassaemia, probably again due to preferential binding of the scarce α chains with the β rather than with the δ counterparts. This is especially clearly seen in HbH disease, where HbA_2 can drop to less than 1% (fig 1).

Method-dependent variables

The presence of Hb variants may interfere with the quantification of HbA_2 on dedicated HPLC systems. In addition to the effect of HbS as mentioned above, HbE and HbLepore may also interfere with HbA_2 because of co-elution. In our experience, the highest HbA_2 level expected in β thalassaemia trait is 9%; the presence of 25–30% "HbA_2" indicates HbE, and about 15% indicates HbLepore. To date, the only routine method able to separate HbA_2 from HbE and HbLepore is probably capillary electrophoresis (Capillarys; Sebia, Paris, France). However, as for carriers of HbS, the accurate quantification of HbA_2 as a β thalassaemia marker is, in the case of HbE carriers, unnecessary, due to the presence of two β gene products. On the other hand, an elevated HbA_2 level is typical in HbE homozygotes, while in HbE/β thalassaemia or HbLepore/β thalassaemia the increased HbF values and the severe phenotype are helpful for the diagnosis.

A number of technical factors must also be taken into account when using HPLC methods. The estimation of the fractions is based on the calculation of the areas, and integration modes (such as valley-to-valley) may be different depending on the manufacturer. Other factors, such as carry-over, sample concentration and batch-to-batch differences might influence the result. Therefore HPLC apparatus must be handled only by well-trained personnel, and critical evaluation of the chromatograms, together with calibration and quality control programmes run on a regular basis, are essential.

Table 1 Main pre-analytical subject-related factors that might modify HbA_2 levels

Effect on HbA_2 value	
↑ HbA_2	↓ HbA_2
Hyperthyroidism	Severe iron deficiency anaemia
Megaloblastic anaemia	Sideroblastic anaemia
Antiretroviral therapy	"Silent" β thalassaemia alleles
Homozygous HbS + α thalassaemia	Interaction between δ and β thalassaemia (δ+β thalassaemia)
Some unstable haemoglobin variants	
Triplicate α gene (ααα)	δβ Thalassaemia
Pseudoxanthoma elasticum	α Chain variants
Hypertrophic osteoartropathy	δ Chain variants
	HbH disease
	Some forms of hereditary persistence of fetal Hb
	HbLepore
	Erythroleukaemia

Hb, haemoglobin.

Sample collection and storage

With the exception of heparin for in vitro globin chain synthesis analysis, EDTA is the anticoagulant of choice for haemoglobinopathy analysis. The best HbA_2 results are obtained on fresh material not older than 1 week. However, HPLC analysis remains fairly stable on samples stored at 4°C for 3 weeks or longer,[23] while methaemoglobin derivatives and other storage artefacts will progressively increase. Lysates can be frozen at −20 or −80°C for longer storage, although precise data on stability under such conditions are not present in the literature. Some manufacturers claim an overall stability of 1 month at −20°C and 3 months at −80°C. Thawing the samples and refreezing is discouraged.

Analytical variables

Analytical goals

Biological variation-based quality specifications are frequently used to set the analytical quality of laboratory procedures. While for total Hb or glycated Hb (HbA_{1c}) such specifications have been defined,[24] none have been reported for HbA_2. Based on a set of assumptions, we have recently calculated a maximum desirable imprecision of 1.4%, a bias of 4.9%, and an allowable total error of 7.3%.[12]

However, from the diagnostic point-of-view, another argument can be pursued, based on the principle that the overall analytical quality must be satisfactory if it is not to lead to misclassification. Indeed, to correctly classify a subject with a true HbA_2 value of 3.6% (besides other variables, such as mean cell volume (MCV) and mean cell haemoglobin (MCH)), the measurement error should not exceed 0.25% (relative total error 7.0%) in order to avoid the possibility of misclassifying an individual on the basis of a HbA_2 measurement as a β thalassaemia carrier ($HbA_2 \geq 3.8$ %) or as a non-β thalassaemia subject ($HbA_2 < 3.3$%).

Therefore, by merging the two different approaches, i.e. the average of the total error calculated from the deductions on biological variability, and the one calculated on the diagnostic needs, a reasonable limit for total error in measuring HbA_2 can be set at 7.15%.

Measurement units

The relative percentage (%) on total haemoglobin is the measurement unit for the expression of HbA_2. This unit, although not in line with the international SI unit system, is well established and uniformly used worldwide, as are other non-approved units of other quantities (eg, partial pressure expressed in mmHg and not kPa).

Reference intervals

As for any laboratory test, all laboratory professionals are theoretically responsible for calculating their own reference intervals for HbA_2 by measuring this Hb fraction in at least 100 adult individuals who are not iron-depleted, and are not carriers of α or β thalassaemia.

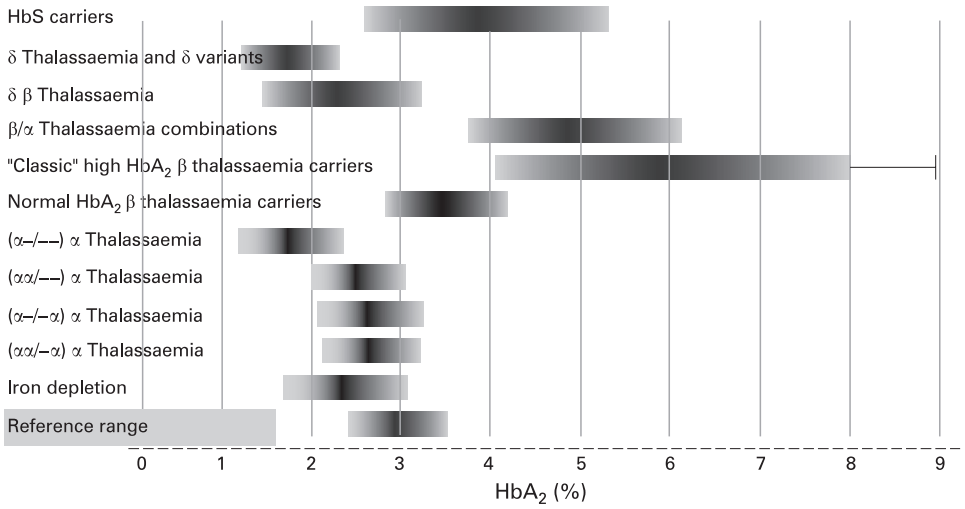

Figure 1 Specificity and overlapping of haemoglobin (Hb)A_2 values measured in different cohort of patients (adapted from Van Delft[25]).

The average value found in the reference laboratory at Leiden University using the Variant HPLC (Bio-Rad Laboratories, Hercules, California, USA) was 2.9%. The relative reference interval (2.5–97.5 centiles) was 2.3–3.5%.

Other laboratories might obtain slightly different figures due to differences in the alignment of the analytical procedures, or characteristics of the local population regarding α thalassaemia carriers or iron deficiency. In a cohort of 473 Sardinian inhabitants the mean (SD) HbA_2 was found to be 2.5 (0.3)% in non-β-thalassaemia carriers and 5.1 (0.7)% in β thalassaemia carriers.[26]

In order to show the variability of HbA_2 levels measured in the clinical setting we report a graphic example of the HbA_2 values measured in substantial cohorts of individuals with different common disorders (fig 1). The data come from 100 non-α-thalassaemic and non-iron-depleted healthy adult subjects, and from more than 670 subjects, tested at Leiden University Medical Center on a Bio-Rad Variant HPLC system. All diagnoses were confirmed by molecular analysis.[25]

With regard to follow-up during the first year of life, it is important to know that after the third month it is possible to diagnose a β thalassaemia carrier by the early rise of the HbA_2 fraction, as well as by other variables (MCV, HbF). HbA_2 reaches a stable level after the first year of life, and HbF reaches adult levels after the second year, as reported in Table 2.

INTERPRETATION OF HbA_2 DATA

Since HbA_2, together with a minimum set of other tests, is useful for the diagnosis of thalassaemia syndromes, a flowchart (fig 2) has been developed summarising HbA_2 levels and the other most relevant measurements (ie, MCV, MCH, HbF and iron status markers). Red cell count is also included, although in many cases it is not very informative. Finally, the most frequently used molecular techniques are given. The use of such techniques may be different depending on the population studied (for instance in Sardinia sequencing of the β gene is rarely required and is mostly essential when genetic counselling has to be performed).

On the right side of fig 2, the classical findings of a subject heterozygous for β thalassaemia are shown. HbA_2 levels in these cases are usually greater than 4%,[23] and very rarely up to 9% of total Hb.

Low HbA_2 levels can be differently interpreted. If the full blood count is normal and no additional HbA_2 fraction is present, then a clinically irrelevant δ thalassaemia is the most likely diagnosis. If a second HbA_2 peak of equal intensity is

Table 2 HbA_2 and HbF values, together with some relevant red cell indices, in normal infants and β thalassaemia carriers during the first year of life

Age	Subject	Number	HbA_2 (%)	HbF (%)	Hb (g/dl)	MCV (fl)	MCH (pg)
At birth	Normal	16	0.4 (0.2)	65.1 (7.5)	18.1 (2.3)	101.3 (6.9)	35.1 (3.5)
	β Thalassaemia	31	0.5 (0.2)	73.8 (10.1)	18.3 (2.3)	98.5 (8.1)	33.8 (2.6)
			NS	p<0.05	NS	NS	NS
3 months	Normal	8	1.7 (0.3)	18.1 (3.6)	11.0 (0.7)	82.5 (3.6)	27.9 (2.0)
	β Thalassaemia	12	3.2 (0.7)	27.0 (10.5)	10.1 (1.1)	69.9 (5.8)	22.8 (1.8)
			p<0.01	p<0.05	NS	p<0.001	p<0.001
6 months	Normal	8	2.5 (0.3)	3.2 (1.1)	11.5 (0.8)	74.7 (2.9)	24.9 (1.4)
	β Thalassaemia	10	4.8 (0.7)	8.2 (4.0)	10.5 (0.8)	59.2 (3.5)	19.2 (1.2)
			p<0.001	p<0.001	p<0.05	p<0.001	p<0.001
9–10 months	Normal	6	2.5 (0.4)	2.6 (1.4)	12.5 (1.0)	76.8 (5.2)	25.9 (1.7)
	β Thalassaemia	14	5.1 (0.5)	4.4 (2.1)	11.1 (0.9)	58.7 (1.6)	19.6 (0.9)
			p<0.001	NS	p<0.005	p<0.001	p<0.001
1 year	Normal	5	2.5 (0.3)	1.4 (0.6)	12.3 (1.0)	74.6 (5.0)	24.8 (2.7)
	β Thalassaemia	8	4.8 (0.4)	4.1 (2.1)	11.2 (0.9)	57.5 (2.4)	18.7 (0.6)
			p<0.001	p<0.02	p<0.005	p<0.001	p<0.001

Table adapted from Galanello *et al*.
Values are means (SD); the statistical significance between subjects was tested by one-tailed Student t test.
Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; NS not significant.

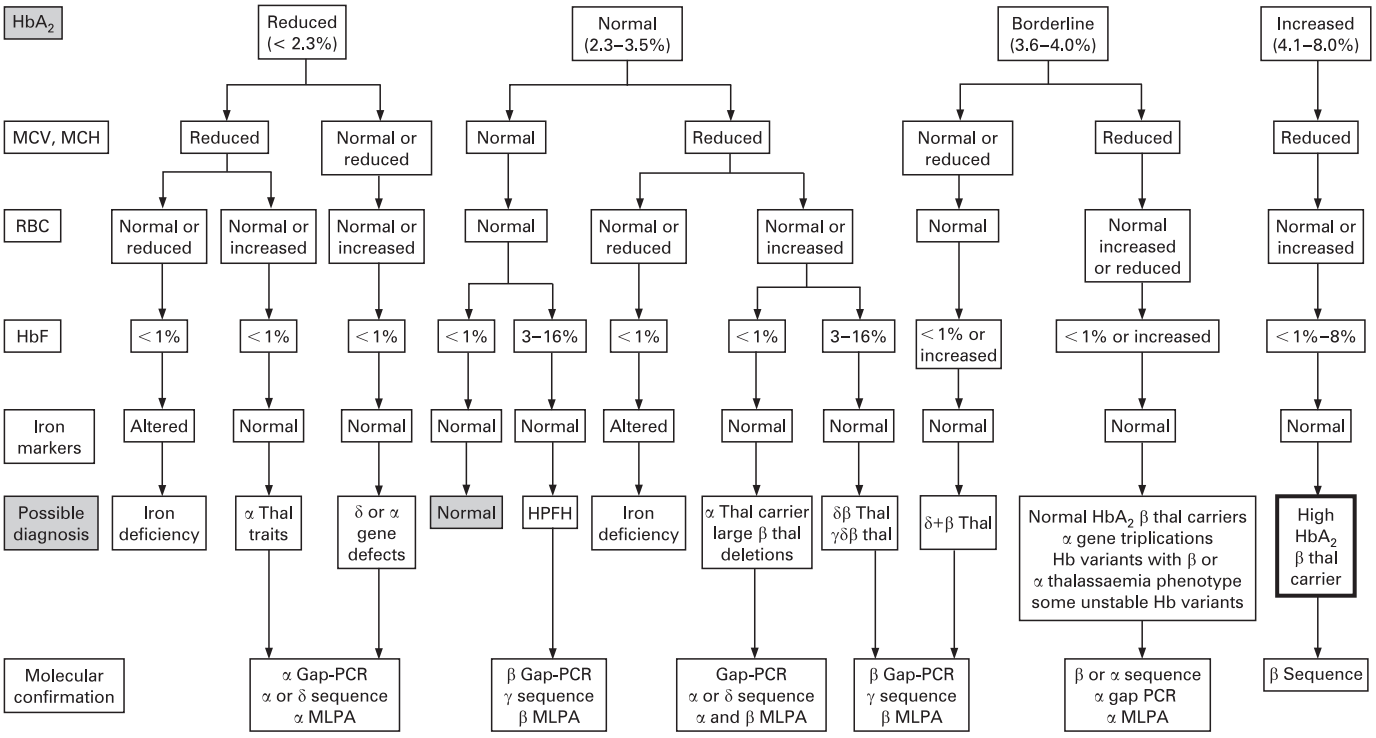

Figure 2 Diagnostic flowchart for the interpretation of haemoglobin (Hb)A_2. The most probable diagnostic conclusions are indicated based on the HbA_2 determination and the observation of the basic haematological and iron parameters. The HbA_2 concentration values may differ depending on the method used, as discussed in the text. The values are those used by the authors, based on high-performance liquid chromatography measurements. HPFH, hereditary persistence of fetal haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; MLPA, multiplex ligation-dependent probe amplification; RBC, red blood cell. Note: elevated RBC counts are characteristic of the thalassaemic phenotype. However, this compensation mechanism is folic acid dependent. A lower Hb value (anaemia) can be substantial or barely present in thalassaemia depending from the type, the compensation by RBC elevation or the presence of HbF. A normal ferritin value does not exclude a coexisting (α) thalassaemia condition, especially in females after more than one pregnancy. Persistent microcytosis after iron therapy should be investigated. Ferritin is an acute phase marker and can be falsely elevated due to a coexisting inflammatory condition.

visible, then a δ chain variant is present. A second HbA_2 fraction present in much lower levels (1/4 of the total HbA_2) in the presence of a second major peak (approximately 20% on the total Hb area) and normal blood counts indicates a stable α chain variant. Low/normal HbA_2 with reduced MCV and MCH might indicate iron deficiency, α thalassaemia or a large deletion involving all of the β globin gene cluster. If HbF is elevated, then a possible δβ or γδβ thalassaemia is present.

Particular care has to be paid to the interpretation of those cases with "borderline" HbA_2 levels (ie, values above the normal reference interval but not in the classical range of β thalassaemia carriers). It has been demonstrated that these values, if associated with microcytosis, are compatible with severe β thalassaemia mutations, such as β39.[26] Cases of normal HbA_2 β thalassaemia may result from the presence of co-inherited δ thalassaemia either in *cis* or in *trans* to a β thalassaemia gene, or from one of the common structural mutants of the δ gene, such as HbB_2 (also know as haemoglobin A_2'), or from mild β^+ thalassaemia mutations, such as −101 C→T, −92 C→T, CAP +45 G→T, CAP +1 A→C, IVS2.nt844 C→G, whether associated with microcytosis or not.[27]

We have recently investigated the incidence of such borderline phenotypes in two centres located in areas with high prevalence of thalassaemic syndromes,[13] showing that the incidence is significant and in some cases as high as 8%. Therefore particular attention has to be paid to the analytical reliability of the determination, especially in the case of risk assessment, when molecular diagnostics should always be implemented.

The flowchart in fig 2 and the data presented in fig 1 are intended as guide for basic diagnostics using a reliable HbA_2 determination, together with other significant parameters. Having identified a potential couple at risk by using these tests, the best predictor of the clinical phenotype is the extended genotype that, in some cases, can be very complex because of multi-ethnicity or possible interactions with secondary or tertiary factors (modifier genes), some of which are predictable by means of family studies, while others are as yet unknown.[28]

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