

The relevance of hemoglobin F measurement in the diagnosis of thalassemias and related hemoglobinopathies

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Abstract

Objectives: The increase in hemoglobin (Hb) F level is variably associated to the presence of beta thalassemia trait, and is more typical in presence of $\delta\beta$ thalassemia and of hereditary persistence of fetal hemoglobin. In normal healthy subjects variable levels of HbF are related to the presence of the polymorphism $C^{\gamma} - 158 (C > T)$. Moreover, HbF can also be variably increased in association with other acquired conditions. The objective of this work is to review the role of the determination of HbF in various conditions.

Design and methods: In the present document we comment on the need for accuracy and standardization, and on the interpretation of the HbF value, reviewing most crucial aspects related to this test.

Results: We present a practical flow-chart summarizing the significance of the HbF estimation in different thalassemia syndromes and related hemoglobinopathies.

Conclusion: The determination of HbF is relevant for the final diagnosis of various physiopathological conditions. In our opinion its importance will increase in the following years, because of the proliferation of novel approaches for the induction of HbF synthesis as a cure for thalassemia syndromes.

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Introduction

Fetal hemoglobin (HbF) is the main hemoglobin component throughout fetal life and at birth, accounting for approximately 80% of total hemoglobin in newborns. HbF is produced from the sixth week of gestation and during the rest of fetal life, replacing the embryonic hemoglobins Gower I, Gower II and Portland. After birth, HbF synthesis rapidly declines and HbF is gradually substituted by HbA in the peripheral blood, so that within the first two years of life, the characteristic hemoglobin phenotype of the adult with very low levels of HbF (less than 1%) is found [1,2]. In normal adults, HbF is heterogeneously distributed among erythrocytes though its synthesis is restricted to a small population of cells, termed F-cells. Approximately 3–7% of red blood cells are F-cells, containing 20–25% of HbF [3].

HbF ($\alpha_2\gamma_2$) is formed by two α - and two γ -globin chains consisting of 141 and 146 amino acid residues, respectively. While α -chains are the same as those contained in adult hemoglobins, i.e. HbA ($\alpha_2\beta_2$) and HbA₂ ($\alpha_2\delta_2$), γ -chains are characteristic for HbF and differ from the similar β -chain by 39 residues. Two type of γ -chains can be present in HbF, G^{γ} and A^{γ} , they are functionally identical but differ for the amino acid presents at position 136 that is either an alanine or a glycine. Moreover, a common variant of A^{γ} chain is $A^{\gamma T}$ that shows an isoleucine to threonine substitution at position 75. G^{γ} and A^{γ} globin chains are encoded by two distinct genes located in the β -globin cluster on chromosome 11. The $G^{\gamma}:A^{\gamma}$ ratio is around 70:30 at birth and usually 40:60 in the trace amount of HbF found in the adult. Changes in this ratio were observed in some hemoglobin disorders [4,5]. Unusual proportions of the two types of gamma chains can be observed in individuals having duplications or rearrangements of these genes [6].

In cord blood, as well in adult life, about 10–20% of HbF is acetylated as a result of a post-translational event that is enzyme

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mediated and occurs on the N-terminal glycine residue of the γ -chains [7,8].

Functionally, HbF differs mostly from HbA because it has a slightly higher oxygen affinity, explained by the low interaction of HbF with 2,3-DPG. This characteristic makes the delivery of oxygen through placenta easier, giving fetus better access to oxygen from the mother's bloodstream [9].

HbF measurement is clinically useful in the study and diagnosis of some important globin gene disorders where HbF levels may vary considerably (mainly, β - and $\delta\beta$ -thalassemia, HPHF) and in a number of acquired conditions associate with mild increases of HbF. Moreover, HbF is known to inhibit the polymerization of HbS and different agents able to increase HbF production have been introduced for therapeutic aim [10]. So, a reliable monitoring of HbF concentration during the treatment and follow up of patients with sickle cell disease is highly required.

Therefore, a correct measurement of HbF is needed. We shall summarize the latest information available in this regard, and will make recommendations to help in the avoidance of pitfalls and in understanding the significance of this laboratory test.

Methodological aspects

The classical method for the determination of fetal hemoglobin is that based on the alkali denaturation [11]. This method relies on the resistance to denaturation by alkali of HbF compared to HbA, the denaturation being activated by the ionisation of buried, weakly acidic side chains (one tyrosine and two cysteines) present in HbA and not in HbF [12]. This is only a relative difference, and the conditions have been there optimized over time in order that during the time of exposure to alkali most of the HbA is denaturated while the HbF is largely unaffected. Before the exposure to alkali, all the hemoglobin forms are transformed in the more stable cyanmethemoglobin form by means of treatment with Drabkin's reagent. An optimized version of the preliminary method has been proposed by Pembrey [13]. It is important to remember that the alkali denaturation is a kinetic test to be performed only under well standardized conditions in order to obtain reproducible results.

Today the most common approach to the quantification of HbF is based on the separation of this hemoglobin from other hemoglobin fractions by cation exchange HPLC on dedicated commercial apparatus [14,15], often with direct loading from the primary tube. Capillary electrophoresis [16] is becoming a valid alternative, while proposed immunochemical methods are still not sufficiently validated. As a matter of fact, it should be recalled that while the by the alkali denaturation resistance all HbF is measured, in HPLC the acetylated fraction is eluted before the main fraction of HbF, thus leading to a value significantly lower. This fact, although not clinically relevant, may be important when performing method comparison analyses.

The analysis of fetal Hb has been recently investigated by electrospray-ionization mass spectrometry (ESI-MS) [17], a technique already widely diffused to the analysis of hemoglobin variants also in screening programmes [18]. The method has a good reproducibility (CV <5%) and can be used to accurately measure the fetal γ -chain masses in neonatal cord blood.

Unfortunately, since no international standardization program has been foreseen for HbF, the data are method-dependent and few EQAS programs (UK NEQAS, ASL Careggi Firenze) include such analyte among the ones offered to the participants.

The determination of red cell containing HbF is used to detect red cells containing fetal hemoglobin in mixtures of cells containing adult hemoglobin. This is useful to define the distribution of fetal hemoglobin in red cells in presence of HPHF, in which the distribution of HbF is almost pancellular, or for the detection of fetal erythrocytes in maternal blood following transplacental hemorrhages. The acid elution test originally proposed by Keihauer is still used [19], although more sensitive and specific method based on monoclonal antibodies and flow cytometry have been recently proposed [20].

Finally, estimation of HbF in neonatal blood has been also proposed by hemoxymetry, through the determination of P_{50} on the HbO_2 dissociation curve, based on linear assumption. This method has never been well validated, and showed significant overestimation respect to HPLC [21].

Pre-analytical variables

Case-related variables

Several pre-analytical factors may influence the HbF levels in blood, and the most relevant of them are reported in Table 1, in which increased levels of fetal hemoglobin can be ascribed essentially to gamma genes defects, to defects in other genes, or to acquired conditions. As a general rule it should be present that HbF levels have always to be interpreted in the general

Table 1
Main pre-analytical subject-related factors that may increase HbF levels (adapted and modified from ref. [1]).

Hereditary disorders	Acquired conditions
Homozygous β -thalassemia	Pernicious anemia
Heterozygous β -thalassemia	Paroxysmal nocturnal hemoglobinuria (PNH)
HPFH, homozygous	Refractory normoblastic anemia
HPFH, heterozygous	Sideroblastic anemia
$\delta\beta$ thalassemia, homozygous	Pure red cell aplasia
$\delta\beta$ thalassemia, heterozygous	Aplastic anemia
Sickle cell anemia	Pregnancy
Some other hemoglobinopathies (HbC, HbE, Hb Lepore, some unstable Hbs) (variable)	Recovery after bone marrow transplant, marrow hypoplasia, leukemia chemotherapy and transient erythroblastopenia; treatment with hydroxyurea, 5-aza-2'-deoxycytidine, butyrates, erythropoietin
Trisomy D	Hyperthyroidism
Hereditary spherocytosis	Juvenile chronic myeloid leukemia
Some Hb variants (Hb Rainier, Hb Bethesda) alkali resistant ^a	Acute leukemias
Hb variants with isoelectric point identical to that of HbF ^b	Erythroleukemia
	Benign monoclonal gammopathies
	Cancer with marrow metastases, hepatoma
Hb variants with retention time similar to that of HbF	Chronic renal disease

^a Pre-analytical effect on the alkali denaturation method.

^b Pre-analytical effect on HPLC methods.

context of the most important hematological conditions (anemia, microcytosis, hypochromia).

Nowadays more than 70 mutations in gamma genes or surrounding regions, can lead to HPFH. Some mutations (such as the $-117\text{ G}\rightarrow\text{A}$ in the $^{\text{A}}\gamma$ gene; $-175\text{ T}\rightarrow\text{C}$ in $^{\text{A}}\gamma$ or in $^{\text{G}}\gamma$; $-196\text{ C}\rightarrow\text{T}$ in $^{\text{A}}\gamma$ and $-202\text{ C}\rightarrow\text{T}$ in $^{\text{G}}\gamma$) are related to relatively high levels of HbF (up to 35%), equally distributed in all red cells. Other mutations (such as the $-202\text{ C}\rightarrow\text{T}$ in $^{\text{A}}\gamma$) are associated with lower HbF levels (up to 15%) distributed heterocellularly, some other (such as the $-37\text{ A}\rightarrow\text{T}$ in $^{\text{G}}\gamma$) are associated with a rare δ^0 -thalassemia with slightly elevated HbF levels around 2–3% [22], and finally other (such as $-158\text{ C}\rightarrow\text{T}$ in $^{\text{G}}\gamma$) with nearly normal HbF levels [23].

In the presence of some hemoglobin variants (HbS, HbC, Hb Lepore and some unstable hemoglobins) and in association with the β -thal trait, a slight increase (1 to 5%) of HbF is found in the heterozygotes, while higher levels can be found in the homozygous. In presence of HbS (1–30%) associated with β -thalassemia the increase in HbF depends on the βS haplotype (Benin, Bantu, Cameroon, Senegal, Arabic–Indian) and is related to the clinical severity of the illness. In the Arabic–Indian haplotype the increase of HbF keeps HbS more soluble in the deoxygenated state, and the illness is less severe [24]. A slight increase in HbF (2–4%) has been reported in hereditary spherocytosis [25]. HbF levels are variable (10–80%) in presence of HbE and β -thalassemia, the important determinants being the age, the presence of α -thalassemia and of genetic determinants of γ -chain synthesis [26]. Moreover, the occurrence of the $-158\text{ C}\rightarrow\text{T}$ polymorphism shown in Fig. 1 clearly increases HbF in normal, not pregnant subjects.

During pregnancy HbF increases up to a maximum level at the beginning of the second trimester, probably because of a response to a placental or fetal inducer of F-cell production entering the maternal circulation, and slowly return to normal values at the end of gestation.

In presence of chronic juvenile myeloid leukemia (a rare syndrome occurring in children less than 5 years old) variable HbF levels (20–80%) have been reported [27], increasing in time during the progression of the disease. Since the HbF is heterocellular and the $^{\text{G}}\gamma/^{\text{A}}\gamma$ ratio looks like during fetal life, probably a cell clone has escaped the “switch” control

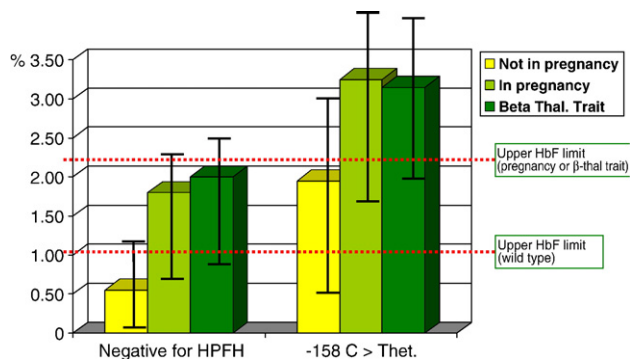


Fig. 1. Hb F typical concentrations in subjects not carriers of HPFH (left) and in heterozygous of the polymorphism $^{\text{G}}\gamma -158\text{ (C>T)}$ (right). Within each group the typical HbF values (median value, 95% confidence interval) are reported for not pregnant women, women in pregnancy, and β -thal carriers.

mechanism leading to a normal differentiation process. Also in other leukemic forms, particularly in the acute leukemia in the remission phase after drug treatment, remarkable increased HbF levels (up to 15%) have been reported [28].

HbF is also increased in congenital aplastic anemia and in erythroid hypoplasias, with heterocellular distribution and $^{\text{G}}\gamma/^{\text{A}}\gamma$ ratio fetal-like. In Fanconi anemia HbF seems to be always increased, even after remission after steroidal therapy [29]. In paroxysmal nocturnal hemoglobinuria (PNH) HbF is also increased, not in the pathological clone, but in the normal ones [30].

Method-dependent variables

The presence of hemoglobin variants may interfere with the quantification of HbF on dedicated HPLC systems. A few variants having retention times similar to HbF have been reported (Hb Camperdown, Hb Dagestan), as reviewed by Prehu et al. [31]. Also high levels of glycosylated hemoglobin (HbA_{1c}) may interfere with the accurate determination of HbF in some HPLC systems.

A number of technical factors must also be taken into account 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 carryover, 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 programs run on a regular basis, are essential.

Sample collection and storage

With the exception of heparin for *in vitro* globin chain synthesis analysis, EDTA is the anticoagulant of choice for hemoglobinopathy analysis. Best HbF results are obtained on fresh material not older than one week. However, HPLC analysis remains fairly stable on samples stored at 4 °C even after 3 weeks [32], while methemoglobin derivatives and other storage artefacts will progressively increase. Lysates can be frozen at -20 °C or at -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 one month at -20 °C and 3 months at -80 °C . Thawing the samples and re-freezing 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 for glycosylated hemoglobin (HbA_{1c}) such specifications are defined [33], none are reported for HbF.

Measurement units

The relative percentage (%) on total hemoglobin is the measurement unit for the expression of HbF. This unit, although not in line with the international SI unit system, is well

Table 2
HbA₂ and HbF values (ranges min–max) in normal infants during the first 2 years of life (adapted from ref. [34]).

Age (mo)	n	Hb A%	HbA ₂ %	Hb F%
At birth	1250	15–40	0–1	58–84
1–3	29	38–70	0.5–1.5	29–61
3–5	85	65–90	1.3–2.1	9–40
5–8	50	83–95	1.6–2.6	3–15
8–12	89	89–96	1.8–2.9	1–10
12–24	222	94–97	1.9–3.0	0.5–3
>24	3550	95–98	2.0–3.3	0.1–1.2

established and uniformly used worldwide, as are other non-approved units of other quantities (e.g. partial pressure expressed in mm Hg and not in kPa).

Reference intervals

As for any laboratory test, all laboratory professionals are theoretically responsible for calculating their own reference intervals for HbF, by measuring this hemoglobin fraction in at least 100 adult individuals who are not iron-depleted, and are not carriers of α or β thalassemia. In case of HbF, because of the low values normally found in the absence of any thalassemia syndrome, the upper limit of normal subjects is usually reported, not really the standard reference interval. The values found in the laboratory of one of the authors (GI) are always $\leq 1\%$, in absence of the polymorphism Xmn – 158 C \rightarrow T, and $\leq 2.0\%$, in presence of such a polymorphism (Fig. 1).

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 thalassemia carriers or iron deficiency.

With regard to follow up during the first year of life, HbF reaches a stable level after the first year of life, and HbF reaches adult levels after the second year [34], as reported in Table 2.

Interpretation of the HbF data and the role of the laboratory

An increased HbF concentration above 1% in a healthy adult subject could be the result of one or more genetic defects, and/or the consequence of some acquired conditions. What is then usually referred as HPFH is just one out of the possible reasons for an increase of HbF.

Therefore, the laboratory can be useful into assessing a final diagnosis in presence of an increased value of HbF by analyzing the globin genes and by choosing the most appropriate reference intervals. HbF being useful for the diagnosis of HPFH and $\delta\beta$ -thalassemia syndromes together with a minimum set of other tests, a flowchart (Fig. 2) has been developed summarizing, besides HbF levels, the other most relevant measurements, these being the hematological data (mostly MCV, MCH), HbF and iron status markers.

Very recently a variant of the BCL11A gene has been found associated with high levels of HbF in β -thalassemia carriers and in subjects with HbS [35], and this may possibly explain also some of the findings listed in Table 1 too.

The flowchart and the data presented in Fig. 2 are intended as guide for basic diagnostics using a reliable HbF determination, together with other significant parameters. A similar and more complex flowchart has been recently presented in order to aid the laboratory professional in interpreting the HbA₂ values [36]. We hope that careful interpretation of these minor hemoglobins, together with an accurate standardization of their analytical

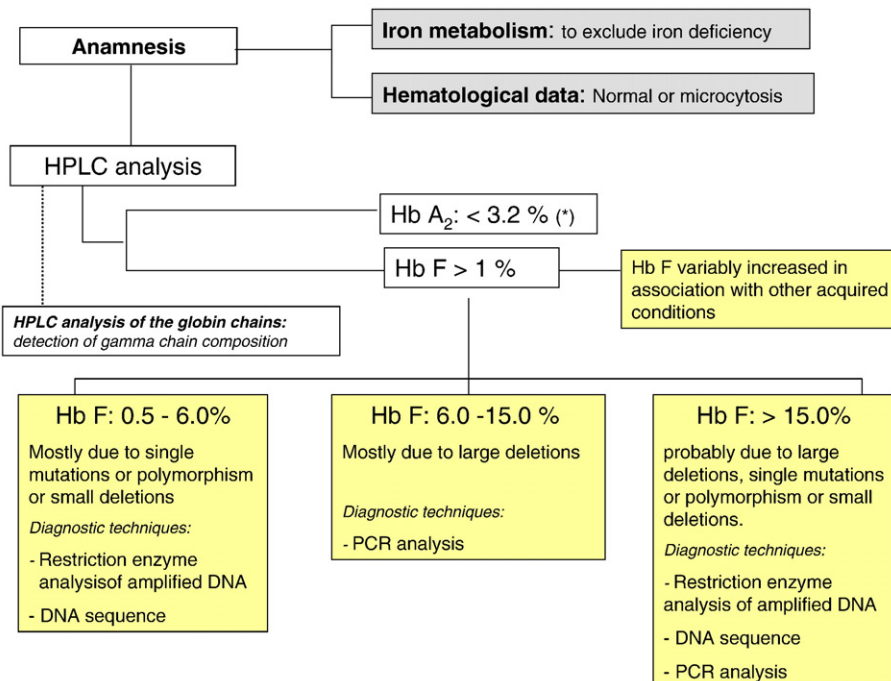


Fig. 2. Flowchart for the possible genetic causes of the increase in Hb F. The analytical approaches for the quantification and the possible molecular characterization steps are illustrated. (*): HbA₂ values typically found in the Author's laboratories.

procedures, will help the detection and care of thalassemia syndromes and related hemoglobinopathies.

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