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# Laboratory Investigation of Hemoglobinopathies and Thalassemias: Review and Update

Gwendolyn M. Clarke and Trefor N. Higgins*

**Structural hemoglobin (Hb) variants typically are based on a point mutation in a globin gene that produce a single amino acid substitution in a globin chain. Although most are of limited clinical significance, a few important subtypes have been identified with some frequency. Homozygous Hb C and Hb S (sickle cell disease) produce significant clinical manifestations, whereas Hb E and Hb D homozygotes may be mildly symptomatic. Although heterozygotes for these variants are typically asymptomatic, diagnosis may be important for genetic counseling. Thalassemia, in contrast, results from quantitative reductions in globin chain synthesis. Those with diminished β-globin chains are termed β-thalassemias, whereas those with decreased α-chain production are called α-thalassemias. Severity of clinical manifestations in these disorders relates to the amount of globin chain produced and the stability of residual chains present in excess. The thalassemia minor syndromes are characterized clinically by mild anemia with persistent microcytosis. Thalassemia intermedia (i.e., Hb H disease) is typified by a moderate, variably compensated hemolytic anemia that may present with clinical symptoms during a period of physiologic stress such as infection, pregnancy, or surgery. The thalassemia major syndromes produce severe, life-threatening anemia. α-Thalassemia major usually is incompatible with extrauterine life; β-thalassemia major presents in infancy and requires life-long transfusion therapy and/or bone marrow transplantation for successful control of the disease. Double heterozygosity for certain structural variants and/or thalassemia syndromes may also lead to severe clinical disease. Several guidelines have been published that outline the required steps for hemoglobinopathy and thalassemia investigation. The availability of HPLC has streamlined many of these requirements, allowing an efficient stepwise diagnostic strategy for these complex disorders.**


Hemoglobin (Hb;[1] $M_r$ 68 000) is the oxygen-carrying moiety of erythrocytes. It is a polypeptide tetramer, globular in structure, and consisting of two pairs of unlike globin chains (i.e., α plus β, δ, or γ), which form a shell around a central cavity containing four oxygen-binding heme groups each covalently linked to a globin chain. In healthy adults, ~95% of the Hb is Hb A ($\alpha_2\beta_2$) with small amounts (<3.5%) of Hb $A_2$ ($\alpha_2\delta_2$) and Hb F ($\alpha_2\gamma_2$) present. During embryonic development, "pre alpha" ζ globin chains contribute to embryonic Hb. During fetal development, β-like globin chains ε and γ contribute to the Hb *(1)*.

The α-globin chain is encoded in duplicate on chromosome 16, and the non-α chains (β, δ, γ) are encoded in a cluster on chromosome 11. A diploid cell therefore has four α-globin genes and two β-like genes. The α and β chains consist of 141 and 146 amino acid residues, respectively. There is some sequence homology between the two chains (64 individual amino acid residues in identical positions), and the β chain differs from the δ and γ chains by 39 and 10 residues, respectively.

Although hemoglobinopathies and thalassemias are two genetically distinct disease groups, the clinical manifestations of both include anemia of variable severity and variable pathophysiology. The thalassemias are characterized by a reduction in the amount of the normal globin chain produced. This diminution in globin chain production may result from gene deletion or from mutations that adversely affect the transcription or stability of mRNA products. The manifestations range from mild anemia with microcytosis (thalassemia trait) to fatal severe anemia (Hb Barts hydrops fetalis or β-thalassemia major). The hemoglobinopathies, or structural Hb variants, are attributable to amino acid substitution in either the α or

Dynacare Kasper Medical Laboratories, 14940 123rd Ave., Edmonton, Alberta T5V 1B4, Canada.
*Author for correspondence. Fax 780-452-8488; e-mail higgins@dkml.com.
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[1] Nonstandard abbreviations: Hb, hemoglobin; CBC, complete blood count; IEF, isoelectric focusing; RBC, red blood cell; MCV, mean corpuscular volume; RDW, red cell distribution width; and CIEF, capillary IEF.

non-α chain. More than 700 hemoglobinopathies have been described to date *(2)*, the majority of which are clinically benign and fortuitously discovered. The clinically significant hemoglobinopathies are attributable to amino acid substitutions, primarily in the non-α chain, that bring about changes in the secondary and tertiary structure of the Hb tetramer. These substitutions are most common at positions in close proximity to either heme group or globin chain attachment sites.

Hemoglobinopathy nomenclature is an assortment of letters (i.e., Hb S, C, and D), place names denoting the site of first discovery or residence of the propositus (e.g., Hb Edmonton), and family names of the index case (e.g., Hb Lepore). A systematic nomenclature that is both logical and informative identifies the chain, the location, and the amino acid substitution on the involved globin chain. Thus Hb Alberta (β101 Ala→Pro) is a substitution of proline for alanine (the normal amino acid) in the 101st position of the β chain. In the heterozygous state, the normal Hb is placed first, followed by the variant, e.g., AS trait.

## Methods of Analysis

The 1975 International Committee for Standardization in Hematology expert panel on abnormal Hbs and thalassemias made diagnostic recommendations regarding the laboratory investigation of these conditions. Initial tests recommended include a complete blood count (CBC), electrophoresis at pH 9.2, tests for solubility and sickling, and quantification of Hb $A_2$ and Hb F. If an abnormal Hb is identified on the preliminary tests, then further techniques were recommended to identify the variants. These techniques include electrophoresis at pH 6.0–6.2, globin chain separation, and isoelectric focusing (IEF). Additional testing, including heat and isopropanol stability tests, was recommended for detection of unstable Hbs or Hbs with altered oxygen affinity.

Although electrophoresis at alkaline and acid pH has been used for many years, cation-exchange HPLC *(3–10)* is emerging as the method of choice for quantification of Hb $A_2$ and Hb F and identification of Hb variants *(11, 12)*. The use of HPLC streamlines the recommended preliminary and follow-up tests for the identification of hemoglobinopathies and thalassemias and provides for rapid and complete diagnostic work up in a majority of cases. The elements of one approach include a CBC, Hb H test, ferritin, HPLC for Hb $A_2$ and F quantification, and detection of any Hb variants followed by electrophoresis at both alkaline and acid pH. The relevance of each of these analytic components is discussed below.

### CBC

Structural hemoglobinopathies may have an impact on the red cell indices, and red cell indices are critical to the diagnosis of thalassemias. The key components of the CBC include: Hb, red blood cell (RBC) number, mean corpuscular volume (MCV), and red cell distribution width (RDW).

The thalassemias generally are classified as hypochromic and microcytic anemias. Hence the MCV is a key diagnostic indicator. Virtually all automated hematology analyzers now provide a measurement of MCV that is both precise and accurate. This cell volume, reported in femtoliters, in most adult populations ranges from ~80 to 100 fL. Thalassemic individuals have a reduced MCV, and one study has suggested that an MCV of 72 fL is maximally sensitive and specific for presumptive diagnosis of thalassemia syndromes *(13)*.

The RDW is a measure of the degree of variation in red cell size. Some causes of microcytic anemia, most notably iron deficiency, are characterized by an increase in RDW. The thalassemias, in contrast, tend to produce a uniform microcytic red cell population without a concomitant increase in RDW. This observation is variable among the thalassemia syndromes, however, with notable increases in RDW in the setting of Hb H disease and δ β-thalassemia minor *(1)*. Therefore, the RDW may provide information useful as an adjunct to diagnosis but is not useful as a lone indicator.

The RBC count is also useful as a diagnostic adjunct because the thalassemias produce a microcytic anemia with an associated increase in the RBC number. Other causes of microcytic anemia, including iron deficiency and anemia of chronic disease, are more typically associated with a decrease in the RBC number that is proportional to the degree of decrease in Hb concentration.

The Hb concentration typically is decreased in thalassemia. The thalassemia minor conditions produce minimal decrements in the Hb concentration, whereas thalassemia intermedia and thalassemia major may be associated with moderate to severe decreases in Hb concentration.

Various indices utilizing these CBC components have been developed with a view to providing a mathematical derivation to reliably differentiate iron deficiency from thalassemia minor. None are useful in all clinical settings, and probably none exceed the value of the MCV alone in selecting cases for subsequent investigations *(13)*.

### Hb H inclusions

Hb H refers to an insoluble Hb tetramer comprising four β-globin chains. Hb H arises in the setting of α-thalassemia where the decreased production of α-globin chains leads to β-globin excess. Oxidation of these tetramers provokes precipitation, which can be visualized microscopically. In vitro generation of Hb H inclusions is accomplished by staining unfixed cells with an oxidative dye such as New Methylene Blue or Brilliant Cresyl Blue. Because batch-to-batch variability in the dye occurs, positive and negative control slides are critical *(1, 14, 15)*.

Various authorities differ with respect to the necessity for freshly obtained blood samples *(14, 15)*. Our own unpublished data suggest that freshly collected blood is

not critical. Blood film examination is undertaken with a search for cells with typical "golf ball" inclusions.

In the setting of Hb H disease, a disorder in which three of four α-globin chain genes are nonexpressed, 30–100% of red cells contain typical inclusions. In contrast, α-thalassemia minor may be associated with as few as 1 inclusion-containing cell in 1000–10 000 cells *(14)*. The absence of Hb H inclusions therefore does not exclude thalassemia trait, but the presence of typical inclusions may be helpful in confirming a presumptive diagnosis.

The Hb H stain is nonspecific in that other nucleic acid or protein precipitates also stain. Reticulin and Howell Jolly bodies do not have the distinctive pattern of Hb H inclusions; however, a brisk reticulocytosis can make identification of a rare H-containing cell difficult.

### ELECTROPHORESIS

Traditionally, electrophoresis has been the method of choice for identification and quantification of variant Hbs. Commercial, rapid electrophoretic methods have been developed that allow for separation at pH 8.4 (alkaline) and pH 6.2 (acid) on agarose gels. These provide a clear background, allowing for quantification of the Hb present by densitometric scanning. Visualization of the Hb bands is made possible by staining with Amino Black and Acid Violet (or similar stains). At alkaline pH, electrophoretic migration of Hb C, Hb E, Hb $A_2$, and Hb O is similar. Hb S, Hb D, and Hb G also comigrate. At acid pH, electrophoretic separation of Hb C from Hb E, and Hb O and Hb S from Hb D and Hb G is accomplished. It is not possible to differentiate between Hb E and Hb O, and Hb D and Hb G using electrophoretic methods.

Electrophoresis, is slow, labor-intensive, and inaccurate in the quantification of low-concentration Hb variants (e.g., Hb $A_2$) or in the detection of fast Hb variants (Hb H, Hb Barts).

The precision and accuracy of Hb $A_2$ measurements using densitometric scanning of electrophoretic gels is poor, especially when compared with HPLC techniques *(8)*. A recent College of American Pathologists *(16)* hemoglobinopathy survey showed a CV of 33.6% for densitometric scanning of electrophoretic gels at a Hb $A_2$ concentration of 2.41%. For column chromatography, the CV was 14.6% at a mean Hb $A_2$ concentration of 3.21%, and for HPLC, the CV was 4.3% at a mean Hb $A_2$ of 3.47% *(16)*.

Despite the imprecision, 296 of 387 laboratories participating in the College of American Pathologists Hemoglobinopathy Survey program reported results for Hb F, Hb $A_2$, and Hb identification using electrophoretic methods. Some laboratories, including ours, use a combination of electrophoresis and HPLC to identify and quantify Hb.

### IEF

IEF is an electrophoretic technique with excellent resolution. Although labor-intensive and time-consuming, it has been used to identify and quantify Hbs *(9, 17)*. IEF is an equilibrium process in which Hb migrates in a pH gradient to a position of 0 net charge. The Hb migration order of IEF is the same as that of alkaline electrophoresis with resolution of Hb C from Hb E and Hb O and Hb S from Hb D and Hb G. In addition, Hb A and Hb F are clearly resolved. The narrow bands obtained on IEF allow for more precise and accurate quantification than standard electrophoresis.

### CAPILLARY IEF

Capillary IEF (CIEF) *(5, 18, 19)* is a hybrid technique combining the capillary electrophoresis sensitivity of detection with the automated sampling and data acquisition of HPLC. Many published works have described the utility of CIEF in the detection and quantification of Hb variants. Separation of the Hb in this method is related to the isoelectric point of the Hb, and this may enhance interlaboratory reproducibility.

CIEF has been used to quantify Hb variants Hb $A_2$ and Hb F *(18)*. One comparison *(5)* of CIEF with cation-exchange chromatography for the qualitative and quantitative analysis of Hb variants found that quantitative data between the two methods were highly correlated and that CIEF gave slightly better resolution of the unusual variants Hb C Harlem and Hb D Punjab.

### HPLC

Cation-exchange HPLC is emerging as the method of choice for the initial screening of Hb variants *(4)* (including neonatal screening where this is mandated) and for quantification of Hb $A_2$ and Hb F concentrations. The Bio-Rad Variant (Bio-Rad Laboratories) is an automated cation-exchange HPLC instrument that has been used to quantify Hb $A_2$, Hb F, Hb A, Hb S, and Hb C. College of American Pathologists studies have shown equivalence or superiority over electrophoretic methods.

HPLC is not without intrinsic interpretive problems. Hbs may co-elute or may elute before instrument peak integration. Using the Bio-Rad Variant β Thal short program, we have found that Hb E, Hb Osu Christianbourg, and Hb G Copenhagen co-elute with Hb $A_2$, making Hb $A_2$ quantification impossible on samples with these Hb variants, a problem noted by others *(6, 18)*.

The measurement of Hb $A_2$ using cation-exchange HPLC is complicated in individuals with Hb S *(20, 21)* because the Hb $A_2$ is falsely increased by the presence of Hb S adducts. A capillary zone electrophoretic method has been discussed for quantification of Hb $A_2$ in the presence of Hb S that eliminates interference from these adducts *(18)*. A micro anion-exchange column methodology that eliminates this interference has also been described *(21)*.

In individuals with Hb D trait *(22, 23)*, the Hb $A_2$ value obtained with the Bio-Rad Variant is falsely decreased because of an integration error. Correction for the rising baseline produces Hb $A_2$ values similar to those obtained by anion chromatography.

The quantification of Hb F is important in the diagnosis of hereditary persistence of fetal Hb, juvenile chronic myelogenous leukemia, and monosomy 7 syndrome as well as for therapeutic monitoring in patients with sickle cell anemia. Published Hb F quantities from standard texts often are the result of alkali denaturation/spectrophotometry methods and are higher than those obtained by HPLC techniques. Immunodiffusion techniques are labor-intensive and relatively insensitive. Densitometric scans of an alkaline electrophoretic gel cannot detect Hb F in healthy adults or in those with marginally increased Hb F.

Reversed-phase HPLC has been used as an enhancement for electrospray ionization spectroscopy in the characterization of Hb Rambam ($\beta$69 Gly→Asp) *(24)*. This represents an additional novel use of HPLC in Hb variant diagnosis. Liquid chromatography/mass spectroscopy has been used to characterize other variant Hbs *(25)*.

HPLC has been used to diagnose thalassemia and hemoglobinopathies, including detection of $\alpha$-thalassemic genotypes in cord blood *(6)*.

The presence of a structural Hb variant may adversely affect the measurement of Hb $A_{1C}$ *(24, 26–29)*. Hb Niigata *(28)* gives inappropriately high Hb $A_{1C}$ concentrations using HPLC and low results using a latex immunoagglutination method. Hb Sherwood Forest *(29)*, Rambam *(24)*, and Raleigh *(27)* produce falsely increased Hb $A_{1C}$ results when measured by cation HPLC.

### DNA analysis

After presumptive identification of hemoglobinopathies and thalassemia syndromes, and particularly for purposes of genetic counseling, defining the mutation or deletion present may be required. Several molecular techniques are available.

DNA from white blood cells, amniocytes, or chorionic tissue may be utilized for diagnosis of various $\alpha$- and $\beta$-globin chain abnormalities. Typically, deletional mutations causing $\alpha$-thalassemia syndromes and some rare $\beta$-thalassemias are diagnosed using Southern blot hybridization of particular restriction enzyme digests to labeled complementary gene probes.

PCR techniques using allele-specific probes after globin gene amplification, allele-specific primers, or deletion-dependent amplification with flanking primers are used in definition of known globin chain mutations/deletions, including those for Hb S, E, D, and O, and several $\beta$-thalassemias *(14, 30–38)*.

For unknown mutations, several PCR-based methods, including denaturing gradient gel electrophoresis and single-strand conformation polymorphism analysis, as well as sequencing of the amplified globin gene DNA may be used *(37)*.

## Clinical Manifestations

Of the many genetic Hb variants and thalassemia syndromes identified, only nine have substantial clinical significance. These syndromes can be further classified according to their clinical effects.

### Sickling disorders

The prototype Hb variant associated with a sickling disorder is Hb S. The point mutation in the $\beta$-globin gene that produces Hb S exerts its effect by causing precipitation and polymerization of the deoxygenated Hb S with resulting sickling of the red cells. These sickled cells lack deformability, occlude the microvasculature, and lead to tissue infarctions, which manifest as painful sickle cell crises. The permanently deformed cells are subsequently removed from the circulation well before the usual 120-day life span of a healthy red cell, contributing to a chronic hemolytic anemia. The clinical manifestations occur only in individuals with homozygous sickle cell disease. Heterozygotes are said to have sickle cell trait and are symptom free. Sickle cell trait is common (8% of the African-American population) and important from a genetic counseling perspective. Double heterozygosity for certain variants (e.g., Hb S/Hb D Los Angeles, Hb Montgomery/Hb S) that occur with appreciable frequency in the same ethnic populations as Hb S may also produce significant sickling disease *(25, 36, 39, 40)*.

Diagnosis of sickle cell trait and disease depends on a typical HPLC (Fig. 1) and Hb electrophoretic pattern. In the case of sickle cell disease, sickled cells may be apparent on peripheral blood film examination, and sickling screens (in which a hemolysate is exposed to a reducing agent) are positive. Sickle cell trait also produces a positive sickle cell screen. The Hb F concentration is variable in sickle cell anemia, and increased proportions of Hb F may mitigate the clinical effects. The focus of several treatment regimens is an increase in Hb F. Ongoing quantitative assessment of Hb F and Hb S concentrations is therefore frequently required. For transfusion therapy, the quantification of Hb A is also monitored.

Hb C, resulting from a separate single base pair mutation in the $\beta$-globin gene, causes a hemolytic anemia of mild to moderate severity in the homozygous form. Heterozygotes are not symptomatically affected. Peripheral blood film analysis may reveal frequent target cells (Target cells are discoid RBCs that have a central hemoglobinized area in the clear center, producing a target appearance. They result from an increase in the ratio of cell surface area to cell volume.) and an increase in polychromasia. HPLC and electrophoretic patterns are diagnostic. Double heterozygosity for $\beta$ C and $\beta$ S Hb leads to a severe sickling disorder.

Similarly, Hb O Arab and Hb D Punjab result from point mutations of the $\beta$-globin gene. Although both heterozygous and rare homozygous individuals may be diagnosed on the basis of HPLC and electrophoretic analysis, clinical manifestations are minimal. The major clinical impact of these hemoglobinopathies results from co-inheritance with Hb S, which occurs in a similar

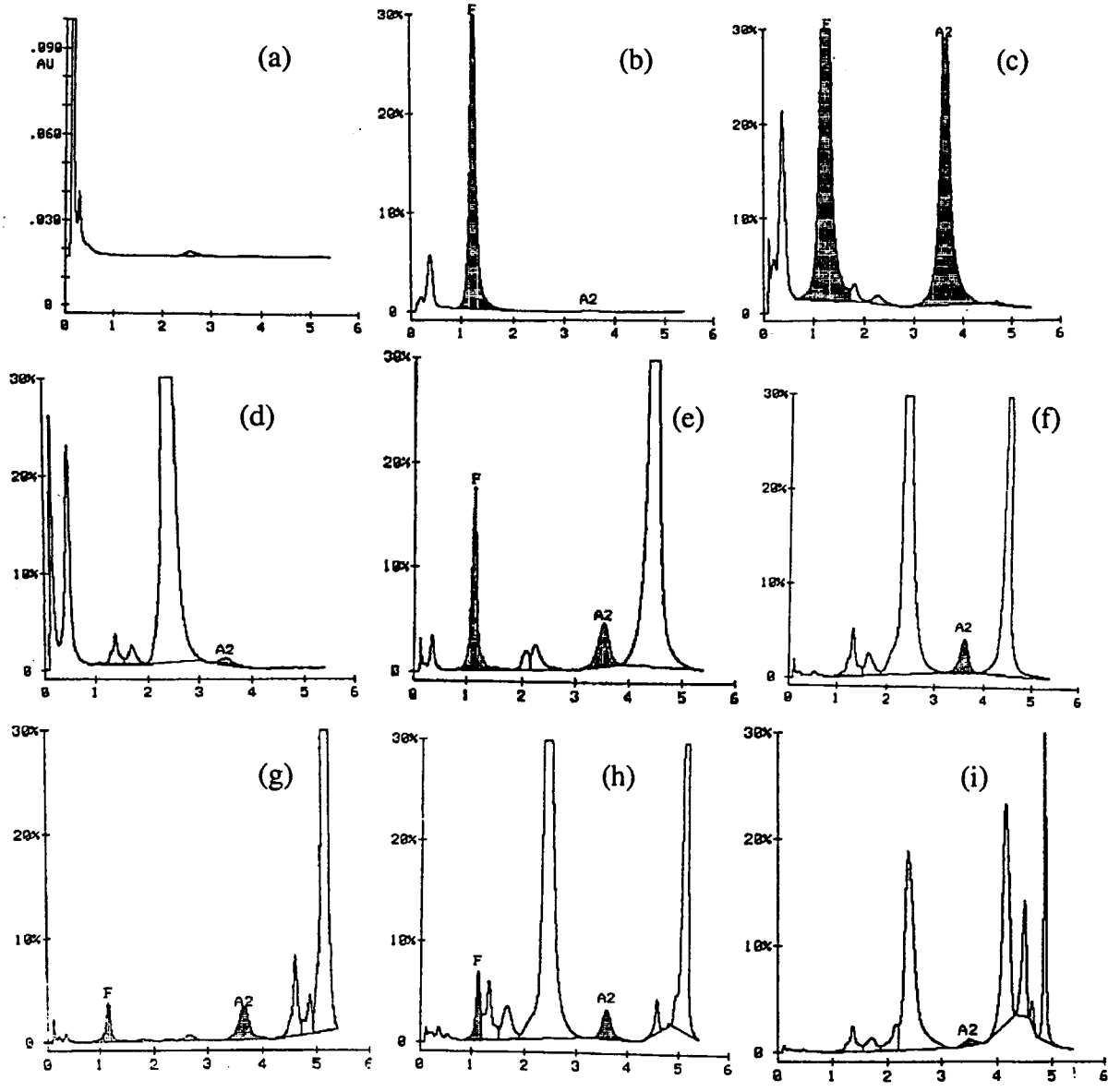


Fig. 1. HPLC chromatograms obtained on the Bio-Rad Variant β Thal short program for Hb Barts (*a*), $\beta^o$ thalassemia major (*b*), $\beta^+$ thalassemia homozygous E (*c*), Hb H (*d*), homozygous S (*e*), S trait (*f*), homozygous C (*g*), C trait (*h*), and Hb S-Hb G Philadelphia (*i*).

population and which if inherited with either Hb O Arab or Hb D Punjab may produce a severe sickling disorder.

### THALASSEMIC DISORDERS

Hemoglobinopathies that lead to decreased production of globin chains (α or β) produce a clinical syndrome characterized by anemia of variable severity with hypochromic and microcytic red cells *(41, 42)*.

The α- and β-thalassemias result, respectively, from deletions or mutations of the α- and β-globin genes that lead to decreased transcription or translation of the gene product. In addition to decreased Hb production, however, manifestations of the thalassemias are complicated by the resulting chain imbalance: In the α-thalassemias, β- and γ-globin chains are produced in excess. These proteins associate to form unstable tetramers (β4 = Hb H; γ4 = Hb Barts), which precipitate in red cell precursors and lead to ineffective erythropoiesis with bone marrow expansion and splenomegaly. In the β-thalassemias, similarly, α-chain excess leads to ineffective erythropoiesis and interference with erythroid maturation. In general, the more genes affected, the more significant the complications of the disorder. In the case of α-thalassemia, a four-gene deletion produces Hb Barts hydrops fetalis, a disorder almost always incompatible with postnatal life. Pregnant women with an affected fetus may present in the late second or third trimester of pregnancy with complications of pregnancy and/or fetal distress. The fetus typically is hydropic and severely anemic, and frequently in these cases, prenatal diagnosis is required.

The HPLC chromatogram is diagnostic, showing a typical early elution peak representing Hb Barts (Fig. 1a). Review of parental blood films shows features typical of α-thalassemia trait with hypochromia, microcytosis, and an absence of Hb variants or any increases in Hb $A_2$ or F. The Hb H preparation typically is strongly positive in the afflicted fetus, with inclusions representing precipitated Hb Barts, whereas parental blood films stained with Brilliant Cresyl Blue would be expected to show only rare H-containing cells.

A three-gene deletion of α-globin produces a moderately severe chronic hemolytic anemia characterized by multiple Hb H-containing cells, hypochromia, microcytosis, and a typical HPLC pattern (Fig. 1d). Once again, family studies are helpful in diagnosis with parental features typical of α-thalassemia trait expected.

A two α-globin gene deletion produces hypochromia and microcytosis with a normal or mildly decreased Hb concentration and, frequently, an erythrocytosis. A single α-globin gene deletion is clinically and hematologically silent.

β-Thalassemias usually result from mutations that affect transcription, translation, or RNA stability *(41)*. When a single β-globin gene is affected, β-thalassemia minor results. Erythrocytosis and a mild hypochromic microcytic anemia characterize this heterozygous condition. HPLC analysis shows an increase in Hb $A_2$ and, in some cases, Hb F. Homozygous β-thalassemia may lead to a marked reduction ($\beta^+$-thalassemia) or absence ($\beta^0$-thalassemia) of normal β-chain production. Affected individuals typically present midway through the first year of life with a severe hypochromic microcytic anemia. HPLC and electrophoretic findings include absence or marked reduction in Hb A with a variable Hb F concentration. Interestingly and unlike the pattern in the heterozygotes, Hb $A_2$ is not increased in homozygous β-thalassemia *(42)*.

Certain structural Hb variants also have thalassemic manifestations. The most common of these, seen almost exclusively in South East Asians, is Hb E. Hb E is synthesized at a slower rate than is normal β-globin; hence an individual with Hb E trait has hypochromic and microcytic red cell indices. HPLC and electrophoretic patterns are diagnostic. The primary clinical importance of Hb E trait is the risk of co-inheritance with the thalassemia syndromes, which occur in the same population groups. An Hb E β-thalassemic double heterozygote exhibits the clinical syndrome of thalassemia major *(42)* (Fig. 1c).

Hb Lepore is another structural Hb variant that leads to decreased globin chain production with a hypochromic microcytic anemia in the heterozygote and a typical electrophoretic and HPLC pattern.

Additional clinical subgroups of the hemoglobinopathies include those producing cyanosis, erythrocytosis, and a small number of variants that produce unstable Hb tetramers. These variants may be apparent on HPLC but typically produce clinical manifestations that would lead to alternative diagnostic testing, including co-oximetry, spectrophotometric assessment for methemoglobin and unstable Hb testing.

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