Introduction to Hemoglobinopathies Diagnostics on HPLC
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The Protein

Hemoglobin is a member of the superfamily of globins which is present in the three kingdoms of life. It serves several functions, from the scavenging of oxygen reactive species to gas transport. In vertebrates, this vital protein transports oxygen from the lungs to the tissues. Human hemoglobins consist of two β- and two α-type chains and their composition changes during embryonic, fetal, and postnatal life. The globin genes coding for the different globin chains are located on chromosomes 16 and 11, respectively (Figure 1).
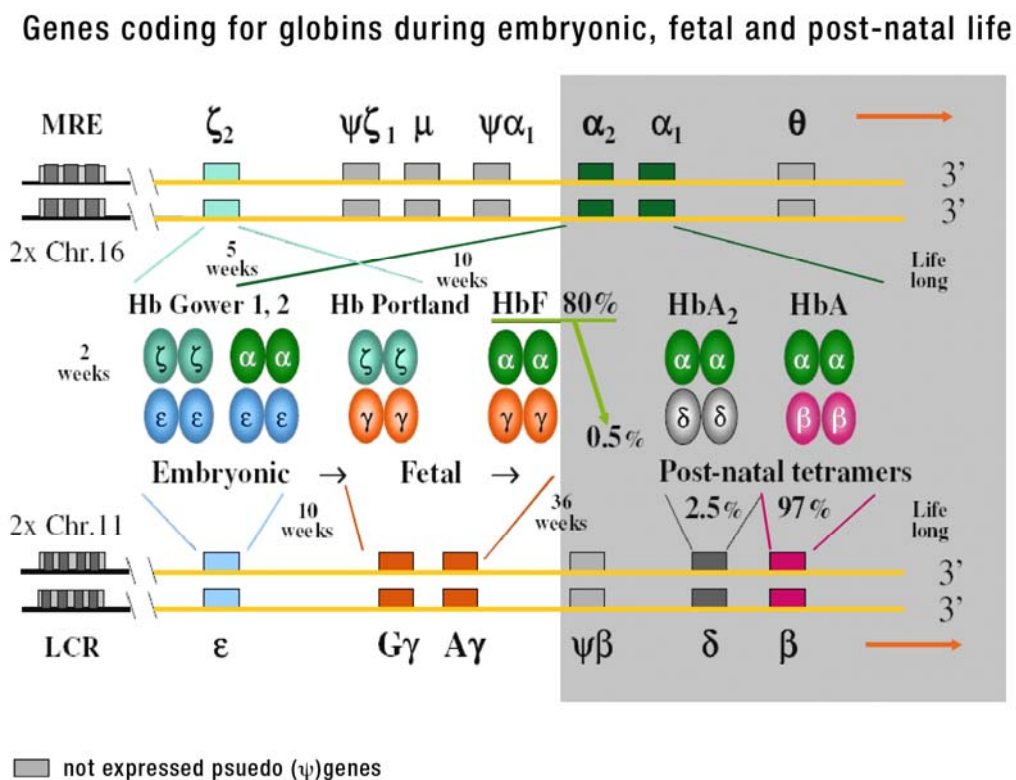

Genes coding for globins during embryonic, fetal and post-natal life

Figure 1: α- *and* β-*globin gene clusters situated on the two chromosomes 16 and 11 from maternal and paternal origin are shown. The LCR and MRE loci, 5' to the embryonic genes regulate the expression of the* β *and* α *genes, respectively. When erythropoietic factors become involved, these regulating elements activate the promoter of the genes, starting gene expression at the right time and in the right tissue. The embryonic genes* ζ₂ *and* ε *are the first to be activated during early embryonic life, producing Hb Gower-1, Hb Gower-2, and Hb Portland. The two* α *genes begin to be expressed later in embryonic life and maintain their expression during fetal life, producing fetal hemoglobin (HbF) in combination with the two* γ-*genes. During adult life, the* α *genes remain active producing HbA and HbA₂ in combination with the* β *and* δ *gene products, respectively. Because of the pre- and*

postnatal expression, pathological genotypes involving the four α genes manifest both in pre- and postnatal life. The two δ- and two β-globin genes are significantly expressed only in postnatal life. Therefore, pathological genotypes restricted to the β genes come to expression only in the after-birth stages of life, when fetal cells have disappeared. The δ gene has a physiologically low expression.

The Disease. Hemoglobinopathies (HBP) are autosomal, recessive selected disorders of the hemoglobin molecule, meaning that carriers are of both genders, usually healthy and protected against malaria. HBP are caused by mutations altering the expression of the β- and α- globin genes, which codes for the globin chains needed for building up the hemoglobin molecule. Mutations may reduce the expression of the genes, causing β- and α-Thalassemia, respectively or change the structure of the gene products, causing abnormal hemoglobins.

Including the γ and δ genes, more than 900 abnormal hemoglobins (Hb variants) have been described thus far. Only a fraction of these variants are more or less clinically relevant, mainly due to instability (hemolysis, Heinz body anemia) which may produce a semi-dominant phenotype in the carrier.

The β Defects

Only a few abnormal hemoglobins are very common, recessive and clinically relevant. These are the HbS, C, E, and D^{Punjab} variants. They are all β gene defects and therefore, do not manifest until approximately six months after birth. HbS causes sickle cell disease (SCD) in the homozygous state and in combination with HbC, E, D^{Punjab}, O^{Arab}, β-Thalassemia, and other less common traits. In contrast, homozygous carriers of HbC suffer from only a mild hemolytic anemia. Homozygotes for HbD^{Punjab} are clinically normal. HbE is a mild β-Thalassemic allele, which may cause β-Thalassemia major in combination with various types of β-Thalassemia mutations.

More than 200 mutations are known to cause β-Thalassemia minor phenotypes in the heterozygous state. It is characterized by a mild, chronic, microcytic-hypochromic anemia in the carrier. Almost all these mutations present with an increased level of the HbA$_2$ fraction in the carrier in postnatal life.

The α Defects

In normal conditions, α-globin chains are coded by four α genes, two located on paternal, and two on maternal chromosome 16. Therefore, it is not the homozygous state of the α defect that is necessarily associated with a severe α-Thalassemia condition, but the degree of the α-globin expression left in the presence of a particular genotype.

α-Thalassemia is mainly caused by a limited number of deletion defects involving one or both α genes on chromosome 16 and by a steadily increasing number of point mutations. Most α-Thalassemia carriers missing the expression of one (-α/αα) or two α genes, either in cis (--/αα) or in trans (-α/-α), are only mildly anemic (Figure 2).

Genotype phenotype correlation in α-thalassemia

α₂ α₁	Genotypes	Phenotypes
	Normal (αα /αα)	Normal
	α⁺ thalassemia heterozygous (-α/αα)	mild microcytic anemia
	α⁺ thalassemia homozygous (-α/-α)	mild microcytic anemia
	α⁰ thalassemia heterozygous (--/αα)	mild microcytic anemia
	α⁺/α⁰ combination (-α/--)	HbH disease, pre- en postnatal intermediate hemolytic anemia
	α⁰ thalassemia homozygous (--/--)	Hb Bart's hydrops foetalis severe prenatal hemolytic anemia

Figure 2: Genotype / Phenotype Correlation in α-Thalassemia

The (-α) allele, also called α⁺-Thalassemia (or α₂-Thalassemia) is the mildest defect; it does not induce a severe phenotype in the homozygous or compound heterozygous state (-α/-α). Conversely, the (--/αα) allele, also called α°-Thalassemia (or α₁-thalassemia) does. When three of the four α genes are not expressed (--/-α) the patient may have moderate to severe hemolytic anemia (HbH disease).
The most severe form of α-Thalassemia (Hb Bart's Hydrops Fetalis (HF)) is caused by the total absence of α- gene expression (--/--) and results in perinatal death. On CE, HbH disease is clearly detectable in postnatal life, when a sufficient amount of this unstable β4 homotetramer is present. Hb Bart's HF is characterized by the absence of HbF, and the presence of HbBart's (γ4) and the embryonic tetramer Hb Portland (ζ2/γ2).

Disease and Carrier Diagnostics
The clear identification of α-Thalassemia by electrophoresis, High Performance Liquid Chromatography (HPLC) or other separation methods is limited to the cases of HbH disease (- -/- α) or Hydrops Fetalis. HbH disease is diagnosed by the presence of this tetramer (β4) in adult or by the equivalent Hb Bart's tetramer (γ4) in newborn. In all other cases of α-Thalassemia, HPLC provides only a reasonable indication when the level of HbA₂ results low or normal in cases of hypochromic patients with normal ferritin levels. In all these cases, molecular diagnostics will be needed starting with Gap-PCR for the detection of the common 7 deletions (α° or α+). In the case of a negative result, molecular analysis might require α gene sequencing, MLPA, and

eventually β gene sequencing, to define the presence of α-Thalassemia due to point mutations or uncommon deletions or the presence of a β-Thalassemia due to a β large deletion or a silent point mutation. All common and most of the less common Hb variants are detectable in the carrier state on electrophoresis and on isoelectric focusing (IEF); however, their expression is only roughly estimated by these methods. Conversely, modern methods such as HPLC provide putative identification and estimation of the fractions in a matter of minutes, allowing a confident diagnosis of high HbA_2 β-Thalassemia and the common Hb variants individually or in more complex combinations. HPLC is a proven reliable method that will contribute to further improvement of carrier diagnostic, the identification of healthy couples at risk, and the prevention of SCD and β-Thalassemia major (Cooley's anemia) worldwide.