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Molecular Basis of Thalassemia

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1. Introduction

Hemoglobinopathies are a heterogeneous group of monogenic disorders widespread overall. They are commonly subdivided into three partially overlapping subgroups: structural variants which comprise the sickle cell anemia syndrome; thalassemias, characterized by a reduced rate of synthesis of one or more globin chains of hemoglobin; conditions of high persistence of fetal hemoglobin in adulthood (HPFH) (Weatherall & Clegg, 2001).

As a group, they are the commonest monogenic disorders in the world population. It is thought that the high prevalence of these defects could be due to selective advantage of the carrier state to malaria infection. However, in spite of epidemiological evidences supporting this hypothesis as well as of extensive hematological studies, the mechanisms underlying this protection still remain unknown. It is, however, evident that as a consequence of this positive selection, these diseases are mostly common in geographic areas extending from the Mediterranean region through tropical countries including Sub-Saharan Africa, the Middle East, India, Southeast Asia and Indonesia, where malaria was or still is endemic (Weatherall & Clegg, 2001). In many of these areas the estimated frequencies of these disorders range from 3 to 10 percent, even though in some specific areas the carrier frequencies may be higher, reaching 80-90% in some tribal populations in India (Harteveld & Higgs, 2010). Because of their high frequencies, different hemoglobin defects may be co-inherited, giving rise to an extremely complex series of genotypes and clinical phenotypes. In fact, in many regions thalassemic defects coexist with structural Hb variants; it is also quite common for individuals from areas at high frequency of thalassemic defects to inherit genes for more than one type of thalassemia. Furthermore, some Hb variants are synthesized at reduced rate or are highly unstable, leading both to functional and structural deficiency of the affected globin chain, thus resulting in a thalassemic condition, generally showing dominant inheritance. These complex interactions contribute to generate a wide range of clinical disorders that, taken together, constitute the thalassemic syndromes (Weatherall, 2001).

The complex and heterogeneous spectrum of molecular defects underlying these inherited conditions is regionally specific and in most cases the geographic and ethnic distributions have been determined, providing support for prevention programs based on screening, genetic counselling and prenatal diagnosis in couples at risk.

On the other hand, as the result of mass migration of populations from areas at high risk, hemoglobinopathies are being seen with increasing frequency even in regions where they were rather uncommon.

In Italy eight point mutations represent about 90% of β -thalassemia defects (Rosatelli et al., 1992) with the remaining 10% being represented by a wide array of molecular defects, some of which very rare. Furthermore, recent intensive immigration flows moving from countries with high incidence of hemoglobinopathies (Middle East, Southeast Asia and Northern Africa) with their own specific pattern of mutations as well, has rapidly increased the molecular heterogeneity of hemoglobinopathies in our region. This condition requires additional efforts to allow rapid and feasible carrier and prenatal screening programs.

2. Organization and structure of human globin genes

Hemoglobin tetramer is composed by two α -like and two β -like globin chains which are encoded by genes localized in two clusters where they are arranged in a sequential mode in the 5'→3' direction, according to their order of activation and expression during ontogenesis (Weatherall & Clegg, 2001). The α -like gene cluster is located in a region of about 30 kb in the telomeric region on the short arm of chromosome 16 (Fig. 1). It includes in the 5'→3' order an embryonal gene (ζ 2), three pseudogenes, ($\Psi\zeta$ 1, $\Psi\alpha$ 2, $\Psi\alpha$ 1), the α 2 and α 1 genes and the pseudogene θ . The β -like gene cluster is located in a region of DNA of about 60 kb on the short arm of chromosome 11 (Fig. 2). It includes in the 5'→3' order the genes ϵ , $G\gamma$, $A\gamma$, the pseudogene $\Psi\beta$ followed by the δ and β genes (Weatherall & Clegg, 2001). All globin genes share a similar structure which includes three coding exons separated by two introns. Conserved sequences critical for gene expression are found in the proximal promoter regions, at the exon-intron boundaries and in the 5' and 3' untranslated (UTR) regions. The fetal globin chains are encoded by two genes, $G\gamma$ and $A\gamma$ which share the same sequence, except in the proximal promoter region and at codon 136, where a glycine residue ($G\gamma$) is replaced by alanine ($A\gamma$). Besides typical promoter and enhancer elements, each globin gene cluster has an upstream regulatory region which plays a crucial role to promote erythroid-specific gene expression and to coordinate the developmental regulation of each gene. In the β -gene cluster this region is known as Locus Control Region (LCR), a relatively large element, encompassing ~20 Kb. It is located approximately 25 Kb upstream of the most proximal ϵ -globin gene and contains five DNase I hypersensitive (HS) erythroid specific sites (HS-1 HS-2 HS-3 HS-4 HS-5). These sites define sub-regions of open chromatin that are bound by multi-protein complexes (Fig. 2). Similarly a regulatory region, known as HS-40, is located in the α -gene cluster, upstream of the embryonal α -like globin gene (Fig. 1) (Cao & Moi, 2002; Ho & Thein, 2000; Weatherall & Clegg, 2001).

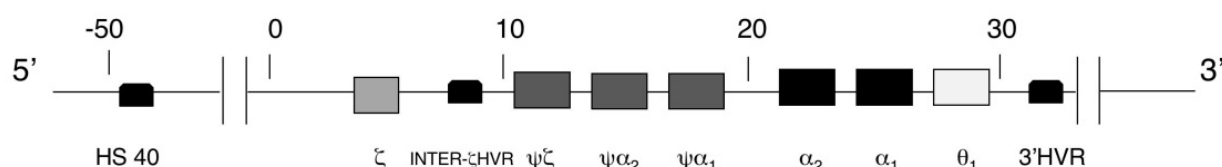


Fig. 1. **Structure of the α -gene cluster on chromosome 16.** The genes are arranged spatially in the order of their expression during ontogeny.

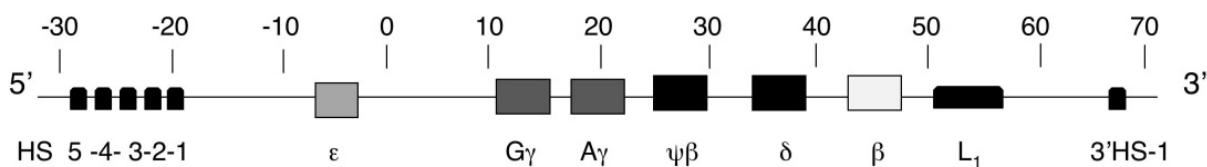


Fig. 2. **Structure of the β -gene cluster on chromosome 11.** The genes are arranged spatially in the order of their expression during ontogeny.

3. Regulation of globin gene expression

The expression of human globin genes is regulated throughout ontogeny by fine and complex mechanisms involving transcriptional, post-transcriptional and post-translational processes. The function of such a tight control is to assure that, at any stage of development, the production of α -like globin chains equals that of β -like globin chains (α /non- α ratio = 1) for the correct hemoglobin assembling (Cao & Moi, 2000). However, this mechanism of control is not able to detect whether a gene which is to be activated is functional or not. Therefore if a mutation that impairs gene expression occurs in any globin gene, it will give rise to an imbalanced globin chain production. When synthesis of α -globin genes is defective, β -like globin chains will be in excess, thus leading to α -thalassemia, whereas impaired β -globin chain output will lead to excess of α -globin chains and β -thalassemia conditions.

Transcriptional control of each globin gene expression requires distant upstream regulatory regions as well as proximal promoter regions. All proximal regulatory elements are located within the first 500 base pairs (bp), 5' to the transcriptional start (Cap) site. The promoters of all the globin genes share high homology but they also show unique sequences that may be responsible for their developmental stage-specific regulation. Three major regulatory elements with minor sequence variations are common to all globin promoter regions: the TATA, CCAAT and CACCC boxes. In the β -globin gene promoter the TATA box is located at positions -28 to -31, the CCAAT box at positions -72 to -76 and the duplicated CACCC sequences at positions -86 to -90 (proximal element) and at position -101 to -105 (distal element), respectively. It is noteworthy that, with respect to the β -globin gene promoter, the γ -globin gene shows a single CACCC element and a duplication of the CAAT box, which may have implications in the different developmental regulation of these genes. All promoter regions also contain binding sites for specific erythroid transacting factors (Cao & Moi, 2002; Ho & Thein, 2000).

All these elements, through direct interactions with the LCR and transcriptional factors, act as positive regulators and are required for optimal transcription. In fact, mutations in these sequences lead to impaired globin gene expression levels.

Several other positive regulatory elements known as enhancers have been identified within gene sequences or in intergenic regions which increase transcriptional activity of certain promoters. In the β -globin gene, enhancers are found in intron 2 and 3' to the gene, 600 to 900 bp downstream of the polyadenylation site. Silencer elements which repress gene expression play a role in the developmental control of globin gene expression, in the switch from embryonal to fetal to adult hemoglobin production. Indeed, these elements

are found in the distal promoter region of the ε -globin gene and in the γ -globin genes (Oneal et al., 2006).

The primary role of the LCR in the β -globin cluster is to confer a tissue specific state of open chromatin at the globin gene loci and also to allow interaction of transacting factors with specific globin gene promoters in a developmental stage-specific manner. Specific binding site for EKLF, GATA-1 and NF-E2, three erythroid-specific transcriptional factors that play critical roles in activation of the β -globin genes, have been described both in the LCR and in the promoters of the globin genes, thus allowing speculations on the complex function of the LCR on globin gene expression. Therefore, the stage-specific expression of globin genes could depend on the location of the genes in the cluster as well as on the availability of stage-specific transcription factors (Cao & Moi, 2002; Ho & Thein, 2000).

4. Switching of globin gene expression

During ontogenesis, physiological changes in oxygen requirements are accompanied by the switching of globin gene expression (Stamatoyannopoulos G. & Gronsveld F., 2001). This process represents one of the most intriguing and studied regulatory mechanisms of gene expression which leads to progressive and sequential changes in the expression of embryonic, fetal and adult globin genes and thus allows to synthesize different types of hemoglobin tetramers. However, the detailed mechanisms that control this process are still not fully understood (Pi et al., 2010; Ross et al., 2009).

Human hemoglobin synthesis requires two switches: from embryonic to fetal hemoglobin at 6 week of gestation and from fetal to adult production at birth (Fig. 3). The first genes to be expressed are those of the ζ -chain (α -like) and ε -chain (β -like), synthesized in the embryonic yolk sac until 4-5 weeks of gestation, which lead to the formation of Hb Gowers I ($\zeta 2\varepsilon 2$). Then, with the change of the liver as the main erythropoietic compartment, synthesis of α and γ chains is activated. At this stage the embryonic Hb Gowers II ($\alpha 2\varepsilon 2$) and Hb Portland ($\zeta 2\gamma 2$) are progressively and completely substituted by the fetal hemoglobin Hb F ($\alpha 2\gamma 2$). Around birth, when the bone marrow becomes the main erythropoietic site, β -globin gene expression is activated to synthesize the adult Hb A ($\alpha 2\beta 2$), which at birth is about 20% of total hemoglobin. The switch from fetal to adult hemoglobin is completed within the first two years of life and leads to the pattern in which adult globin expression HbA ($\alpha 2\beta 2$) comprises about 97%, HbA2 ($\alpha 2\delta 2$) 2-3% and HbF ($\alpha 2\gamma 2$) less than 1% of total hemoglobin, respectively (Stamatoyannopoulos G. & Gronsveld F., 2001).

The control of tissue and developmental expression of specific globin genes is exerted by physical interactions between the different globin gene promoters and the LCR through binding of both ubiquitous and erythroid-specific transacting factors. The sequential expression of different globin genes requires coordinated mechanisms of gene silencing and gene competition for the LCR sequences, as well as chromatin remodelling and complex chromosomal looping and tracking processes (Pi et al., 2010; Ross et al., 2009).

The switching of the expression of β -globin genes is not only a fascinating and complex model used for studying regulation mechanisms of gene expression in space and time, but its full understanding could also have important therapeutic implications in the treatment of sickle cell anemia and β -thalassemia. Indeed, the clinical picture of these conditions can improve in the presence of sufficiently high levels of HbF: in β -thalassemia syndromes, in

fact, hereditary persistence or drug-mediated reactivation of γ -globin chain output may result in a reduction of the α /non α globin chain imbalance which represents the main pathogenetic factor influencing the severity of these conditions, whereas in sickle cell anemia an increase in HbF contributes to ameliorate the severity of disease by inhibiting the polymerization of sickle hemoglobin and its related pathophysiological effects (Fathallah & Atweh, 2006).

Persistent expression of fetal hemoglobin may be associated with specific genotypes (as described below in detail) or induced by appropriate drug treatments. In fact, fetal globin genes can be reactivated by demethylation of regulatory sequences generated by hydroxyurea or 5-azacytidine or by histone deacetylation induced by treatment with short-chain fatty acids (Fathallah & Atweh, 2006). However, besides toxic side effects of these drugs, response to treatment is transient and highly variable. Thus, a better understanding of the switching processes and regulatory mechanisms of fetal globin genes may indicate new therapeutic approaches in the treatment of thalassemia and sickle cell anemia by means of a permanent reactivation of the γ -globin genes.

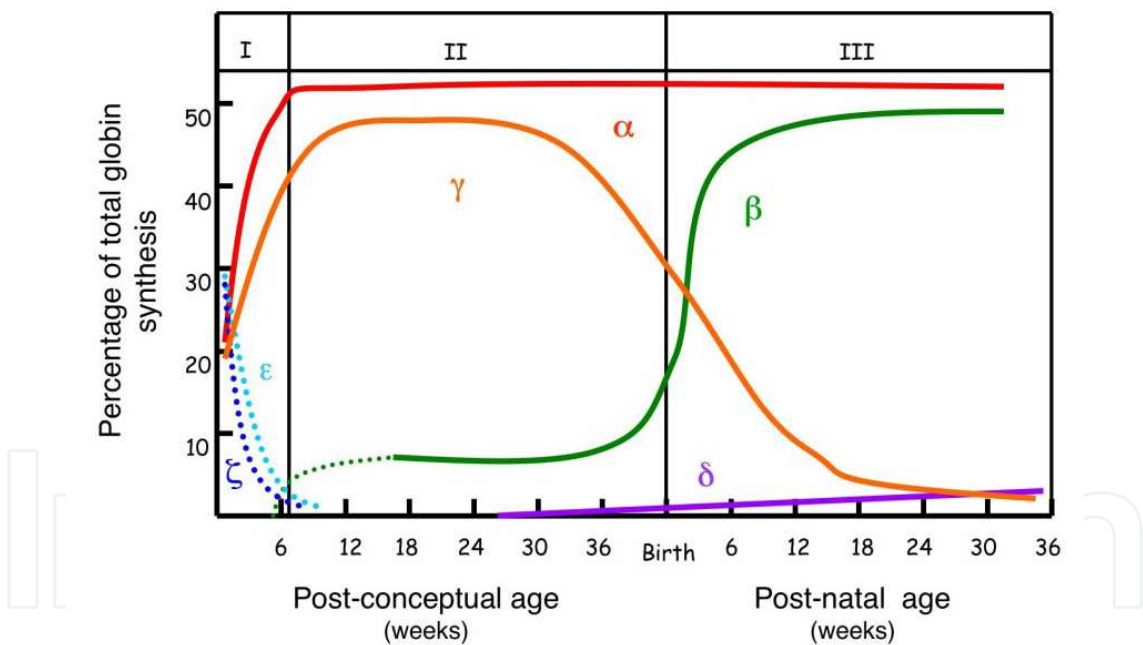


Fig. 3. **Changes in globin gene expression profile during ontogeny.** The x-axis represents the age of the fetus in weeks. The y-axis corresponds to the expression of each globin gene as a percentage of total globin gene expression. Time of birth is denoted with a vertical line. The embryonic genes are expressed during the first six weeks of gestation. The first switch from ϵ - to γ -globin occurs within 6 weeks after conception, and the second switch from γ - to β -globin occurs shortly after birth.

5. Molecular basis of hemoglobinopathies

With few exceptions, molecular defects affecting the globin genes are transmitted in an autosomal recessive manner and can result in:

1. Structural variants, characterized by the production of abnormal globin chains;
2. Thalassemias due to a quantitative defect in the synthesis of one or more globin chains;
3. Hereditary persistence of fetal haemoglobin (HPFH), a heterogeneous group of defects in the switch from fetal to adult globin gene expression which leads to persistent fetal hemoglobin synthesis in adult life. This condition, without any clinical relevance, is of great interest because it represents a useful model for studying the regulation of globin gene switching during development and because of its potential therapeutic role, since high HbF levels can ameliorate the severity of clinical phenotypes associated with some structural hemoglobin variants or thalassemias.

5.1 The structural variants

Over 900 hemoglobin variants have been identified so far (Weatherall & Clegg, 2001). Although their frequencies vary greatly in different ethnic groups, only three of them occur at high frequency in different populations: the Hb S, responsible of the sickle cell anemia which is distributed in the sub-Saharan region, in the Mediterranean area, in Middle East and in some Indian regions; the Hb C which is present in West Africa and certain parts of the Mediterranean area; the Hb E which occurs at very high frequency in Indian and Southern Asian populations.

The majority of human Hb variants result from single amino acid substitutions in one of the globin chains. Some rarer variants are instead characterized by elongated or shortened globin chains. Another type of structural variant is due to unequal crossing-over events with the formation of hybrid or fusion globin chains, as in the case of the Hb Lepore which involves the δ - and β -globin genes. All variants have the $\alpha_2\beta_2$ tetrameric structure, with the exception of the non-functional Hb Bart's and HbH, which are γ_4 and β_4 homotetramers, respectively.

5.2 The thalassemias

The thalassemias are a heterogeneous group of inherited disorders of hemoglobin synthesis, all characterized by the absent or reduced output of one or more globin chains. They are classified into α -, β -, $\delta\beta$ -, $\gamma\delta\beta$ - and $\epsilon\gamma\delta\beta$ -thalassemias, according to the particular globin chain(s) which is ineffectively synthesized. However, since the prevalent hemoglobin tetramer in adulthood is composed by α - and β -globin chains ($\alpha_2\beta_2$), the most relevant clinical forms are thus α - and β -thalassemias, respectively. In recent years, the molecular basis of the thalassemia syndromes have been described in detail, revealing the wide range of mutations encountered in each type of thalassemia (Galanello & Ortiga, 2010).

5.2.1 The β -thalassemias

The β -thalassemias are subdivided into the β^0 -, β^+ and β^{++} groups, to designate a complete, severe or mild defect in β -globin chain synthesis, respectively (Weatherall & Clegg, 2001). This results in excess of α -globin chains and, consequently, various degrees of imbalanced α /non α chain output, which is the main determinant of the typical hematological phenotypes and the clinical severity of these conditions (Cao & Moi, 2000; Weatherall, 2001).

Molecular basis of β -thalassemia are extremely heterogeneous. So far, more than 200 different β -thalassemic mutations have been described. Most of them are point mutations (single base changes, small deletions or insertions), whereas only a minority are due to large deletions encompassing the β -globin cluster (a comprehensive database of thalassemia and other globin gene defects is available at <http://globin.cse.psu.edu/>).

These mutations may occur in exon or intron sequences, as well as in the promoter or the 5' and 3' flanking UTR sequences (Fig. 4). As a consequence of the type and the position in which these defects fall, they have been reported to affect expression of the β -globin gene at the following stages:

- transcription efficiency, for mutations occurring in the promoter region, i.e., recognition sequences for proteins involved in transcriptional or post-transcriptional mechanisms such as the conserved TATA, CCAAT and CACCC boxes. Generally, such mutations are of β^+ or β^{++} types, thus resulting in mild forms of β -thalassemia;
- maturation of pre-mRNA, if they fall into splicing or polyadenylation sites. RNA-splicing mutations are fairly common and represent a large portion of all β -thalassemic mutations. These mutations affect the splicing process at variable degree, depending on the position in which the mutation occurs. Mutations that affect either of the invariant dinucleotide at the intron-exon junction (the GT motif at the 5' or donor site and the AG motif at the 3' or acceptor site) completely abolish normal splicing and result in β^0 -thalassemia. Mutations occurring in the splicing consensus sequences are instead of β^+ type, resulting in variable degrees of defective splicing and causing milder types of β -thalassemia. Other mutations occurring in exon or intron sequences may activate a cryptic splicing site, thus leading to abnormal mRNA processing. Even in these cases defective splicing occurs at variable degrees, resulting in phenotypes that range from mild to severe;
- RNA stability, if they occur in the 5' UTR, Cap site, 3' UTR or the polyadenylation site. These mutations are generally associated with mild β -thalassemia phenotypes. In particular, mutations occurring in the 5' UTR are so mild that they act as silent β -thalassemic alleles which generally show normal hematological phenotypes in heterozygotes.
- mRNA translation, if they generate premature nonsense codons. Premature termination of globin chain synthesis generally leads to the production of short, nonviable β -chains or to nonsense mediated decay (NMD) of abnormal mRNA. In all these cases mutations are of β^0 -type and result in severe thalassemia;
- protein instability, if they give rise to truncated or elongated globin chains which tend to form insoluble tetrameters.

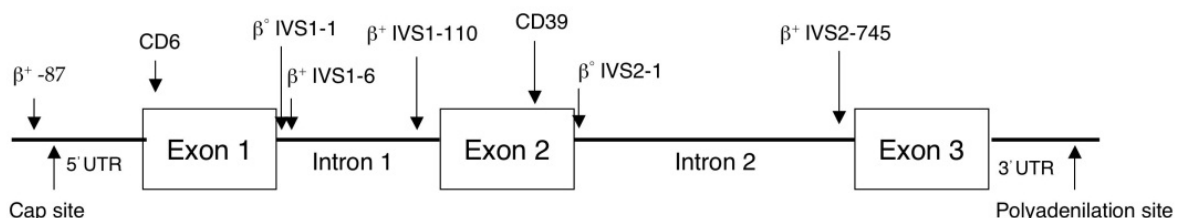


Fig. 4. **Schematic representation of the β -globin gene.** The arrows show the positions of the most frequent β -thalassemic mutations in the Mediterranean area (Rosatelli et al., 1992).

5.2.2 The α -thalassemias

Alpha thalassemias are characterized by absent (α^0 -thalassemia) or reduced (α^+ -thalassemia) output of α -globin chains, thus resulting in globin chain imbalance. As a consequence, there is a relative excess of γ - and β -globin chains which aggregate to form the homotetramers Hb Bart's and HbH, respectively, and are responsible of a severe hemolytic anemia. The two main groups of α -thalassemia can be further subdivided into deletional and non-deletional forms, according to the specific type of the underlying molecular defect. In fact, the majority of the α -thalassemia defects result from deletions involving one or both α -globin genes on the same chromosome whereas point mutations affecting the functional expression of one of the two α -globin genes (α_2 or α_1 globin gene) are less common (Harteveld & Higgs, 2010; Higgs & Gibbons, 2010). The cause of the increased susceptibility to such deletional defects for the α -globin cluster with respect to the β -globin cluster is due to the presence of highly homologous regions scattered within this cluster which predispose to events of unequal recombination. Normal individuals have four α -globin genes since each chromosome 16 carries two α -globin genes (Fig. 1); therefore, normal genotypes can be written as $\alpha\alpha/\alpha\alpha$. Deletions so far reported result in loss of one ($-\alpha$) or both ($--\alpha$) of the duplicated α -globin genes from the same chromosome (Kattamis et al., 1996). The clinical picture of α -thalassemia is determined by the number of the remaining functional genes. The deletional loss of the α_2 or α_1 gene (namely, the $-\alpha^{4.2}$ and $-\alpha^{3.7}$ deletion, respectively) are the most common molecular defects responsible for α -thalassemia. These two mutations have been found, even with different frequencies, in all populations in which thalassemia defects are common. The unequal crossing-over events responsible for their origin give also rise to the corresponding triplicated or quadruplicated α -gene arrangements which are referred to as $\alpha\alpha\alpha^{\text{anti}3.7}$ and $\alpha\alpha\alpha^{\text{anti}4.2}$ or $\alpha\alpha\alpha\alpha^{\text{anti}3.7}$ and $\alpha\alpha\alpha\alpha^{\text{anti}4.2}$, respectively. In α^0 -thalassemias large deletions almost entirely remove the α -globin cluster region. The two most common α^0 -thalassemias, the $--_{\text{SEA}}$ and $--_{\text{MED}}$ occur in Southeast Asia and Mediterranean area, respectively (Sessa et al., 2010; Harteveld & Higgs, 2010; Kattamis et al., 1996; Mesbah-Amroun et al., 2008) (Fig. 5).

Non-deletional α -thalassemias (indicated at the heterozygous state as $\alpha\alpha^T/\alpha\alpha$) are due to point mutations that, similarly to the mutations responsible for β -thalassemia, occur in genomic regions critical for normal expression of the α -genes. Furthermore, as for the β -thalassemia defects, they may be classified according to the level of gene expression that is affected and also their distribution is population-specific. However, point mutations affecting the α_2 gene are able to impair more greatly α -globin gene expression since in normal conditions the α_2 globin gene expression is about three times greater than that of the α_1 gene. Therefore, such mutations have more relevant effects on phenotype and it is expected that they could provide a greater selective advantage with respect to malaria infection. It is thus evident that they are more common than those occurring in the α_1 gene. On the other hand, non deletional α -thalassemia mutations have also a greater effect on phenotype than $-\alpha$ deletions. In fact, the $-\alpha^{4.2}$ deletional form of α -thalassemia which involves the α_2 -globin gene results in a compensatory increase in the remaining intact α_1 gene, whereas no increased expression in the remaining functional gene is detected when the α_2 globin gene is inactivated by a point mutation.

Alpha thalassemia point mutations so far detected may have effects either on RNA processing, as in the case of α^{Hph} mutation, or on RNA translation, as for the α^{Nco} defect, or

on protein instability, as for the Hb Suan Dok or the Hb Evanston. Some point mutations affecting the termination codon give rise to elongated α -globin chains, as in the case of Hb Constant Spring which is found with relatively high frequency in Southeast Asia (Harteveld & Higgs, 2010; Weatherall, 2001).

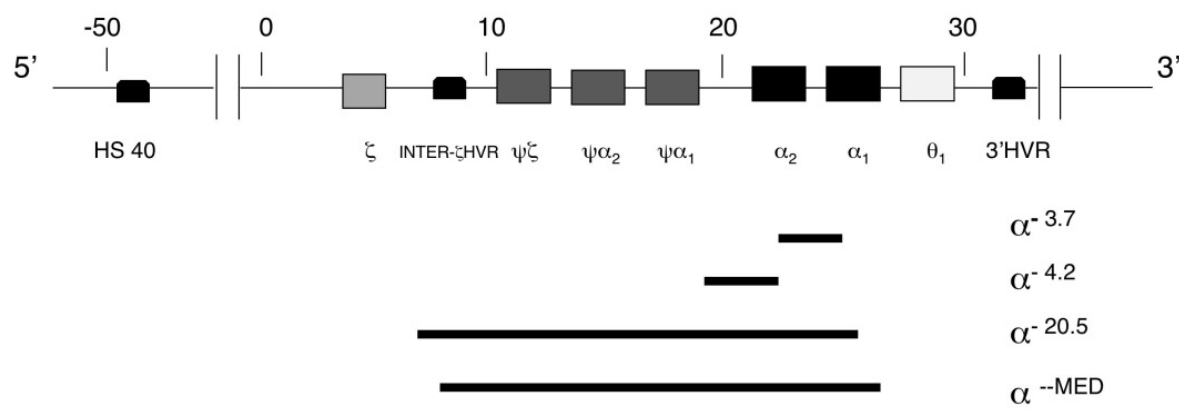


Fig. 5. Schematic representation of the α -globin cluster showing the regions removed by the most frequent α -thalassemic deletions in the Mediterranean area (Harteveld & Higgs, 2010).

5.3 High persistence of fetal hemoglobin (HPFH)

Generally, fetal hemoglobin production declines rapidly within few months after birth and in adult life it is detected only in traces (<1% of total hemoglobin). This production is normally confined to a particular small subpopulation of red cells, called F cells (fetal cells), in which the fetal profile of globin gene expression is still active. Persistent expression of fetal hemoglobin in adulthood represents a group of conditions which are referred to as HPFH (high persistence of fetal hemoglobin). The mechanisms and the molecular basis underlying these conditions are very heterogeneous. In conditions of hematopoietic stress, such as β -thalassemia, an increased number of F cells may be detected along with higher HbF levels. In these cases the γ -chains have an ameliorative effect on the thalassemic phenotype, since they reduce the excess of α -globin chains. Consequently, the erythroid precursors which produce high γ -chain levels undergo positive selection, thus increasing the number of circulating F cells.

However, because the number of F cells is largely under genetic control, the HbF levels vary considerably in these patients and this contributes to the remarkable diversity in the severity of these diseases (see also the 6.1 paragraph, for further discussion). HbF levels vary considerably not only in thalassemic patients but also in healthy adults, where it represents a benign and clinically silent condition (Thein et al., 2009; Weatherall, 2001).

Besides epigenetic factors such as gender and age (Chang et al., 1995), the molecular basis of this variation largely depends on genetic determinants which may be linked or unlinked to the β -globin cluster. Forms of HPFH may be caused by large deletions within the β -globin cluster or point mutations in the proximal or distant promoter regions of the fetal globin genes (Cao & Moi, 2000). These rare forms, in which all red blood cells show increased levels of HbF, are referred to as pancellular HPFH and are transmitted in a simple Mendelian manner. Large deletions involving intergenic γ - δ sequences, the structural δ - and β -globin genes as well as regulatory regions at the 3' end of the β -globin cluster (Thein et al., 2009)

are associated with reactivation of fetal globin gene expression. These types of rearrangements may indeed cause a loss of regulatory regions involved in globin gene switching or may result in enhancer regions brought in apposition to the γ genes as well. Examples of HPFH deletions in the β -globin cluster are the Sicilian $\delta\beta^0$ deletion (Esposito G. et al., 1994) and the Corfù deletion (Bank A. et al., 2005). A similar but less marked effect on HbF increase is found in the Hb Lepore, a $\delta\beta$ hybrid hemoglobin variant caused by a deletion originated from a mechanism of non homologous recombination between the δ - and β -globin genes. It is thought that this deletion also removes putative elements involved in globin gene switching located between these two genes, thus leading to persistent expression of fetal hemoglobin (Weaterall & Clegg, 2001).

Point mutations responsible for this form of HPFH are thought to modify the binding of transacting factors involved either in the mechanisms of globin gene switching or in γ -globin gene silencing. Among these defects, the most common mutations are those occurring in the proximal promoter region of the $\zeta\gamma$ gene at position -202 (C→G) and -175 (T→C) or in the proximal promoter of the $\Delta\gamma$ gene at position -196 (C→T), -175 (T→C) and -117 (G→A) (Olave et al., 2007). A relatively common C→T polymorphism at position -158 in the $\zeta\gamma$ -globin gene, altering a Xmn I recognition site, is associated with increased HbF levels in conditions of hematopoietic stress whereas it has no or little effects in normal individuals. Its presence has also been associated with a delayed decline of γ -globin gene expression in infant age (Grosso et al., 2007).

In heterocellular HPFH, a more common set of conditions, the HbF is distributed in an uneven fashion among F cells. Heterocellular HPFH forms are generally characterized by a structurally intact β -globin cluster and are inherited as a quantitative genetic trait (Thein et al., 2009). Extensive linkage studies have so far identified three major quantitative trait loci (QTLs) involved in the heterocellular HPFH phenotype: the Xmn I-158 $\zeta\gamma$ polymorphism, the HBS1L-MYB intergenic region on chromosome 6q23 and the BCL11A on chromosome 2p16 (Craig et al., 1996; Manzel et al., 2007). The role for one of these QTLs has been recently described. It had been found that the BCL11A locus codifies for a transfactor acting as repressor of fetal globin genes (Xu et al., 2011). Recently, another repressor of fetal globin genes, the Cold Shock Domain Protein A or CSDA, has been identified and characterized (Petruzzelli et al., 2010). Therefore, both these two factors may be directly involved in the switching of globin gene expression through silencing of the transcriptional activity of γ -globin genes in adult life, although additional studies are required in order to define better their role in the regulation of this complex mechanism of gene expression.

6. Clinical phenotypes

6.1 β -thalassemias

The hallmark of β -thalassemias is the quantitative defect in the production of β -globin chains which leads to imbalanced α -/non α -globin chain ratio and an excess of α -chains. This condition is the main determinant in the pathophysiology of β -thalassemia. Alpha-globin chains in excess precipitate in red-cell precursors, causing oxidative membrane damage, abnormal cell maturation and erythroid premature destruction in the bone marrow with consequent ineffective erythropoiesis. These abnormalities are responsible for the subsequent erythroid marrow expansion and characteristic skeletal deformities. Marrow

expansion leads ultimately to increased iron absorption and progressive deposition of iron in tissues (Weatherall & Clegg, 2001). Severity of clinical conditions is thus clearly related to the degree of globin chain imbalance which gives rise to a wide array of extremely diverse hematological phenotypes. The most severe forms are represented by β -thalassemia major, a set of conditions characterized by severe anemia requiring regular blood transfusion treatments for survival since early childhood. These forms most often result from homozygosity or compound heterozygosity for β -globin gene mutations and, in rarer cases, from heterozygosity for dominant mutations. In some cases, however, the same genotypes may lead to milder conditions, referred to as thalassemia intermedia. These intermediate forms show very heterogeneous clinical pictures that range in severity from the asymptomatic condition to transfusion-dependent anemia, generally only slightly less severe than thalassemia major. Since, as above discussed, severity of disease is related to the degree of globin chain imbalance, the milder clinical phenotype of these conditions can be explained by coinheritance of genetic factors that are able to reduce the excess of α -globin chains, such as α -thalassemia that reduce the α -globin chain production, or genetic determinants that lead to persistent expression of γ -globin chains in adulthood, thus increasing the β -like globin chain production. Alternatively, these attenuated phenotypes may also be due to homozygosity for mild mutations or compound heterozygosity for a mild or silent mutation and a more severe defect in the β -globin genes. On the other hand, triplication or quadruplication rearrangements of α -globin genes may produce a more marked imbalance in globin chain production thus leading to a clinical picture of thalassemia intermedia even in simple heterozygotes for β -thalassemia, who are otherwise generally clinically silent (Cao & Moi, 2002; Thein, 2005; Weatherall, 2001; Wong et al., 2004). This latter condition, also referred to as β -thalassemia minor, is characterized by mild anemia and morphological changes of the red blood cells, which are typically hypochromic and microcytic. Other typical features are represented by increased HbA₂ levels and slight increase of HbF (Steinberg & Adams J.G. III, Weatherall & Clegg, 2001). Finally, a rarer form of β -thalassemia trait is characterized by normal HbA₂ and thalassemia-like red cell indices (Moi et al., 1988). This condition may represent co-inheritance of mutations, associated or not on the same chromosome and decreasing both β - and δ -globin gene function. Increased HbF levels are usually detected in $\delta\beta$ -thalassemia characterized by deletions involving both δ - and β -globin genes.

6.2 α -thalassemias

The clinical phenotypes of α -thalassemia are classified in four types that range from mild to severe conditions, depending on the degree of defective α -globin gene output. The wide heterogeneity in clinical and hematological pictures is largely related on the wide spectrum of molecular defects which may lead to a great variety of genotypes characterized by loss of one, two, three or all four α -globin genes. In general, the loss of only one α -gene leads to very mild clinical conditions, with the $-\alpha^{3.7}$ producing milder phenotypes respect to the $-\alpha^{4.2}$. Non deletional mutants involving the α_2 gene are responsible of a more pronounced phenotype, as already discussed, whereas deletional loss of both α genes leads to more severe conditions (Higgs & Gibbons, 2010; Weatherall, 2010).

Deletion of one α gene ($-\alpha/\alpha\alpha$) is associated to the milder clinical forms of α -thalassemia, referred to as silent carrier state, which are characterized by slight imbalanced α /non- α globin chain production, normal values of HbA₂ and mild microcytosis, with or without anemia. The α -thalassemia trait is instead characterized by mild or moderate microcytic and

hypochromic anemia, which is clinically asymptomatic and is generally diagnosed during regular health checks or prenatal screening. This condition is commonly generated by loss of two α genes ($-\alpha/-\alpha$ or $--/\alpha\alpha$ genotypes). On the other hand, the homozygous condition of the non deletional form of α -thalassemia responsible for the synthesis of Hb Constant Spring ($\alpha\alpha^{CS}/\alpha\alpha^{CS}$) causes a more severe phenotype respect to the α -thalassemia trait. This condition is characterized by severe anemia, typical thalassemic changes in hematological indices, moderate jaundice and a variable degree of hepatosplenomegaly, a clinical picture more similar to the HbH disease than to the mild thalassemic trait. The HbH disease is most frequently the result of compound heterozygosity for α^+ and α^0 mutations ($--/-\alpha$) and is therefore predominantly found in Southeast Asia and in the Mediterranean region, where these defects are more common. HbH disease forms produced by non deletional defects are more severe than those caused by the more common deletional α -thalassemic types. The clinical conditions resemble those of β -thalassemia intermedia. Similarly to these intermediate conditions, the HbH disease is characterized by considerable variation in the severity of hematological conditions. The predominant features are variable degrees of anemia, with hemoglobin levels ranging from 2.6 to 12.4 g/dl, and amounts of HbH from 2 to 40% of total hemoglobin. Hb Bart's is occasionally detected in the peripheral blood. HbH patients usually have hepatosplenomegaly, jaundice in variable degrees, gall stones and acute hemolytic episodes induced by infections or drug treatments. In fact, with respect to the β -thalassemia conditions characterized by ineffective erythropoiesis, in α -thalassemia the main mechanism of anemia is due to hemolysis. The most severe deficiencies in α -globin chain production lead to Hb Bart's hydrops fetalis syndrome, which is commonly the result of inheritance of two α^0 determinants, although it may also result from compound heterozygosity for a severe non deletional determinant with a deletional α^0 mutant allele. In this syndrome most of the circulating hemoglobin is constituted by the non functional homotetramers γ_4 and β_4 , with also variable amounts of the embryonic Hb Portland, the only functional type of hemoglobin in these patients. The severity of anemia conditions and cardiac failure, along with the other prominent features of this syndrome, often leads to death *in utero* (23-38 weeks of pregnancy) or soon after birth (Weatherall & Clegg, 2001).

7. Unusual forms of thalassemia

Over the last two decades, our group has been involved in a prevention program for hemoglobinopathies based on screening and molecular characterization of carriers and on prenatal diagnosis in couples at-risk. In the course of this activity we have defined the molecular basis of several atypical thalassemic phenotypes which have been found to be associated with complex and unusual interactions of mutations affecting the expression of one or more globin genes. Our study provides further indications on the complexity and heterogeneity of molecular basis of thalassemia in our region. Our experience also highlights the potential pitfalls in genetic counselling in areas where globin gene disorders are most common. Some of the most intriguing cases are now being discussed in detail.

7.1 A $\delta\beta$ -thalassemia phenotype associated with a complex interaction of mutations in the γ -, δ - and β -globin genes

In this case the propositus was a 2 year-old girl of Italian descent showing a mild hypochromic microcytic anemia (Fig. 6) (Grosso M al., 2007). The peripheral blood film showed anisopoikilocytosis and marked microcytosis; she had normal serum iron values and increased

osmotic fragility; the hemoglobin analysis, carried out by cation exchange HPLC, revealed normal Hb A₂ (2.5%) and increased Hb F (6.4%) levels. This condition was consistent with a δβ-thalassemia trait. To confirm this hypothesis, hematological studies were extended to all family members. Unexpectedly, the father was found to be a typical β-thalassemia carrier with a mild increase of Hb A₂ (3.5%) and no Hb F, while the mother showed normal red blood cell indices and iron balance with a low Hb A₂ level (1.5%) and no HbF. Her two siblings, a 4 year old brother and a 13 month old sister, had both normal red blood cell indices and iron balance with HbA₂=2.1% , HbF=1% and HbA₂ 0.8%, HbF 6%, respectively. Molecular analysis was performed for all family members on genomic DNA. The propositus and her father were heterozygotes for the β⁺IVS I-6 (C→T) mutation. The propositus, her mother and the younger sister were all carriers of the δ+27 (G→T), the Mediterranean most common δ-thalassemia defect (Pirastu et al., 1983) and the -158 G_γ gene polymorphism (C→T) (Fig. 6). The molecular study thus showed that in the propositus co-inheritance of the β⁺IVS I-6 with the δ+27 mutation was responsible for the normalization of the HbA₂ level, whereas the HbF increase was associated with the -158 G_γ-globin gene polymorphism. The δ- and γ-globin gene defects had been co-inherited *in cis* on the maternal chromosome. However, the mother had normal HbF values. In fact, carriers for the -158 G_γ- gene polymorphism have increased HbF levels only in conditions of erythropoietic stress, which may be caused by a β-thalassemic trait, as found in the propositus and not in her mother. This case also allowed us to detect a novel *in cis* association of the -158 G_γ polymorphism with a δ-thalassemic defect, thus providing further evidence of the complex effects on the hematological phenotype when different thalassemic defects are co-inherited.

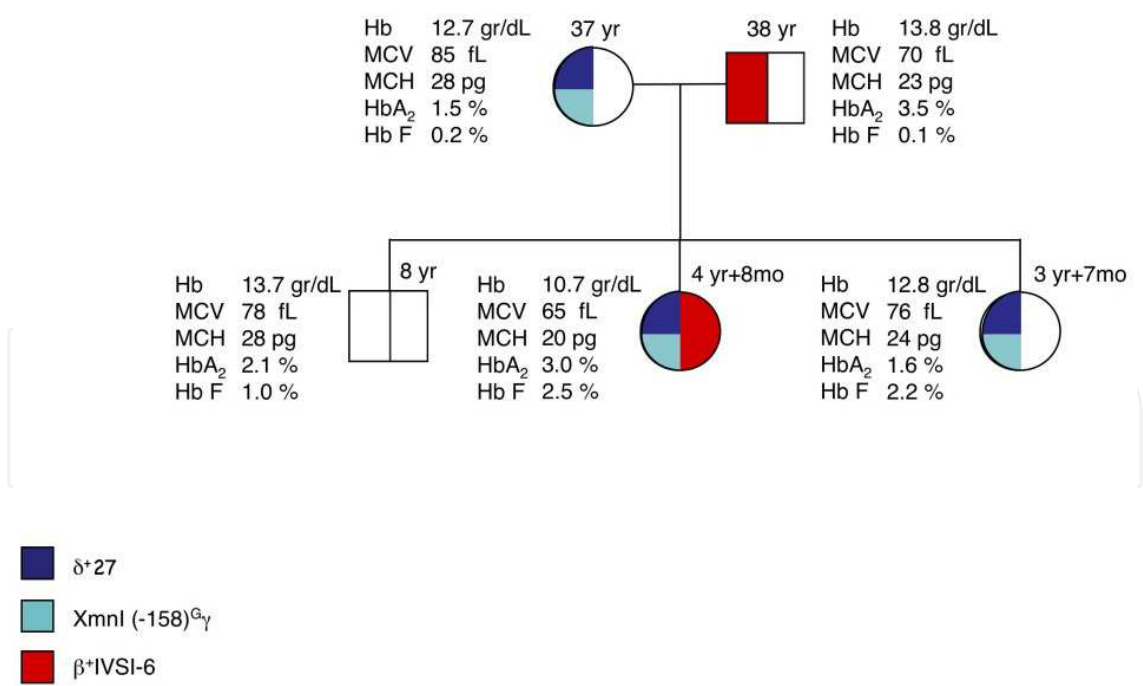


Fig. 6. **Complex interaction of mutations in the γ-, δ- and β-globin genes.** Pedigree with hematological data of the propositus and her family. The propositus had inherited the β-thalassemic defect from her father and the maternal chromosome bearing both the δ+27 mutation, which is responsible for the normalization of the HbA₂ level, and the -158 (C→T) G_γ-globin gene polymorphism associated with increased HbF levels.

7.2 An unusual δβ-thalassemia phenotype associated with a complex interaction of mutations in the δ- and β-globin genes

We investigated the molecular basis of a mild hypochromic microcytic anemia in a 5-year-old boy from Naples, Italy (Grosso et al., 2001). The patient had normal HbA₂ (2.5%) and no HbF. Osmotic resistance, serum iron and transferrin concentrations were within normal values. His father had normal red blood indices and iron balance, low HbA₂ (1.5%) and normal HbF (0.3%) levels. His mother had mildly hypochromic microcytic red blood indices, normal HbA₂ (2.7%) and HbF (0.6%). This phenotype could have been explained by either double heterozygosity for δ- and β-thalassemia or heterozygosity for α-thalassemia. Globin chain synthesis analysis allowed to exclude the α-thalassemia carrier status since the α/ non-α globin chain ratio was 2.27 for the patient, and 1.66 and 0.96 for his mother and father, respectively. Reverse phase HPLC performed in the course of globin chain synthesis analysis revealed in the patient and his mother an anomalous β-globin peak that showed features comparable to those of Hb Neapolis (beta 126 (H4) Val→Gly). Hb Neapolis is a rare unstable hemoglobin variant undetectable by conventional hematological HPLC screening methods and showing mild thalassemic features. Subjects heterozygous for this variant are characterized by mild microcytosis and slightly increased Hb A₂ levels (Pagano et al., 1991; Pagano et al., 1997). DNA sequence analysis confirmed the presence of the mutation causing the synthesis of Hb Neapolis in the propositus and his mother.

Our study also revealed the δ⁺27 (G→T) mutation in the heterozygote state, in the patient and his father.

These results indicated that the atypical δβ-thalassemia phenotype was determined in this patient by coinheritance *in trans* of δ⁺27 (G→T) and β-globin codon 126 (T→G) mutations. The δ-thalassemic trait completely normalized HbA₂ concentration, thus almost completely silencing the mild β-thalassemic phenotype produced by Hb Neapolis (Fig. 7).

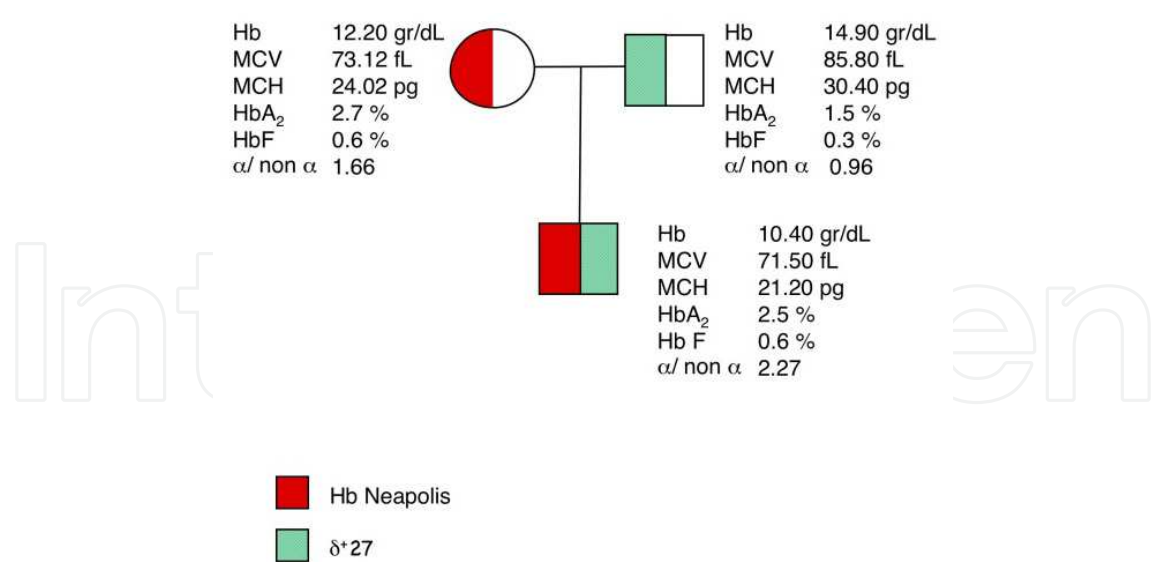


Fig. 7. An unusual δβ-thalassemia phenotype associated with a complex interaction of mutations in the δ- and β-globin genes. Pedigree with hematological data of the propositus and his family. The propositus was found to be a compound heterozygous for two rare mutations: a rare hemoglobin variant, the Hb Neapolis, associated with a mild thalassemic phenotype, inherited from his mother, and a δ-thalassemic defect (δ⁺27) of paternal origin, which was responsible for the normalization of the HbA₂ level (2.5%).

It is noteworthy that the HbA₂ level detected in the mother is lower comparable to that expected for carriers of the Hb Neapolis variant. In this case the slightly increased HbA₂ level reported for this mutation was reduced to normal values by coexistence of iron deficiency (Moi et al., 1988). This condition might have masked the underlying mild β -thalassemia carrier status, thus strengthening the importance of accurate evaluation of hematological features in families at-risk for hemoglobinopathies.

7.3 Interaction of α - and β -globin gene mutations in a couple at risk for thalassemia

A couple at risk for hemoglobinopathies, originated from Nigeria, was referred to our Prenatal Centre for genetic counselling. The woman showed slight microcytic indices with normal iron and ferritin serum values. Cation exchange HPLC analysis of hemoglobin showed mild increase of Hb F and normal HbA₂ level, along with an abnormal peak consistent with the presence of the HbS variant at the heterozygous state. The Hb S is characterized by the replacement of the glutamic acid residue at position 6 with a valine residue in the β -globin chain. At the DNA level a point mutation (A→T) at codon 6 modifies the codon GAG (glutamic acid) into GTG (valine) (Weatherall & Clegg, 2001). Molecular analysis confirmed the presence of this mutation and excluded other β -globin gene defects.

Her partner showed a slight microcytic anemia, normal HbA₂ values (2.9%) with both serum iron and ferritin low levels (Fig. 8).

We hypothesized that the hematological phenotype in both cases could be explained by the presence of α -thalassemia defects. Indeed, both partners were found to be heterozygous for the $-\alpha^{-3.7}$ deletion, one of the most common *thalassemic* defects in their country of origin along with the hemoglobin variant HbS (Galanello & Origa, 2010). Transmission of both these defects does not represent a factor of risk for hemoglobinopathy since when combined with α -thalassemic determinants, the HbS defect remains in a carrier state (Cao & Moi, 2000).

However, it is known that iron deficiency contributes to slightly reduce HbA₂ levels. Therefore, we could not exclude that the normal HbA₂ value detected in the male could have been the result of a complex combination of both genetic and epigenetic factors. We thus performed extensive β -globin gene sequence analysis also in this subject, in order to verify the hypothesis that the α -thalassemic defect associated with iron deficiency could have masked a mild β -thalassemic trait. Indeed, if this was the case, co-inheritance of the paternal β -thalassemic allele with the maternal HbS defect, which also occurs within the β -globin gene, would affect the production of functional hemoglobin with varying degree of severity and cause a disease known as HbS- β thalassemia (Weatherhall & Clegg, 2001).

This analysis showed the presence at the heterozygous state of a single nucleotide substitution at the position 108 in the first intron of the β -globin gene (IVS1-108), a defect reported to be associated with a mild β -thalassemic trait phenotype in a Cuban patient of European origin (Muñiz et al., 2000). It has been hypothesized that this point mutation may activate an intronic cryptic branch site, resulting in defective β -globin gene splicing efficiency.

Our study thus allowed us to define the risk of this couple having a child with HbS- β thalassemia disease which, on the basis of the mild β -thalassemic defect, we might expect would present with mild or moderate symptoms.

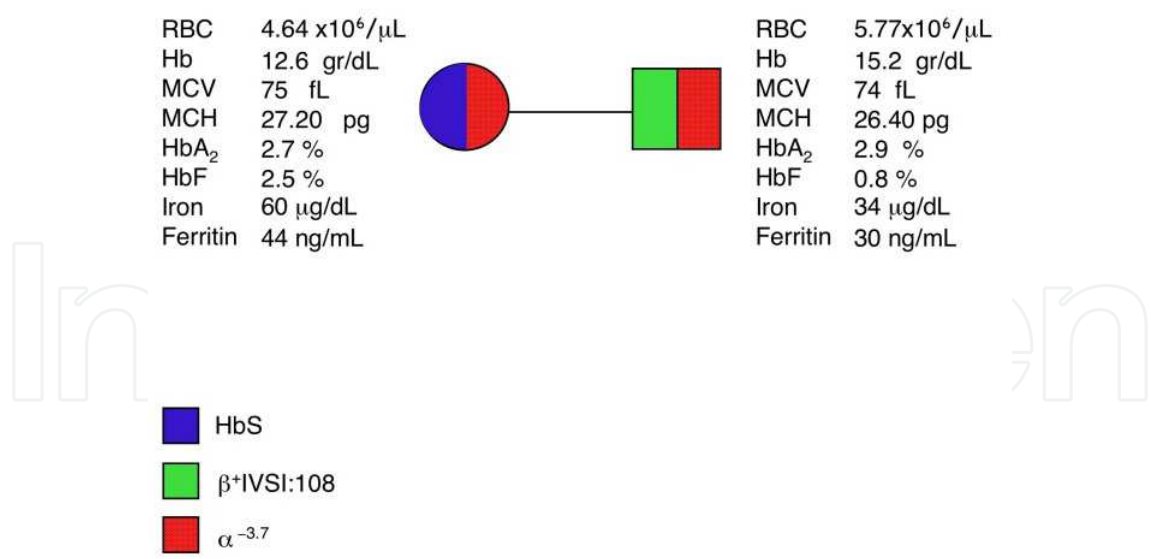


Fig. 8. **Interaction of α - and β -globin gene mutations in a couple at risk for thalassemia.** Genotypes and hematological phenotypes of the couple at-risk for hemoglobinopathy. Serum iron and ferritin values are also reported, showing a mild hyposideremic condition in the male partner.

7.4 Rare α -thalassemic genotypes detected in a family at-risk for thalassemia.

A couple of Italian descend at risk for thalassemia was referred to our Prenatal Centre for genetic counselling and further investigations. The woman who was at the 8th week of pregnancy showed slight microcythemia (MCV=78.40 fl) with normal values of HbA₂ and Hb F. This phenotype was consistent with a typical α -thalassemia carrier state. However, the proband was found negative for the most common deletional forms of α -thalassemia. Since her partner was a typical carrier of β -thalassemic trait, extensive molecular studies were performed in the woman to exclude the presence of any silent or mild β -thalassemia defect. Furthermore, to investigate the molecular basis of this very mild phenotype, we extended our study to her parents.

We found that whereas her mother had normal hematological indices, her father showed a more relevant reduction of both MCV and HbA₂ values with respect to her daughter (Fig. 9). In this subject, analysis of α -thalassemic deletions revealed the heterozygous condition for the thalassemic $-\alpha^{3.7}$ defect. Sequencing analysis performed on the α -globin genes revealed in both the propositus and her father the heterozygous state for a deletion of five nucleotides in the 5' donor site of the first intron (-TGAGG) of the $\alpha 2$ -globin gene that abolishes a restriction site for the Hph I restriction enzyme. This mutation, like other non deletional α -thalassemic determinants, has a more severe effect on the hematological phenotype with respect to deletions of single α -globin genes. Our study thus allowed us to define the complex α -globin gene genotypes in this family: the propositus was a carrier of a non deletional defect ($\alpha\alpha^T/\alpha\alpha$) whereas her father had a more complex genotype ($\alpha\alpha^T/-\alpha$), consistent with his more prominent α -thalassemic phenotype.

These investigations also allowed us to exclude the risk for thalassemia and the requirement for prenatal diagnosis in this couple since, as already discussed, co-inheritance of α -thalassemia trait has ameliorative effects on the β -thalassemic phenotype.

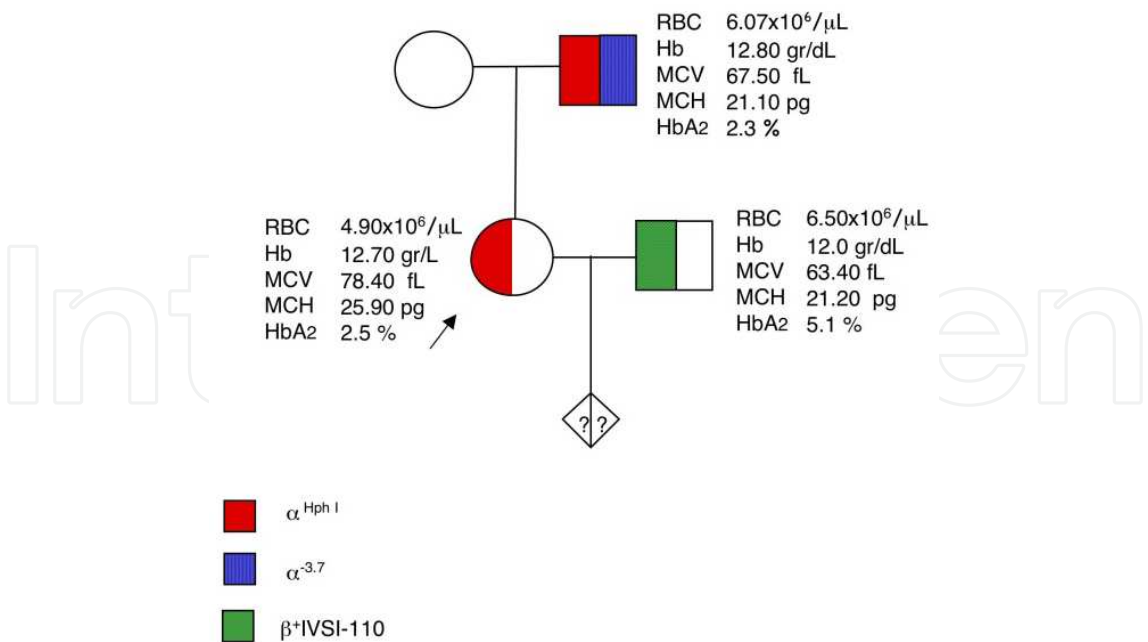


Fig. 9. **Rare α -thalassemic genotypes detected in a family at-risk for thalassemia.** Pedigree with hematological data of the proband and her family. The arrow shows the proband who was found to be a carrier of a rare point mutation in the α -globin gene ($\alpha^{\text{Hph I}}$), inherited from her father who instead is a compound heterozygous for a common deletional defect ($\alpha^{-3.7}$) and the rarer non deletional $\alpha^{\text{Hph I}}$ mutation.

8. Conclusions

The thalassemias, together with sickle cell anemia, are the most common group of inherited monogenic disorders in the world. Their high incidence is related to selective advantage of the carrier state to malaria infection. As a result, these diseases are mostly common in geographic areas extending from the Mediterranean region through tropical countries including Sub-Saharan Africa, the Middle East, India, Southeast Asia and Indonesia, where malaria was or still is endemic. Their clinical severity varies greatly, from asymptomatic hypochromia and microcytosis conditions to life-threatening ineffective erythropoiesis and hemolytic anemia. The more severe conditions require intensive medical treatments throughout life, even though, as a result of advances in transfusion, iron chelation and bone marrow transplantation therapies, expectancy as well as quality of life have increased very considerably in the last years.

Among the first diseases to be studied at the molecular level, the thalassemias still remain a paradigm for understanding the pathogenetic basis of inherited disorders, as well as the molecular mechanisms involved in the regulation of gene expression. In fact, since late 70's when DNA recombinant technologies emerged as a powerful tool for the identification of the molecular defects of the human inherited diseases, the experimental strategies and the methods firstly developed to study hemoglobinopathies were subsequently applied to define the molecular basis of other genetic diseases. These studies have also contributed to define the complex pathophysiological mechanisms underlying these syndromes and have made possible prevention programs based on large-scale screening and prenatal diagnosis in populations at high risk for hemoglobinopathies.

Molecular and clinical investigations have also provided powerful insights into the relationships between the molecular basis of thalassemias and their clinical diversity and have contributed to clarify the effects of the interactions among different genetic determinants on the thalassemic phenotypes, providing in the meantime the basis for accurate genetic counselling and preventive medicine services.

More recently, many efforts have been made toward the definition of the molecular basis of globin gene switching which represents a fascinating and unique model to study the mechanisms of gene expression regulation in space and time. Furthermore, these studies are also expected to provide novel therapeutic targets in the treatment of sickle cell anemia and β -thalassemia, as conditions of high persistence of fetal hemoglobin (HPFH) or drug-mediated reactivation of fetal globin gene expression have considerable ameliorating effects on the severity of these conditions.

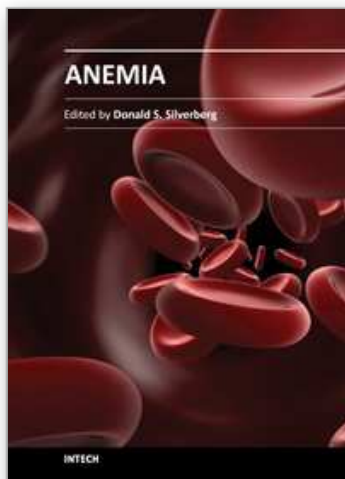
A vast body of knowledge has been gained so far and great progress has been made in the understanding of these mechanisms. It is expected that these advances will rapidly lead to novel molecular approaches to the treatment of hemoglobinopathies, before definitive gene therapy strategies will enter clinical practice.

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Anemia

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This book provides an up- to- date summary of many advances in our understanding of anemia, including its causes and pathogenesis, methods of diagnosis, and the morbidity and mortality associated with it. Special attention is paid to the anemia of chronic disease. Nutritional causes of anemia, especially in developing countries, are discussed. Also presented are anemias related to pregnancy, the fetus and the newborn infant. Two common infections that cause anemia in developing countries, malaria and trypanosomiasis are discussed. The genetic diseases sickle cell disease and thalassemia are reviewed as are Paroxysmal Nocturnal Hemoglobinuria, Fanconi anemia and some anemias caused by toxins. Thus this book provides a wide coverage of anemia which should be useful to those involved in many fields of anemia from basic researchers to epidemiologists to clinical practitioners.

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