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Cellular and biomolecular technologies for stratification of β thalassemia patients: applications in theranostics

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Introduction

1. Hemoglobin and hemoglobinopathies

The hemoglobin (Hb) is a globular chromoprotein (Kendrew 1962; Bianco 1998), in red blood cells, that deals with transportation of oxygen from the lungs to various tissues through the bloodstream. It is a tetramer composed of two types of subunits designated α and β, with a stoichiometry $\alpha_2\beta_2$ (Silvestroni and Bianco 1948; Straus, Gordon et al. 1969; Weatherall 1974). The hemoglobin consists of a fraction of high molecular weight, globin, and a fraction of non-protein low molecular weight, the heme group binding oxygen molecules in the pulmonary alveoli and then disposes in various tissues (Efimov 1979). Each individual heme molecule contains one Fe2+ atom. In the lungs where oxygen is abundant, an oxygen molecule binds to the ferrous iron atom of the heme molecule. This interaction produces a conformational change creating a three-dimensional structure, where the different subunits of hemoglobin can interact in different ways with other subunits and neighboring molecules.

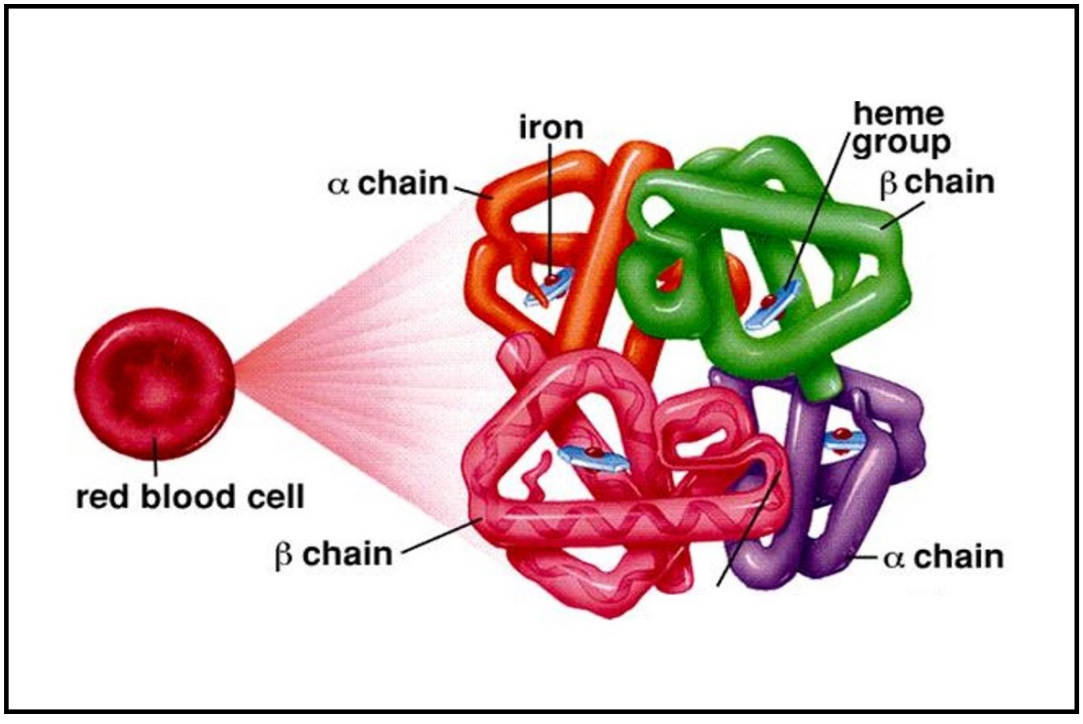

Figure 1 Structure of adult hemoglobin. Hemoglobin is a globular protein consisting of two α chains and two β-chains . It is located within the red cells where It has the function to transport oxygen from the lungs to the tissues due to the presence of a prosthetic group called heme containing an iron ion.

These additional interactions determine the formation of the quaternary structure of the protein (Ranney HM. and Beutler E 1991; Fronticelli, Sanna et al. 1995). The variations in the interactions between the different subunits are trasmitted from the surface to the heme group of a second subunit. This cause a more facilitated access of the oxygen to the ion iron leading to a greater affinity of the haemoglobin for a second molecule of oxygen. **(Figure1)** (Saroff 1970).
Defects in this protein can lead to various pathological complications broadly documented and characterized as hemoglobinopathies or thalassemias.

1.1 Hemoglobinopathies

The hemoglobinopathy is a genetic defect that results in an abnormal structure in one of the globin chains of the hemoglobin protein. The genetic defect may be due to the substitution of one amino acid for another eg. HbS, HbC and other altered hemoglobins, delection of a portion of the amino acid sequence, uncorrect hybridization between two chians, or abnormal elogation of the globin chian (Old 1996; Giardine, Borg et al. 2014). The resultulting chain may be the α-chain (eg. $HbG_{Philadelphia}$), β-chain (eg. HbS, HbC), γ-chian (HbF_{Texas}), or δ-chain ($HbA_{2Flatbush}$) (Weatherall 1974). These abnormal hemoglobins may involve a wide variety of physiological effects, but the most severe (HbS or HbC) are characterized by hemolysis (Weatherall, Clegg et al. 1974).

1.2 Specific Hemoglobinpathies: HbS and sicle cells disease

The HbS gene was detected for the first time in population of native African origin. The incidence of the gene in this population reaches 40%, in native Afro-Americans the incidence is 8%. Its presence, although at much lower frequency, was also documented in the non-Indo-European aboriginal populations of India as well as Middle East. In rare case have been reported in Caucasians of Mediterranean descent. In 1944 in Italy, Silvestroni and Bianco, submitted the first case in the world of β-thalassemia association with HbS, which they called micro-drepanocytosis. In Italy, the most affected region is Sicily, where HbS is present with a frequency of 2-5% followed immediately by Calabria. Its' frequency in other areas of Italy appears to be very low (Silvestroni and Bianco 1948). However, the new migratory waves from Africa and the Middle East could, in the next ten years

significantly change the geographical distrubution of this gene and related diseases (Old 1996; Patrinos, Kollia et al. 2005).

The expression of the HbS gene is mostly present under certain populations, perhaps given more to climatic and environmental conditions. In fact, its expression in heterozygosity (sickle cell trait) represents a kind of protection against the infestation of Plasmodium Falciparum and reduces the severity of related clinical manifestations (Flint, Harding et al. 1998; Clegg and Weatherall 1999). Unfortunately, however homozygous expression produces sickle cell disease characterized by a chronic hemolytic anemia and vaso-occlusive condition that in most cases leads to the death of the patient (Andemariam, Owarish-Gross et al.; Nur, Biemond et al.; Kato, McGowan et al. 2006).

From the molecular perspective, the HbS is characterized by a point mutation GAG→GTG in codon 6 of the β-globin gene that results in the substitution in 6 position on the β chain of a glutamic acid residue with one of valine (Rees, Williams et al.). This structural variation changes the surface of hemoglobin causing in deoxygenation conditions hydrophobic interaction between the hemoglobin tetramers, leading to the formation of polymers organized in parallel structures of fiber bundles (tactoids). The polymerization of hemoglobin is associated with significant changes in the membrane erythrocytes, which become less malleable and more fragile (Ballas 2002; Vekilov 2007). These alterations of the membrane, initially reversible, culminate with the formation in the red blood cells of the classical sickle form (Samuel, Salmon et al. 1990). This gives the name to the pathology associated with the presence of HbS, sickle cell anemia.

Clinical manifestations associated with the presence of Hb are associated with different genotypes: i) homozygosity for Hb(S/S) that causes sickle cell anemia classic; ii) double heterozygosity with β-thalassemia (S/β such), (the form that most frequently found in Italy (Silvestroni, Bianco et al. 1950); iii) double heterozygosity with some hemoglobin variants eg. S/C, D/D Los Angeles, S/O-Arab. The clinical demonstrations include both acute and chronic manifestations.

In individuals homozygous for the gene Hbs, the 97% of hemoglobin is Hbs in childhood, the remainder being the shortest normal hemoglobin, HbA_2 (α_2 δ_2). Several coexisting genetic abnormalities prevalent in African populaitons may ameliorate the course of the disease:

1. <u>α-thalassemia carriers,</u> which comprise 20% of African Americans, have a lower Mean Corpuscolar Haemoglobin Concentration (MCHC) than normal individuals. It

has been suggested that a low MCHC produce a benefit in decreasing of the vasoocclusive properties of sickled cells. These sickle cell patients live longer and have a milder disease with respect to non-thalassemic patients (Akinsheye, Alsultan et al.; Steinberg 2009).

2. Hereditary Persistence of Fetal Hemoglobin (HPFH) has established itself prominently in the black population. These people in the fetal hemoglobin (HbF) gene does not "turn off" in infancy but persists indefinitely. With the accurance of the HbF gene, there is a dilution of HbS, which reduces sickling in population (Thein, 2009)

3. G6PD deficiency has been suggested as an ameliorative condition for sickle cell disease (Badens, Martinez di Montemuros et al. 2000; van Schaftingen and Gerin 2002). This is however controversial.

1.3 Thalassemia

The thalassemia is a genetic defect that results in the production of an abnormally low quantity of a given hemoglobin chains (Silvestroni and Bianco 1948; Clegg and Weatherall 1999; Canali 2008). The defect may affect the α, β, γ, δ chain, or may affect some combination of thre β, γ and δ in the same patient. The result is an imbalanced production of globin chians and the production of an inadequate number of red cells. These cells are hypochromic/microcytic and contain a surfeit of the unaffected chains, which cannot bind the thalassemic chains in stoichiometrically manner (Williams Hematology Ernest Beutler 1995). These unrelated chains produce various kinds of damage inside the red blood cells and which leads to destruction of red cells within the marrow (ineffective erythropoiesis) and in bloodstream (hemolysis) (Cazzola 1996).

1.4 Globin clusters

To better understand thalassemia, the genes that code for the different globin chains should identify and characterize.

The gene coding for the different types of globin chains present in the hemoglobin are organized in two groups known as globin clusters (α and β); each cluster consists of structural genes and some regulatory regions constituted by highly conserved nucleotide sequences (Bianco 1998).

This α-cluster of about 30 Kb is located in the distal short arm of chromosome 16 and includes an embryonic gene (ζ), a fetal-adult block consisted of the α_1 and α_2 genes although producing different quantities of mRNA, which have identical globin chains (Myers, Tilly et al. 1986), a few pseudo-genes (ψξ, $\psi\alpha_1$, $\psi\alpha_2$) and the θ gene, which is considered a pseudo-gene, because while producing a mRNA does not express any globin .

The cluster β (or named non-α), approximately 70 kb is located in the distal portion of the short arm of chromosome 11and contains five structural genes ε, Gγ, Aγ, δ and β and the pseudo gene ψβ (Efstratiadis, Posakony et al. 1980). The genes present in β cluster produce during the embryonic period the haemoglobins Gower I ($\zeta_2\varepsilon_2$), Portland ($\zeta_2\gamma_2$) and Gower II ($\alpha_2\varepsilon_2$); in the fetal period HbF ($\alpha_2\gamma_2$) and in the adult period HbA ($\alpha_2\beta_2$) and HbA2 ($\alpha_2\delta_2$) (**Figure2).** (Lawn, Efstratiadis et al. 1980; Thein 2004; Thein 2005).

The expression of the globin chains at different stages of development depends on the activation of several genes and them switching off through methylation and demethylation processes (Ho and Thein 2000; Harju, McQueen et al. 2002; Levings and Bungert 2002; Qiliang Li 2002). The first gene switch already happens at the fifth week of gestation and produce the switching off of embryonic hemoglobin genes in favor of the activation of fetal hemoglobin genes. In this step, zeta globin (ζ) gene expression decrease and the levels of alpha globins gene (α) increase. Alpha globins get to the maximum level at the twenty-fourth week of conception and remain constant throughout life. Epsilon globins (ε), instead, are replaced by the gamma globins (γ). The second gene switch determines a drastic reduction of γ-globins, which are replaced by beta-globins (β) within the first year of age. However, during adulthood, a small amount of γ-globins are expressed, so there are three types of hemoglobins: HbA ($\alpha_2\beta_2$) that represents 97% of hemoglobin total, HbA_2 ($\alpha_2\delta_2$) and 0-2% HbF present in very low amounts. The HbF content depend on several factors such as age, sex and genetic characteristics and on the presence of point mutations in the β cluster, which could produce an increase of HbF (Bank 2006; Sankaran and Nathan 2010).

The fetal hemoglobin, compared to the adult form, has greater affinity for oxygen and this makes it more efficient oxygen transfer from the maternal to fetal blood (Efstratiadis, Posakony et al. 1980).

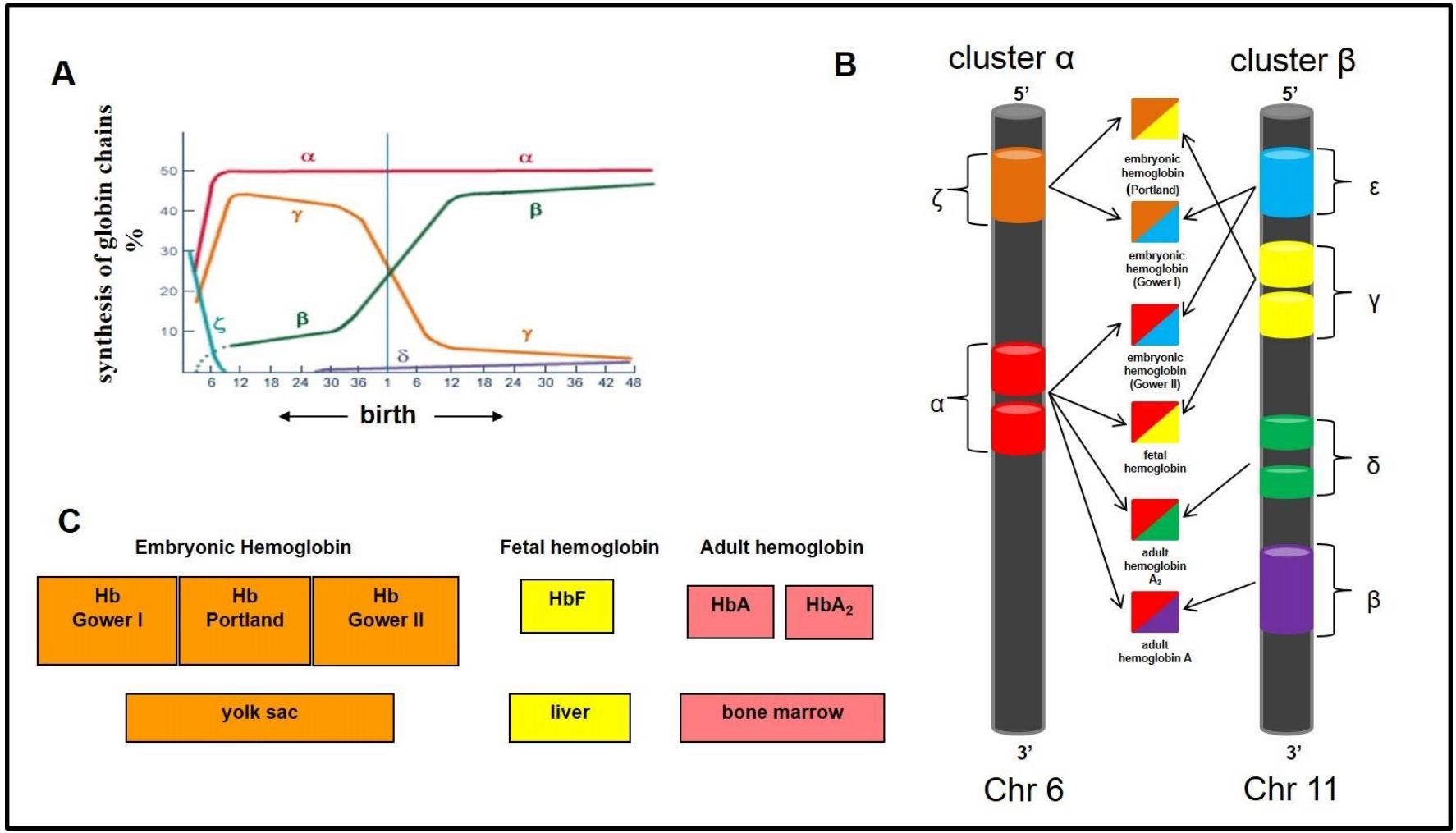

Figure 2 Hemoglobin switching. A) Schematic representation of the production of the hemoglobins during the stages of development from the embryonic stage to adulthood: B) Schematic representation of the α-cluster on chromosome 16 and β-cluster on chromosome 11. There are also represented the tetramers of hemoglobins synthesized during the different stages of development. C) The organs and tissues, where occur the hemoglobins synthesis during the different development stages, are reported with different colors, as indicated

In each clusters the structural genes are sorted in the same order to reflect the gene of expression during ontogenesis (Hanscombe, Whyatt et al. 1991). Their transcription is finely regulated by different protein elements (transcription factors) that activate or repress the globin genes through specific regulatory sequences placed within the globin cluster: i) promoters, ii) enhancer sequences, iii) the Locus Control Region (LCR). In particular the latter is located upstream of the gene for the ε-globin and it is able to modulate the gene transcription interacting with the promoters of the single globin genes. LCR region contains five domains, hypersensitive to DNase 1, named HS1, HS2, HS3, HS4, HS5, only the first four are specific to erythroid cells (Hardison, Slightom et al. 1997; Wilber, Nienhuis et al.). Among these the most important is certainly HS2, which performs the function of the enhancer or activator of transcription, it contains the binding sites for several transcription factors including: Sp1 (Specificity Protein 1), NF-E_2 (nuclear factor E2), GATA-1 (erythroid cell and megakaryocyte specific trascription factor 1) and USF (Upstream Stimolatory Factor) (Elnitski, Miller et al. 1997; Harju, McQueen et al. 2002). The domains HS3 and HS4 are involved in chromatin remodeling so as to facilitate the transcription factors binding. The role of HS5 is still uncertain. Some studies attribute to him the role of silencer or insulator (Wai, Gillemans et al. 2003). The insulator is an element able to block the activity of the histone deacetylase (Hardison, Slightom et al. 1997), so LCR plays an important role in chromatin remodeling (Levings and Bungert 2002; Qiliang Li 2002).

Initial studies about the regulation of the globin genes were mainly focused on the relationship between the LCR and the β-like genes, through the analysis of the function of the locus large fragments in transgenic mice (Myers, Tilly et al. 1986). Experiments carried out on transgenic mice, transduced with constructs containing both portions that the entire locus for the human β-globin gene, demostrated the presence of a cis-control of globin switching (Dillon, Trimborn et al. 1997). Studies about factors which control the globin gene expression have revealed a variety of activators and repressors factors that act at different stages of development. Unlike the LCR, the cis-acting elements play local function on neighboring regions of chromatin; in contrast, LCR acts on long distances to activate the expression of the globin genes (Dillon, Trimborn et al. 1997). These elements cis-elemnt could be:

1. Silencer. It binds protein complexes interfering with the promoter activity and causing the reduction of gene expression (Ramchandran, Bengra et al. 2000). A

silencer element positioned distally in the promoter for the ε-globin gene controls the repression of its expression during the fetal and adult stage. The GATA1 and YY1 proteins constitute at least two of the components included in the repression complex (Raich, Clegg et al. 1995; Ramchandran, Bengra et al. 2000) In addition, two elements DR (Direct Repeat) in the ε-globin promoter, bind a protein recently identified DRED (direct repeat definitive erythroid-binding protein), which interferes with the factor EKLF (erythroid Kruppel-like factor) binding causing the final silencing of the ε-globin gene in adulthood (Nuez, Michalovich et al. 1995).

2. Insulator. It forms independent functional domains blocking the adverse effects of heterochromatin surrounding without stepping or activate gene transcription. It disturbs the interaction between the promoter region and other regulatory element, interposing between these sequences. The elements insulators can also block the activity of histone deacetylase. Finally, the insulators facilitate activity of elements enhancers located into the region of open chromatin. One hypothesis is that the LCR could have function of insulator, in particular, the hypersensitive 5'HS5 LCR site (Harju, McQueen et al. 2002).

3. MARs (matrix attachment region) and SARs (scaffold attachment region).These DNA elements promote the binding to the nuclear matrix obtaining the formation of loops in contiguous sequences of DNA. These items can be a barrier protecting the locus from the effects of surrounding chromatin and impose a restriction structural chromatin remodeling; for these reasons, the loop of DNA could be a transcriptional target. The MARs have the function to protect DNA from the effects mediated by the elements, which act in cis in adjacent loops when the chromatin it widens, or have the function of keeping together with the cis-regulatory elements of the neighboring loops. They can also promote the juxtaposition of cis-regulatory elements and gene promoters in the same loop. Since the 5'HS-5 region presents homologies with the regions MARs can attribute this activity; this hypothesis is further supported by the fact that, when the LCR is positionally inverted, 5'HS5 could isolate the globin genes by interaction with the LCR, causing the silencing of their expression. Although the role of 5'HS5 remains controversial, one can compare its function to that of a silencer element rather than insulator.

4. Boundary elements. They can be positioned at various levels inside the locus and take the limiting gene expression when associated proteins. These elements are characterized by some main properties: their association with insulator elements, the maintenance of a balance between the open and closed

conformation of chromatin. (Harju, McQueen et al. 2002). Multiple mechanisms of action have been proposed to explain the function of the LCR; the first models based on experiments conducted on transgenic mice, suggesting that switching globin happen following mechanisms of competition and the that genes proximal had preferential interaction with the LCR sequence.

Currently have been proposed four different models of interaction of the LCR region with the different globin genes (Harju, McQueen et al. 2002).

Looping model. LCR and regulatory elements for transcription are folded to form a loop interacting with the interested gene promoter.

Tracking model. The transcription factors and cofactors bind LCR to form an active complex, which slides along the DNA helix, until reaching the gene of interest. At this point other cofactors bind the protein complex activating gene transcription.

Facilitated traking model. Transcription factors bind the LCR sequence and form a loop which slides along the DNA until it reaches the correct gene.

Linking model. Transcription factors in precise sequential order are arranged to reach the promoter region of a particular gene of interest. The chromatin structure is maintained in an open conformation so the different transcription factors may interact activating gene transcription. (Qiliang Li 2002).Below the most important transcription factors are reported involved in the globin expression regulation. **(Table1)**

A possible deletion of LCR region inevitably produces an inhibition of the expression of all proteins encoded in the globin loci resulting in severe forms of thalassemia. (Curtin, Pirastu et al. 1985; Driscoll, Dobkin et al. 1989).

The thalassemias have been described for all four chains α, β, γ, δ we will focus on β-thalassemia.

Table 1 Main transcription factors involved in the regulation of the globin genes expression

Transcription factor	Mechanism of Action	Effects
NF-E_2 (nuclear-factor erythroid derived2)	It binds to the AP-1 domain of the HS2 LCR.	It remodels the chromatin.
GATA-1 (Erythroid cell and megacaryocyte specific trascription factor 1)	It binds to the promoter of ε and γ globin genes with activator activity. It binds to the region of the silencer ε gene in the presence of the protein YY1 with repressor activity.	It check the process of erythroid cells maturation. It controls the expression of the ε and γ globins.
GATA-2 (erythroid cell and megacaryocyte specific trascription factor 2)	Recognizes the same DNA sequences of GATA-1.	It regulates the erythroid differentiation process.
FOG (Friend of GATA1)	It presents zinc-finger domains with which binds to GATA-1 without binding directly to DNA.	It checks the globin expression.
NF-E_4 (nuclear-factor erythroid derived 2)	p22 binds to a ubiquitous transcription factor (CP2) recalling the polymerase 2. p14 binds to CP2 and the transcription factor NF-E_2 preventing the formation of the protein complex that activates γ-globin gene transcription.	It regulates the expression of γ-globin gene.
SOX 6 (Sex Determining Region Y)-Box **6**)	It binds the promoter of the γ-globin gene inhibiting the transcription of the latter. It binds to the promoter of the γ-globin gene only in the presence of BCL11A.	It checks the maturation and proliferation of erythroid cells.
FOP (friend of protein arginine methyltransferase 1)	It works by reducing levels of the transcription factor FOP, indirectly increasing the levels of fetal hemoglobin.	It controls the expression of γ-globin genes.
COUP-TF2 (chicken ovalbumin upstream promoter trascription factor interacting protein)	It binds to the γ-globin promoter.	Represses transcription of γ-globin gene.
DRED-TR_2-TR_4	It binds to the promoter of the ε-globin in the vicinity of a sequence referred to as DR.	It is involved in silencing of the fetal globins.
KLF-1 (erythroid Kruppel-like factor)	It binds to the CACCC sequence present in the β-globin gene. It binds to the BCL11A, activating gene transcription and consequently reducing expression of fetal hemoglobin.	It checks chromatin remodeling and gene transcription It is necessary to adjust hematopoiesis process. It check γ-globin expression
SP1 (Specificity Protein 1)	It recognizes and binds to GC-rich regions through zinc-finger motifs that bind to highly conserved regions.	It takes part in the differentiation of erythroid cells processes. It regulates the globin switching process
BCL11A (B cell limphomaleukemia 11A)	In combination with other transcription factors, it binds to the γ-globin promoter.	It regulates the expression of the β-globin gene and consequently the levels of fetal hemoglobin.

1.5 The β-thalassemia

The β-thalassemia is an autosomal recessive disorder caused by mutations within and near the β-globin gene. All the mutations result in either the absence of the synthesis (β^0-thalassemia) or reduction in synthesis (β^+-thalassemia) of β-globin chains. (Olivieri 1999).

The first description was written by Dr. Cooley in 1925. The name thalassemia was coined to reflect the geografic origin of the target population ("thalassa" is the classical Greek name for the Mediterranean Sea) (Haldane 1949) (Weatherall, Clegg et al. 1974; Weatherall and Clegg 1996). It is estimated that currently about 1.5% of the world's population is healthy carrier (Figus, Lampis et al. 1989; Ristaldi, Pirastu et al. 1989). The patients are approximately 400,000 and there are about 60,000 new cases each year (Galanello and Origa 2010). The β-thalassemia is present in several countries of Mediterranean, Middle east, Central Asia, India, Southern China, Far East as well as countries of Africa and South America **(Figure3).** The highest incidence is reported in Cyprus (14%). In Italy, the most affected areas are Sicily, the river Po delta and Sardinia, where there is an incidence of approximately 95.7% (Silvestroni, Bianco et al. 1950; Hill 1987; Rosatelli, Dozy et al. 1992; Canali 2008). Migration flows and marriages between different ethnic groups are introducing the thalassemia in new geographies such as North Europe (Flint, Harding et al. 1998).

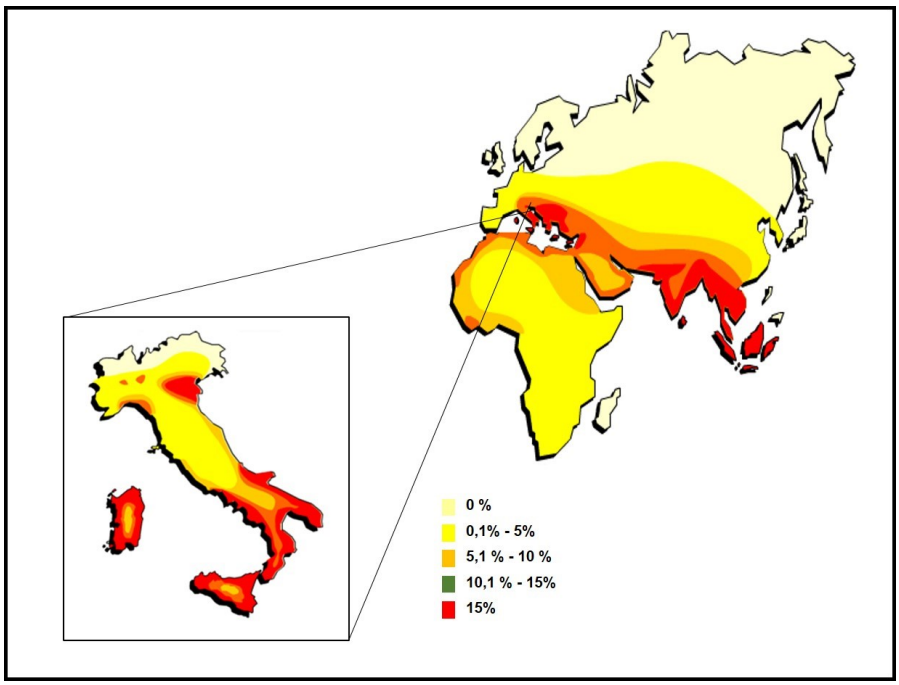

Figure 3 . β-thalassemia distribution. The areas with a high incidence of this disease are highlighted in red. In Italy stand out the areas of the Po Delta, Sardinia, Sicily and the coast of Southern Italy, where the incidence is very high**.**

In general, β-thalassemia can be summarized in four main clinical conditions:

1) Thalassemia silent: this term is used to identify a condition of β thalassemia minor, which is hardly identified as hematological parameters and levels of HbA2 and the HbF are almost normal in heterozygousity; it can be shown only a slight imbalance in the synthesis of the chains in vitro (Gonzalez-Redondo et al, 1989; Moi et al, 2004)

2) Thalassemia Minor or microcytemia, also called β-thalassemia carrier or β-thalassemia trait. It is caused by a single mutated allele (β°/β or β^+/β). Clinically asymptomatic, patients show a slight anemia and high levels of HbA_2 associated with increased HbF. However, in most cases, patients show clinical signs asymptomatic and are defined 'carriers' (Bianco 1998; Moi, Faa et al. 2004).

3) Thalassemia Intermediate: affected individuals with significant anemia which does not require blood transfusions, however. This anemia is caused by abnormalities of both genes β, but one or both mutations are mild (β°/β or β^+/β^+) (Bianco 1998; Thein 2004).

4) Thalassemia Major or Cooley's disease also known as Mediterranean Anemia: the sick people have a severe clinical presentation that implies regular blood transfusions. This form of β-thalassemia is caused by total lack of adult hemoglobin HbA. There are both mutated alleles (β°/β°, β^+/β^+ or β°/β^+) (Thein 2004).

1.6 The β-globin genes

The β-globin gene has a size of 1600 bp and coding for 146 amino acids. The gene consists of three exons (coding regions) and two introns or IVS (non-coding sequences) (Efstratiadis, Posakony et al. 1980; Bianco 1998).

The general structure of the β-globin gene **(Figure4)** is typical of other globin loci and like all other globin genes consists of:

- three exons (coding regions) and two introns or IVS (non-coding sequences).
- promoter region: placed upstream of the gene, essential for transcription, is comprised of several groups of nucleotides (box). Also, in humans there are two other hexanucleotide sequence located downstream of the transcription start site involved in the transduction of mRNA (messenger RNA).
- untraslated Region (UTR) present at the 5' and 3' ends. UTRs are essential for translation of mRNA, they are transcribed but never translated. The 3'UTR constitutes the region between the termination codon and the poly(A) tail.

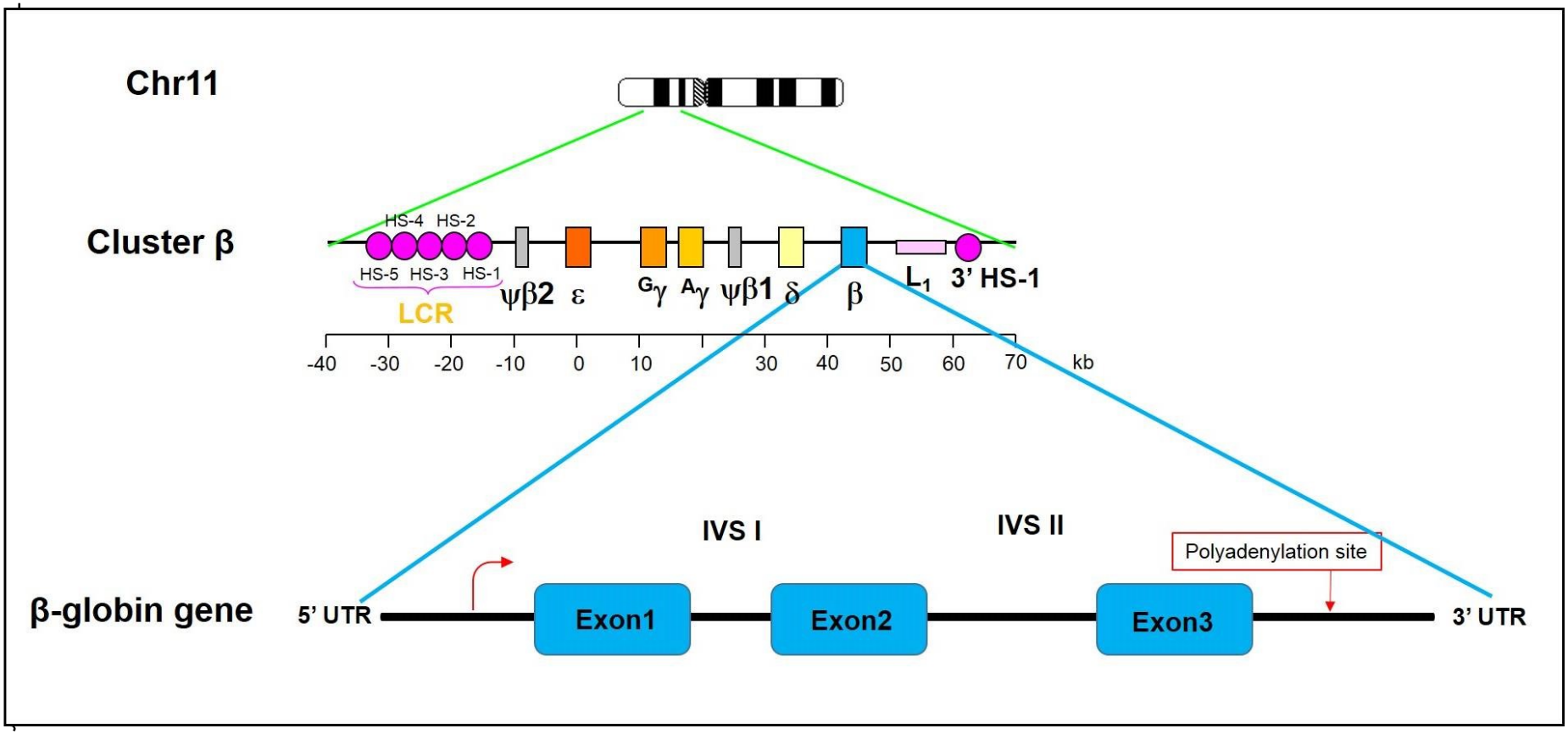

Figure 4 β-cluster and schematic structure of the β-globin gene. β-cluster is located on Chromosome 11. In the structure of β-cluster, the LCR region is located upstream of the globin genes and contains the hypersensitive sites (HS) displayed as pink dots. The genes are located reflecting the activation order during the development.. At the bottom of picture, the representation of the β-globin gene is reported. In blue, the three exons spaced by two intronic regions.

cleavage of the 3' end of the primary transcript and addition of the poly(A) tail which confers stability on the processed mRNA and enhances translation (Weatherall DJ 1991). The 5'UTR occupies a region of 50 nucleotides between 'cap' site of the β-globin mRNA and the initiation (ATG) codon. There are two prominently conserved sequences in the 5'UTR of the various globin genes, one is the CTTCTG hexanucleotide found 8 through 13 nucleotides downstream from the cap site, and the second conserved sequence is the CACCATG, in which the last three nucleotides form the starting codon (Bianco 1998).

There are more than 800 mutations (Giardine, Borg et al. 2014) in the β globin gene. They are mainly point mutations (involving a single nucleotide) and deletions (removal of gene sequence or regulatory regions) (Smetanina, Gu et al. 1997; Higgs 2004; Patrinos, Giardine et al. 2004; Patrinos, Kollia et al. 2005).

Promoter mutations. In the great majority of cases there are point mutations in the promoter region. These kinds of mutations involve a reduction of efficiency of transcription. In particular, the β-globin chains are present, but much smaller than those produced in healthy subject (β^+-thalassemia). Such changes may be described as "slight" (Meloni, Rosatelli et al. 1992; Rosatelli, Dozy et al. 1992).

Non sense mutations: involve an incomplete translation, and therefore the production of a no functional protein. They consists in the substitution of a base in a DNA codon with premature stop codon generation and, thus, termination of translation (β°-thalassemia) (Liebhaber, Trecartin et al. 1981; Trecartin, Liebhaber et al. 1981).

Frameshift mutations: consist of deletions or inserrtion of one or more nucleotides, which causes a shift in the reading frame of the mRNA (frameshift) alterating the termination of translation (Bianco 1998).

Defects in the mRNA maturation: splicing defects and polyadenilation defects (Olivieri 1999).

- Splicing mutations: they are a very large and important group of mutations which are responsible of a lot of β-thalassemias cases. Usually the defects consist in a point mutations that, with various mechanisms, alter the normal process of splicing. These mutations can occur either within an intron or within an exon. In the first case, they may cancel a regular site of splicing destroying the donor site GT or the acceptor site AG, reduce the efficiency of a splicing site altering its consensus sequence or create a new site of splicing, producing a new signal GT or AG in a cryptic site. When, however, mutations affecting the exons, the only thing that can

happen is the creation of a new donor or acceptor site of splicing, thus producing an abnormal mRNA (Orkin, Kazazian et al. 1982; Dominski and Kole 1993).

- Mutations in the cut and polyadenylation site of the pre-mRNA: if the AATAAA sequence is mutated, the cut and polyadenilation process occurs much more downstream. It produces mRNA much longer (several hundred nucleotides) and very unstable. The resulting phenotype, in heterozygous, is a mild β^+-thalassemia with only a little lower HbA together with an increase of HbA_2 (Huisman 1997).

Deletion of the β-globin gene: this type of mutation in β-thalassemia is quite rare. Individuals with this kind of mutation present hematologic characteristics very similar to a person with microcytemia. In these cases we can have levels of HbF normal or slightly higher, and a ratio α/β>1 produced by large deletions removing the β-globin gene, but leaving intact δ-globin gene. With few exceptions, the deletion usually involves the region immediately upstream of the β-globin gene increasing the HbA_2 content (Bianco 1998).

2. HPFH phenotype

The clinical picture resulting in patients affected by β^+-thalassemia or β^0-thalassemia may be improved by co-heritance of mutations related to other genes of the β-globin cluster (Orkin, Kazazian et al. 1982; Thein 2005).

Particularl conditions have observed in subjects affected by hematological disorders with HPFH phenotype (Hereditary Persistence of Fetal Hemoglobin), observed for the first time in some African patients affected by sickle cell anemia (Thein and Craig 1998). High levels of HbF significantly improve the health status in patients (Thein and Menzel 2009; Galanello and Origa 2010; Cao, Moi et al. 2011). In the HPFH condition the HbF, normally expressed at high levels only during the fetal stage of human development, continues to be expressed at high levels in adult erythroid cells. The synthesis of γ-chains in the post fetal period allows to recruit α-chains to perform the HbF hemoglobin ($\alpha_2\gamma_2$) (Weatherall DJ 1991). This greatly reduces the number of free alpha chains therefore decrease the α/non α-globin chain imbalance. In patients affected by thalassemia or other hemoglobinopathies, the presence of HbF reduces the severity of the disease and of all its related clinical complications.

2.1 The γ-globin genes

The γ-globin genes (HBG1 and HBG2) are normally expressed in the fetal liver, spleen and bone marrow. The transcripts of the two γ-globin genes differ only by one amino acid residue in 136 position. In fact, the Glycine present in this position in the G-γ chain (HBG2 gene) is replaced with an Alanine in the A-γ chain (HBG1). The G-γ chains are the predominant transcripts at birth.

The expression of the HBG1 and HBG2 genes, which encode the γ isoforms of HbF, is normally suppressed shortly before birth and replaced by expression of the β or δ chains, which encode HbA. Normally adults have less than 1% HbF, whereas the thalassemic patients and in other subjects affected by hematological disease, but heterozygotes for HPFH, have variable levels (5-30%) of HbF. Homozygotes for HPFH can express HbF in up to 100% of red blood cells (Thein and Craig 1998).

The ratio of G-γ to A-γ is fairly constant (about 7:3) during the fetal period. The ratio decrease progressively during the postnatal γ-to-β switch, leading to an average value of 2:3 in the small residual amount of HbF detectable in normal adult blood. This switch in the γ ratio occurs together with the γ-β switch (Comi, Giglioni et al. 1980). Since the 60-70 years many researchers have studied about γ-globin genes extending the understanding of the different molecular mechanisms that regulating the activation, the expression and regulation of these genes. However, many of these regulatory mechanisms appear still unclear.

In 2004 Li et al. have identified the minimum promoter sequence for the down regulation of γ-globin gene (Li, Han et al. 2004). By a series of γ promoter truncations the CACCC box was largely responsible for the down regulation of the γ-globin gene in adult erythropoiesis. The CACCC box is a common element in the proximal promoters of many housekeeping and lineage-specific genes. All mutations or deletions of this box alter the expression of the involved genes suggesting the CACCC box functions as a positive transcriptional element.

2.2 The γ-globin gene expression

The expression of the globin genes is regulated by several transcription factors. As for the β-globin genes two transcription factors, named KLF1 (Krupper-like factor 1) and *BCL11A* (B cell lymphoma-leukemia 11A), have a crucial role (Armstrong, Bieker et al. 1998). However, BCL11A and KLF1 cooperate with numerous other erythroid-specific and chromatin modifiers factors to carry out their action.

BCL11A, also known as EV19 (Ecotropic Viral Integration Site 19) is a zinc finger transcription factor necessary for B lymphopoiesis and T (Liu, Keller et al. 2003). Several studies demonstrated that BCL11A is a negative regulator of the expression of γ-chains in human erythroid precursors and knockout mice (Sedgewick, Timofeev et al. 2008). Currently are known some BCL11A isoforms generated by alternative splicing. Four are the main and best characterized: isoform BCL11AXL (835 amino acids), isoform BCL11AL (773 amino acids), isoform BCL11AS (243 amino acids), isoform BCL11AXS (142 amino acids). The amino acids encoded by the triplets of the first three exons are the same in these isoforms, but they contain different carboxy-terminal end.

BCL11AXL and BCL11AL act as repressors of the γ-globin expression in human adult erythroid cells (Uda, Galanello et al. 2008). BCL11A binds the upstream locus control region (LCR), ε-globin, and the intergenic regions of the β-globin locus, but does not results associated with the promoter of the γ-globin genes indicating that its regulation depends on a complex process. In fact, BCL11A interacts with transcription factors such GATA1, FOG1 and SOX6 and it is required for recruitment of the NuRD and LSD1/CoREST chromatin remodeling complexes to the β-globin locus in primary erythroid cells. BCL11A tied SOX6 DNA inducing curvature. Therefore, SOX6 may perform its function as an architectural factor by organizing local chromatin structure and assembling other DNA-bound transcriptional factors into biologically active and sterically defined multiprotein transcriptional complexes (Xu, Sankaran et al.; Sankaran, Menne et al. 2008; Sankaran, Xu et al. 2009; Sankaran and Nathan 2010). KLF1 is a member of a large gene family encoding erythroid-specific transcription factors, characterized by the presence of zinc-finger domains. The zinc fingers is an amino acid sequence containing four conserved amino acids including cysteine and/or histidine binding a zinc atom (Armstrong, Bieker et al. 1998). KLF1 binds specific consensus site (CACCC) on the β-globin gene promoter. Mutations in this sequence prevent the binding of KLF1 to the promoter and determine the phenotype of a mild β-thalassemia (Orkin, Kazazian et al. 1982; Moi, Faa et al. 2004). Finally, recent studies in knockout mice for KLF1 gene and in families with persistence hereditary HbF (HPFH) have demonstrated that KLF1 plays a role in the regulation of HbF, also through an other mechanism. Studies about the regulation of the encoding KLF1 gene in human erythroid precursor cells carry out using shRNA KLF1 (short interfering RNA, RNA molecule double helix containing

21-22 nucleotides blocking the translation of messenger RNA target), demonstrated an increased expression of the γ-globin genes associated to a strong reduction of BCL11A, which is an inhibitor of the γ-globin gene expression. In addiction, it was observed in studies carried out using specific antibody versus transcriptional factor lied to DNA ChIP quantitative, and playing a direct rule in the regulation of BCL11A expression (Zhou, Liu et al.).

Studies of G-WAS (genome-wide association studies) identified other loci able to influence the production of HbF. Among these we note the region *HBS1L-Myb* located on chromosome 6q23.3. Myb is a proto-oncogene that is encoding the c-MYB transcription factor, which plays a crucial role in erythroid differentiation. The absence of c-MYB in mice is lethal due to failure to develop hematopoiesis in fetal liver. Disruption of the *HBS1L-Myb* intergenic region in mice suppresses *Myb* levels and leads to elevated expression of embryonic globins and hematopoietic parameters similarly to human HPFH phenotype. Moreover, down regulation of MYB transcription factor through over-expression of microRNAs, as miR-15a and 16-1, results in an increase of HbF levels. The *HBS1L-Myb* intergenic polymorphisms (HMIP) are present in three linkage disequilibrium (LD) blocks with most of the effect on HbF levels and numbers of F cells contributed by the second block (Thein, Menzel et al. 2007; Wahlberg, Jiang et al. 2009).

So, to summarize, in the fetal period the expression of KLF1 is reduced in a manner to not be enough to stimulate for induce the β-globin gene and BCL11A expression (Zhou, Liu et al.). This is the typical profile of fetal β-globin gene and expression with low levels of β-globin and BCL11A, together with high levels of γ-globin genes. Similar situation occurs in the presence of molecular defects of the KLF1 gene. In postnatal life we observe an increase of expression of KLF,which determines the activation of both the β-globin gene and BCL11A, inhibiting γ-globin expression.

Recent experiments carried out in $CD34^+$ cells from patients with β-thalassemia transduced with shRNA *versus* the BCL11A mRNA showed that its silencing can determine a significant increase of HbF, equal to 30% of the total (Wilber, Hargrove et al.). In summary the pharmacological modulation of BCL11A can activate the production of HbF and could be represent a therapeutic approach in β-thalassemia and sickle cell anemia.

We can resume two potential alternatives for pharmacologically activation of HbF production:

- expression inhibition of KLF1 to reduce the of BCL11A levels and to increase the HbF production;
- gene silencing of BCL11A to increased the expression of γ-globin genes.

Currently here are two possible startegie for the down regulation of BCL11A. One involves the use of siRNA (small interference RNA). The siRNA binding to the target mRNA are able to degrade the mRNA reducing the gene expression and the protein production. Another strategy to down regulate BCL11A is using microRNAs, small molecules of single-stranded RNA, physiologically present in the cells. These molecules are able to negatively regulate the expression of several genes. MicroRNA binds the 3'UTR of the mRNA encoding target protein. This binding produces in some cases the inhibition of protein synthesis, in others, the mRNA degradation. In both cases there is a decrease of protein product.

So far we have described some of the molecular mechanisms that are involved in the γ-globin expression. We can see as an alteration in each of these systems leads to a deregulation of gene expression and in this case the increase of fetal hemoglobin content.

We can briefly distinguish three situations which involve increase of HbF:

- The β-thalassemia mutation per se increase the γ-glonbin chain output. This occurs in the following situations: 1) $\delta\beta^0$-thalassemia is caused by large deletions in the HBB cluster; 2) deletion remove only 5' upstream region of the HBB promoter, which results in high levels of HbA_2.
- Co-transmission of HPFH phenotype, which is the result of single point mutation in the hemoglobin Gγ (HBG2) and hemoglobin Aγ (HBG1) gene promoter.
- Co-heritance of heterocellular HPFH phenotype linked or not linked to the HBB gene cluster. As we have previously described, recent studies using G-WAS have identified two quantitative trait loci (QTLs), BCL11A on chromosome 2p16 and *HBS1L-MYB* intergenic region on chromosome 6q23, that account 20%-30% of the common variation in HbF levels in healthy adults and subjects affected by β-thalassemia and sickle cells anemia (Thein, Menzel et al. 2007; Uda, Galanello et al. 2008; Thein, Menzel et al. 2009).

Higher expression of HbF is often termed 'pancellular,' whereas lower expression of HbF is often termed 'heterocellular'(Forget 1998).

However HPFH phenotype can also be caused by other genetic alterations:

- 'deletional': HPFH result from deletions within the β-globin gene cluster on chromosome 11p15 (Ottolenghi and Giglioni 1982; Forget 1998).

- 'non-deletional': HPFH result from point mutations in the promoter regions of the γ globin genes HBG1 and HBG2, these alterations keep intact β cluster (Forget 1998).

Regarding HBG1 gene about 58 mutation have been identified. Ten of these are particularly interesting because they are related to the HPFH phenotype. In HBG2 gene there are about 73 mutations reported in HbVar (Giardine, Borg et al. 2014). Also in this case, some mutation seem to be related to the HPFH.

These mutations occur especially in the regulatory regions at the binding sites of transcription factors, that are ubiquitous or erythro-specific. They alter the binding of the proteins to their consensus sequence and could cause an increased affinity for transactivating factors; an affinity reduction for transcriptional repressors, or a combination of the two mechanisms. Through structural studies it was possible to note that non-deletion HPFH point mutations are grouped in three regions of the γ-globin promoter. They are located close to the positions -200, -175 and -115 upstream to transcription starting site. The region -200, particularly rich in GC, is known to be the target of the different point mutations, which affect Gγ promoter in the position -202 (C→G) and the Aγ promoter respectively in -202 (C→T), -198 (C→G) and -195 (C→G) positions. In *vitro* studies have investigated as these mutations influence the binding of some proteins to DNA. Indeed, mutations at position -202, -198, -196, -195 increase the Sp1 transcriptional factor binding affinity (Motum, Deng et al. 1994). In *vitro* experiments have shown that -175 Aγ/Gγ point mutation prevents the binding with Otc-1 protein (Octamer Binding Factor 1) and modifies the binding site for GATA-1 (erythroid cell and megakaryocyte-specific transcription factor 1); in both cases activating the γ-globin gene expression (Liu Lr and JW. 2004).

Similar point mutations are identified in subjects with HPFH phenotype in different ethnic groups. For example, the -196 (C→T) Aγ mutation was found in Sardinia and its association with the $\beta^0$39 mutation in the β-globin gene produces an F-thalassemia. This condition presents HbF levels increase up to 10-20% by improving the clinical status of the disease (Galanello and Origa 2010) (Cao, Moi et al. 2011).

The -117 Aγ/Gγ, -114 Aγ/Gγ mutations and a deletion of 13 bp in Aγ promoter involve several sites for transcription factors including the CCAAT sequence present in duplicate; in this case, are involved sites of different proteins: CP1

(poly(C)-binding protein-1), CDP (CCAAT displacement protein), GATA-1 and NF-E3 (Nuclear Factor erytroid derived 3) (Ronchi, Bottardi et al. 1995).

The -158 (C→T) point mutation in the Gγ promoter is known also as the XmnI G-γ polymorphism (*rs*7482144). This sequence is recognized and cut by restriction enzyme XmnI. This mutation is localized in Saudi Arabia and in other ethnic groups such as Americans and Africans. It is an example of a genetic alteration which is expressed only in hematopoietic stress condition. In fact, an high level of HbF is present in patients who have a chronic hemolytic stress condition, but it is completely absent in healthy parents (Thein 2005). Unlike the other non deletional HPFH mutations, transcription factors capable of recognizing the sequence XmnI are not yet known (Gilman and Huisman 1985; Labie, Pagnier et al. 1985).

So, mutations in the HBG1and HBG2 **(Table 2)** promoter regions are able to condition several transcription factors binding involved in the HbF *versus* HbA switch, that induce HbF expression favor HPFH phenotype in the adulthood. Recently, G-WAS conducted on different ethnic groups, have shown the involvement of outside β cluster region with HbF regulation.

Table 2 Selected BCL11A and HBS1L-MYB intergenic region SNPs

A γ-globin gene–HBG1	
nd-HPFH	-113 (A→G)
Georgia nd-HPFH	-114(C→T)
Greek-Sardinian HPFH	-117 (G→A) and IVS-I-110
Black-Greek nd-HPFH	-117 (G→A) and IVSII-745 (C→G)
Cretan	-158 (C→T)
Black nd-HPFH	-175 (T→C)
Brazilian nd-HPFH	-195 (C→G)
Italian nd-HPFH	-196 (C→T)
British nd-HPFH	-198(T→C)
Black nd-HPFH	-202 (C→T)
Venezuelan nd-HPFH	-211 (C→T)
13bp deletion	(-114 -102)

Gγ-globin gene–HBG2	
Czech nd-HPFH	-110 (A→C)
Algerian nd-HPFH	-114 (C→A)
Japanese nd-HPFH	-114 (C→T)
Australian nd-HPFH	-114 (C→G)
Black nd-HPFH (XmnI)	-158 (C→T)
Black nd-HPFH	-161 (A→G)
Black-Sardinia-British nd-HPFH	-175 (T→C)
Black nd-HPFH	-202 (C→G)
-250 (C-->T)	-250 (C→T)
-386 (A->G)	-386 (A→G)
Iranian American nd-HPFH	-567 (T→G)

Over the years various regions were investigated but two have led to interesting results. It was shown that on 6q23 SNPs in the intergenic region *HBS1L-Myb* influence HbF levels. Studies in Caucasians have identified three significant SNPs. As for 2p16 region, in the second intron of the *BCL11A* gene, several mapped SNPs were implicated strongly in the HbF modulation. In particular three SNPs *rs*11886868, *rs*4671393, *rs*7557939 have an higher frequency in different populations (Sankaran, Menne et al. 2008).

However, these SNPs often are present with a different frequency depending on the population taken into account. The links between SNPs and HbF has been amply demonstrated and validated in different groups of patients with hemoglobinopathies.

For example SNPs rs28384513, rs9399137, rs4895441 (*HBS1L-Myb*) and rs4671393 (*BCL11A*) were identified in sickle cell anemia patients (Lettre, Sankaran et al. 2008), while rs 11886868 (BCL11A) was recognized as a major factor amiliorating the status of homozygotes $\beta^0$39 thalassemia patients in the Sardinian population (Uda, Galanello et al. 2008). **(Table3)**

All aspects so far described are very interesting as they can help to clarify the molecular basis for the genotype/phenotype correlation and prove useful for the prognostic purposes.

A polymorph framework which modulating the HbF synthesis is able to influence the disease clinical condition, therefore can be considered a positive prognostic impact element for the patient. This offers numerous reflections on possible therapeutic strategies for the treatment of hemoglobinopathies.

The International HapMap Project is an organization which aims to develop a haplotype map (HapMap) of the human genome to describe the common patterns of human genetic variation. HapMap is a key resource for researchers to find genetic variants affecting health, disease and responses to drugs and environmental factors too. The information produced by the project is made freely available to researchers around the world (Project 2003). The International HapMap Project is a collaboration among researchers of academic centers, non-profit biomedical research groups and private companies in Canada, China, Japan, Nigeria, the United Kingdom, and the United States. It officially started with a meeting on October 2002, and was expected to take about three years.

Table 3 Selected BCL11A and HBS1L-MYB intergenic region SNPs

Ref SNP	Origin of population (Cohort)
BCL11A	
*rs*11886868	African Americans SCD (Cooperative Study of Sickle Cell Disease (CSSCD), Brazil SCD, Sardinians , French β-thalassaemia
*rs*4671393	African Americans SCD (CSSCD), Brazil SCD, North Europeans, Chinese β-thalassaemia
*rs*7557939	African Americans SCD (CSSCD), Brazil SCD
*rs*6732518	North Europeans, African Americans SCD (CSSCD), Africans Hong Kong β-thalassaemia heterozygotes heterozygotes or HbE heterozygotes, African Americans SCD , SCD (Silent Infarct Transfusion (SIT) Trial), Thai β-thalassaemia
*rs*10189857	African Americans SCD (CSSCD), Sardinians β -thalassaemia
*rs*6545816	heterozygotes or HbE heterozygote Thai and Thai-Chinese β -thalassaemia/HbE, Thai β-thalassaemia
*rs*7599488	African Americans SCD (CSSCD)
*rs*1427407	North Europeans, Sardinians β -thalassaemia
*rs*766432	North Europeans, Chinese (Hong Kong) β-thalassaemia heterozygotes, Thai and Thai-Chinese β -thalassaemia/HbE, Chinese β- SCD, Hong Kong β-thalassaemia heterozygotes, African Americans SCD (CSSCD), Africans SCD (SIT Trial), African Americans thalassaemia, Thai β-thalassaemia heterozygotes or HbE heterozygotes
*rs*10184550	African Americans SCD
*rs*7606173	Africans SCD (SIT Trial)
*rs*6706648	Africans SCD (SIT Trial)

HBS1L-MYB	
*rs*28384513	African Americans SCD (CSSCD), Brazil SCD
*rs*7776054	African Americans SCD (CSSCD) , Brazil SCD
rs9399137	African Americans SCD (CSSCD), Brazil SCD, North Europeans, Sardinians β^0 -thalassaemia ,Thai and Thai-Chinese β^0 -thalassaemia/HbE , French β-thalassaemia, Chinese (Hong Kong) β-thalassaemia heterozygotes, Africans SCD (SIT Trial)
*rs*9389268	African Americans SCD (CSSCD), Brazil SCD
*rs*4895441	African Americans SCD (CSSCD) , Brazil SCD , Sardinians, Sardinians β -thalassaemia, Thai and Thai-Chinese β - thalassaemia/HbE, North Europeans, Chinese β-thalassaemia, Africans SCD (SIT Trial), Chinese β-thalassaemia heterozygotes
*rs*6929404	North Europeans
*rs*9402686	African Americans SCD (CSSCD) , North Europeans , Chinese β-thalassaemia
*rs*1320963	North Europeans
*rs*6904897	North Europeans, Sardinians β -thalassaemia
*rs*35959442	Chinese β-thalassaemia
*rs*9376090	North Europeans ,Chinese β-thalassaemia
*rs*4895440	North Europeans, Chinese β-thalassaemia
*rs*9494142	Chinese β-thalassaemia
*rs*9402685	North Europeans, Chinese β-thalassaemia
*rs*11759553	North Europeans, Chinese β-thalassaemia
*rs*6934903	Chinese β-thalassaemia heterozygotes

It comprises two phases, the complete data obtained in Phase I were published on October 2005. The analysis of the Phase II dataset was published in October 2007. The Phase III dataset was released in spring 2009.

Galarneau et al. (2010) resequenced 175.2 kb to fine map HbF association signals at the *BCL11A*, *HBS1L-Myb* and β-globin loci investigated in 190 individuals including the HapMap European CEU, Nigerian YRI founders and 70 African Americans with sickle cell anemia. The authors discovered 1489 sequence variants, including 910 previously unreported variants. Using this information and data from HapMap, Galarneau et al. (2010) selected and genotyped 95 SNPs. The XmnI polymorphism *rs*7482144 in the proximal promoter of HBG2 marks the Senegal and Arab-Indian haplotypes and is associated with HbF levels in African Americans with sickle cell disease (Lettre et al., 2008). Galarneau et al. 2010) replicated the association between *rs*7482144 and HbF levels. p=3.7x10(-7). However, rs10128556, a T/C SNP located downstream of HBG1 (*rs*142200), was more strongly associated with HbF levels than rs7482144 by 2 orders of magnitude (p=1.3x10(-9). When conditioned on *rs*10128556, the HbF association result for rs7482144 was not significant, indicating that rs7482144 is not a causal variant for HbF levels in African Americans with sickle cell anemia. The results of a haplotype analysis of the 43 SNPs in the beta-globin locus using rs10128556 as a covariate were not significant (p=0.40), indicating that rs10128556 or a marker in linkage disequilibrium with it is the principal HbF-influencing variant at the beta-globin locus in African Americans with sickle cell anemia.

3. The hematopoiesis and erythroid cell methods of culture.

The cells forming the blood have a short relatively lifetime, for this reason, the human body contains systems to ensure a continuous cell turnover. The hematopoiesis is the process of production of all blood cells from stem cells pluri-potent and it takes place, in the adult, in the bone marrow. The site of hematopoiesis varies in different stages of our life. The bone marrow is about 5% of total body weight and it is distributed mainly in the flat bones and long in childhood and youth, while in later life you it happens at the level of the ribs, vertebrae, sternum and pelvis. During intrauterine life the hematopoiesis happens instead, first in the yolk sac and then in the liver and spleen, while from 7-8 months of gestation, the hematopoiesis occurs gradually in the bone marrow in special areas called "niches" represented by 1-2% of the population bone marrow.

As the site of erythropoiesis changes the process regarding the red cells production, the globin genes being expressed also change, progressing from

embryonic to fetal and finally to adult **(Figure5)**. Thus, globin gene regulation serves both as a model for developmental gene regulation during ontogeny and for the molecular mechanism playing a role in cellular differentiation (Pope, Fibach et al. 2000).

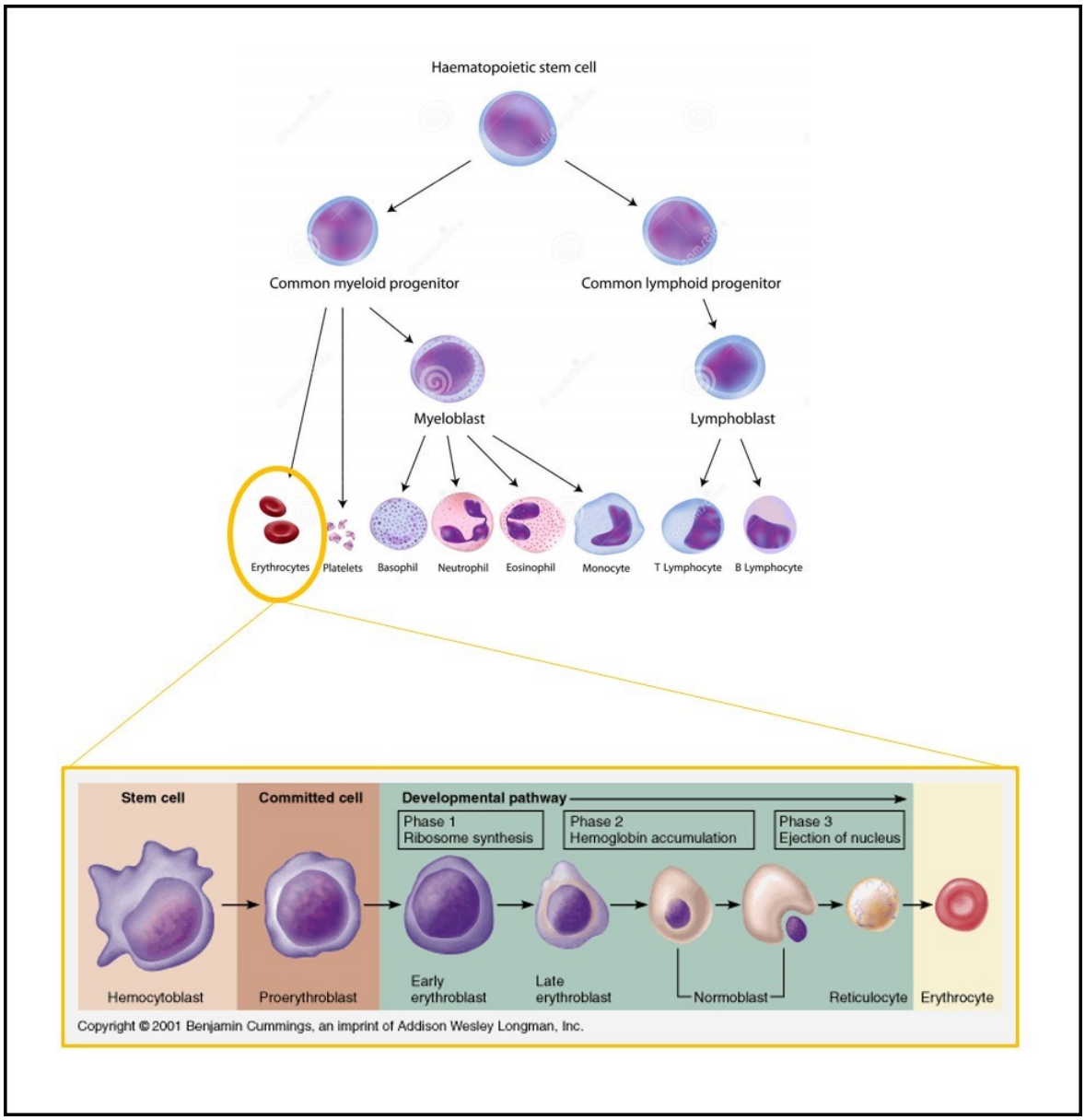

Figure 5 Hemopoiesis. All lymphoid and myeloid cells are originated from a single multipotent hematopoietic stem cell. At the bottom are represented the steps leading to the formation of a mature red blood cell.

Hematopoietic stem cells are also found in the blood, but in very low percentage, approximately 0.05% of peripheral blood cells (Isgro, Marziali et al. 2009). Hematopoietic stem cells are characterized by the presence of specific antigens located on the cell surface. In particular, the $CD34^{+}$ antigen identifies all

hematopoietic stem cells and bone marrow progenitors; this is a transmembrane glycoprotein of 110 kDa single chain; the presence of many glycosylation sites, the lack of tyrosine kinase activity and the very low frequency of synthesis and degradation suggests that $CD34^+$ is a receptor involved in cell-cell or cell-extracellular matrix interaction. His expression varies with the degree of differentiation and proliferative potential of hematopoietic cellular elements: it is highest in the most primitive population of the bone, represented by the "long-term culture-initiating cells" (LTC-IC), and it is thought that it is lost gradually during the cell proliferation and differentiation. This marker is expressed by 1-3% of the cells of the bone, from 0.01-0.1% of peripheral blood cells and by 0.1-0.4% of the cells of the umbilical cord (Isgro, Marziali et al. 2009). One of the first steps of differentiation of hematopoietic stem cells is to continue the path of differentiation in myeloid or lymphoid sense. Together they will give rise to all blood cells. Stem cells still have the capacity to divide, but which are not able to differentiate along all the different ways and guide its development only to a specific line are defined hematopoietic committed, these cells are intended for differentiation according to one line of differentiation. They proliferate in differentiating morphologically identifiable precursors, which are subject to greater maturity, through which acquire the functions highly specialized and lose their ability to regenerate yourself (Amit and Itskovitz-Eldor 2006). The latter, in their turn, give rise to cells that can differentiate further producing (Gurdon, Byrne et al. 2003) myeloblasts, from which they originated granulocytes; erythroblasts, from which derive reticulocytes, and then the red blood cells; megakaryocytes, from which are formed platelets. The process of the differentiation, and in particular the specialization of cells is made possible by a system of gene regulation that repressing the not specific-genes of that cellular sector. Gene regulation is ensured by chemical signals internal to the same cell and by signals from outside the cell, for example from adjacent cells. The early stages of the hematopoietic process are controlled by a group of growth factors called cytokines, produced and secreted not only by the marrow cells, but also by cells of the immune system. Among the cytokines mainly involved in the differentiation process are the SCF (stem cells factor), interleukin-3, GM-CSF (Granulocyte-macrophage colony stimulating factor). Then there are growth factors such as EPO (erythropoietin) which acts on already specialized cells. EPO binds to protein receptors with tyrosine kinase activity activating a mechanism of phosphorylation of the target proteins (Box et al., 1996). The whole process

leading to the production of erythrocytes starting from stem cells is called erythropoiesis. This process requires in addition to the presence of cytokines and erythropoietin and the presence of folic acid, vitamin B_{12}, iron and trace elements such as copper, cobalt and nickel too.

The first erythroid-committed progenitors are defined functionally as burst-forming units (BFUe) (Clarke and Housman 1977). They can be isolated from bone marrow and peripheral blood. The BFU-E can be maintained in culture and after 10-15 days give rise to colonies of erythroid precursors. Erythroid units colony-forming (CFU-E) are more mature cells, capable of producing of small dimensions cellular clones. The formation of erythrocytes from the committed cells is regulated by the EPO that interacting with specific receptors present on the membrane of committed cells and leads to the formation of precursor said pro-normoblasts (also called pro-erythroblasts). The time required for this process is usually about a week (at least four cell divisions) and during this period occurs the reduction the size of the nucleus and the increase in the synthesis of hemoglobin (Cuneo, Balboni et al. 1994). Following the last cellular division, the nucleus is pyknotic ousted from erythroblast and in this way, creates the reticulocyte. Reticulocytes remain for about two or three days in the bone marrow and then be placed in the blood circuit, where they assume their final morphology (loss of mitochondria and ribosomes). After 24 hours the erythrocytes take a structure similar to biconcave and oval disks with a diameter of about 6-8 uM and a maximum thickness of 2 uM, is totally free of cellular organelles and has the ability to transfer large amounts of gas (especially oxygen and CO_2) facilited by ratio between surface and volume. The developmental aspects of erythropoiesis have been studied in culture using in *vitro* established cell lines that can be induced to express erythroid characteristics, or using primary cultures of cells derived from progenitors present in the bone marrow or in peripheral blood.

Erythroid cell lines have been used extensively as models to study β-thalassemia and to develop new therapeutic strategies. In addition, primary cultures of erythroid cells can be readily established from a lot of normal and individual patients and their growth in *vitro* forming blackberries. Until today, the erythroid cells were cultured either in semi-solid medium, where they develop into discrete colonies, or in liquid medium where they grow as single cells or clusters in suspension (Fibach, Manor et al. 1989) (Leimberg, Konijn et al. 2003) (Fibach and Prus 2005). In all these systems erythropoietin (EPO) is essential for full

development of hemoglobin containing erythroid cells (Fibach, Bianchi et al. 2003). Here will be describe these two cellular culture systems.

Semi-solid cultures. This method permit to evaluate the quantity and quality of the grown progenitors, but theis immobilization in the semisolid medium results in several disadvantages that make it technically difficult to carry out quantitative analyses of the developing cells. The culture of erythroid precursors performed in this manner could be provide for suspension of single hematopoietic cells, derived from bone marrow, peripheral blood or from other sources, such as the fetal liver, cord blood or neonatal spleen adults, and are dispersed in semi-solid media usually containing methyl cellulose or plasma clot. With this method it's possible to study the colonies grown from a single cell *in vitro.* Colonies begin to appear after 3-4 days of incubation, and reach their final size and haemoglobinization after 1-2 weeks. Each colony is a clone derived from a progenitor erythroid-committed. On the basis of the final size of the colonies and the time required for theircan be distinguished hemoglobinization various types of progenitors. The colony forming units erythroid late (CFUe) reach the final size and hemoglobinization after 1 week and then disappear, while the early erythroid burst-forming units (BFUe) develop after 2 weeks. Erythroid colonies can be distinguishes from other (myeloid) colonies by their red colour or by their positive reaction with heme-specific reagents. The fact that in this culture system the cells are immobilized in semi-solid medium results in several disadvantages. The cell yield *per* colony and the total cells *per* culture are low making it technically difficult to carry out biochemical, molecular and immunological characterizations of the developing cells. Also, it is a one-step continuous culture and addition of the tested agents during the culture is difficult. In addition, the HbF produced in colonies grown in semi-solid medium is significantly higher than that produced in vivo by the same donor of the cells (Fibach, Bianchi et al. 2003).

The two-phase liquid cultures. The culture procedure yields populations that are large, relatively pure, and synchronized (in terms of differentiation), and which mimic *in vivo* erythropoiesis. Since the cells are grown in suspension, samples of cells can be remuved every time without disturbing the cultures and assayed for various parameters, e.g., morphology, size, number, viability, apoptosis, cell cycle, surface antigens, and gene expression. Cultures can be initiated with cells derived from a whole unit of blood obtained from normal donors (or polycythemia patients undergoing phlebotomy). The starting material, i.e., the “buffy-coat” fraction, is

readily available, since it is routinely separated and discarded by the blood bank during preparation of packed red blood cells (RBC) and platelet-rich plasma. Anemic patients commonly show high frequencies of erythroid progenitors; cultures from anemic patients (e.g., thalassemia, sickle cell anemia) are usually initiated with cells taken of 20 ml as blood sample.

Peripheral blood cells are employed in this procedure for the following reasons: (a) the availability of peripheral blood; and (b) the homogeneity of the peripheral blood erythroid progenitors, namely early BFUe (Clarke and Housman 1977), as opposed to bone marrow, which contains progenitors at various developmental stages.

The culture procedure is divided into two phases is been described from Fibach. The first is an EPO-independent phase, where peripheral blood mononuclear cells are isolated and cultured in liquid medium supplemented with conditioned medium derived from the 5637 human bladder carcinoma cell line and cyclosporine that killing lymphocytes, helps to select the erythroid progenitor cells. This conditioned medium contains a variety of growth factors, not including EPO (Myers, Katz et al. 1984). During this phase, early erythroid-committed progenitors, called burst-forming units erythroid (BFUe), proliferate and differentiate into colony-forming unit erythroid (CFUe)–like progenitors. After one week in phase I, the cultures contain some adherent cells (mainly macrophages) and nonadherent cells (mainly lymphocytes). The nonadherent cells are harvested, washed and cultured in fresh phase II medium supplemented with EPO. In the absence of necessary cytokines to support their proliferation and differentiation, nonerythroid progenitors stop their development. As these cells multiply, they form clusters, then large aggregates reaching hundreds of cells. When these cells are addresed to differentiation, they decrease in size and accumulate hemoglobin, and the aggregates assume a reddish color. The globin expression in the maturing erythrocyte during the two-phase system mimics expression patterns, which occurs in ontogenesis with the fetal to adult hemoglobin switch. This is seen in the reciprocal relationship between γ- and β-globin gene expression, in which γ-globin content declines together a β-globin increasing in early phase II and then amount high levels in late phase II. As expected, α-globin also reaches maximum expression when total hemoglobin increases in the late stage (Pope, Fibach et al. 2000). This culture system has been used extensively for screening and studying drugs that can modulate the production of hemoglobins, in particular HbF (Fibach 2001). The distribution of the

cultured erythroid cell population, with respect to intracellular Hb, such as HbF can be analyzed by flow cytometry using monoclonal antibodies directed against specific Hb subtypes (Amoyal, Goldfarb et al. 2003). The Hb content of the cultured cells can be quantified by HPLC analysis (Huisman 1987). The globin mRNA can be quantified by various techniques including reverse transcription and real-time quantitative polymerase chain reaction (RT-PCR) (Fibach et al., 2003).

Given the importance of human hematopoietic stem cells (hHSCs), research is developing methods and land for their expansion and maintenance, aimed at their use in the medical field (Watanabe, Kawano et al. 1999).Good results can be obtained with cells derived from other sources, includind $CD34^+$ cells purified by immunomagnetic bead technologies.

Hematopoietic stem cells (HSCs) are isolated from peripheral blood by means of particular methods (such as the method MACS), able to recognize and select the receptor $CD34^+$ located on the cell membrane, allowing the exclusion of monocytes, macrophages, and of most of the lymphocytes and other cell types (Zuccato, Breda et al.; Watanabe, Kawano et al. 1999; Wang, Hsu et al. 2001). Starting from a peripheral blood sample of about 20-25ml, the two methods, successive and complementary, are applied to obtaining a cell culture as much as possible pure. The Ficoll Hypaque, is used to "separate" lymphocytes, macrophages, fibroblasts and erythroid precursors from the remaining blood's cellular component; the MACS method (Magnetic Activated Cell Sorting), which allowed us to obtain a population of $CD34^+$ cells as pure as possible (80-90%). In this step the cells are incubated with magnetic microbeads having on their surface small structures similar to receptors able to bind $CD34^+$ cells. Years ago several groups of researchers are developing various innovative protocols aimed at optimizing the selection of $CD34^+$ cells and their expansion *ex vivo*. In 1998 Malik et. al. had described a system for culturing erythroid precursors starting from an enrichment of $CD34^+$ cell (Malik, Barsky et al. 1998). Other important contributions were made to the field over the last 20 years by different groups who have developed a cocktail of factors containing stem cell factor (SCF), erythropoietin, and dexamethasone named SED, that can be used to induce extensive proliferation of a subset of erythroid progenitors (Carotta, Pilat et al. 2004; Dolznig, Kolbus et al. 2005; Leberbauer, Boulme et al. 2005). Olivier et al. recently has presented evidence that the SED cocktail could be used to induce proliferation of $CD34^+$ cells derived from hESCs (Olivier, Qiu et al.). In particular the last years by

Breda et al. has been proposed a different composition of the culture medium which represents a modified version of the medium developed by Leberbauer (Breda, Casu et al.2012; Leberbauer, Boulme et al. 2005). This medium provides that the isolated cells are maintained in two different types of culture medium. It is composed of a serum-free StemSpan containing bovine serum albumin (BSA), hunam recombinant, insulin and human transferrin (iron-saturated), 2-Mercaptoethanol, Iscove's Modified Dulbecco's Medium. At the medium is added a cytokine cocktail (Flt-3 Ligand, stem cell factor, IL-3, IL-6), EPO, dexamethasone and 1% penicillin and streptomycin. As described by Breda et al. in 2012, the advantage production of this method has is a huge number of undifferentiated $CD34^+$ cells that can be employed to different application i.e frozen, thawed, infected for gene therapy. The $CD34^+$ cells in this condition continue slowly their multiplication process reaching the maximum level of expansion after about 10 days maintaining the cells at a concentration of about $1x10^6$ cells/mL. If it's possible to control the status of erythroid differentiation. Several experiments have shown that maintaining the cells in close contact, reduces the time required for erythroid differentiation. After these 10 days the cells can be frozen (de Sio, Naldini 2009; Wang, Hsu et al. 2001) or used for experiments. At this point the cells that are still at a level of undifferentiation are transferred into phase II medium containing α-modified essential medium supplemented with 30% fetal calf serum and 10^{-5}M β-mercaptoethanol. Also in this step it must be used erythropoietin to stimulate erythroid differentiation. This protocol expansion and freezing of $CD34^+$ cells has been developed by (Breda, Casu et al.). To carry out the experiments of gene therapy with β-thalassemia lentiviral vector AnkT9W. Although the cells have undergone a shock temporary post thawing, it was possible to evaluate the increase of HbA following the lentiviral vector treatment.

The ability to cryopreserve different rates of $CD34^+$ cells derived from a single subject is a very tempting prospect for the entire scientific community. Have available rates of frozen cells harvested from patients affected by hematological disease, stimulates researchers to look more and more innovative applications to take advantage of these resouces to seal the deal it increasingly and help to renforce the core concept for developing 'ad personam' therapy.

4. Therapeutic approaches for β-thalassemia and sickle cell anemia

The hemoglobin disorders are an inherited genetic diseases, characterized by the reduction (β^{+}-thalassemia) or absence (β^{0}-thalassemia) of the β-globin chains in adult hemoglobin (Silvestroni and Bianco 1948; Clegg and Weatherall 1999). The shortage of β-globin chains is combined with an α globin chains free accumulation, this causes a red blood cells premature death in the bone marrow defined as 'ineffective erythropoiesis'. The rapid destruction of red blood cells is associated with splenic enlargement and skeletal changes. In fact, the bone marrow is subjected to an abnormal work to try to compensate the red blood cells loss. The many scientific advances aimed at the discovery of new therapeutic strategies have greatly improved the expectations and the life style of patients affectd by hemoglobin disorders.

In summary, we can identify three typologies of therapeutic approach shown below.

4.1 Definitive therapies

We find in this group some therapies aimed at solving the disease permanently including bone marrow and umbilical cord blood cell transplantation. About bone marrow transplantation (BMT), the first successful BMT for thalassaemia major was performed in 1982 and now over 1500 transplants have been performed worldwide with the most experience from Pesaro, Italy (Lawson, Roberts et al. 2003). Just in Pesaro, in 1989, following analysis of potential negative factors such as low quality of chelation therapy before the transplantation, the marked hepatomegaly and portal fibrosis, were established three classes of risk, in which splitting transplant patients and according to them, several protocols of action were developed (Lucarelli, Galimberti et al. 1998). Clearly, the essential prerequisite to make bone marrow transplantation is the availability of a compatible donor. After an ablative treatment of immunosuppression the patient is ready to be transplanted by infusion of bone marrow into a peripheral vein (Lucarelli, Andreani et al. 2002). Early after transplantation may occur rejection of organ transplantation. In this case the patient thalassemia will again be subject to undergo transfusion therapy. Another adverse event post transplantation is the appearance of a disease known as graft *versus* host disease (GVHD), which can occur in acute and chronic. In both cases, complications can occur in different anatomical areas leading to death (Robbins 1997). In this type of therapy it should

not be overlooked the harmful effects produced bydisease in the body patient before transplantation. The best outcome of transplant will have in enough young patients (Lucarelli, Andreani et al. 2002). However, often after the transplant it's necessary to put the patients to procedure of chelation therapy to remove all the iron overload in the vital organs. The patients can not be subjected to this treatment, can be treated with subcutaneous infusions of deferoxamine (Lucarelli, Andreani et al. 2002).

4.2 Maintenance therapies

As the term maintenance, this class of treatments tries to balance the clinical effects produced by the disease, but not solve them completely.

(i)Trasfusion therapy

With transfusions, its provides the amount of hemoglobin required to perform all body functions. The transfusions allow reduce the bone marrow expansion, the bone alterations and limit the spleen activity. Unfortunately, various drawbacks to the blood transfusion are correlated, both in terms of compatibility and safety (risk of viral infections such as the B and C hepatitis and HIV virus), but also with regard to the significant quantities of introduced iron (about 200-250 mg every 200 ml of transfused red blood cells) in the body causing toxicity due to formation of reactive oxygen species.In the body there are tissues having a high number of receptors for transferrin and those NTBI (not trasferrin bound iron) such as liver, heart and endocrine organs; transfused thalassemia patients are subjected to iron chelation therapy To limit this damage. In addition in some cases it is necessary performe surgical procedure as splenectomy. Unfortunately, often this leads to a weakening of the immune system, exposing patients to infections sometimes of severe entity.

(ii) Iron chelation therapy

Deferoxamine (Desferal®), patented drug in the ‘70s, is able to mask the six coordination sites of the iron. It is administered subcutaneously or intravenously, and has a 'short half-life in fact infusions are sustained for 8-12 hours, with the use of an infusion pump constant. This drug is not able to cross the cell membrane and binds iron only in the blood and bile. The chelated iron is excreted equally in urine and stool (Schrier and Angelucci 2005). In recent years, new iron chelators, orally active, were developed. Among these drugs, the most used is deferiprone (Ferriprox®), theoretically it has the potential to be more effective than

deferoxamine in removing intracellular iron from some tissues (Piga, Gaglioti et al. 2003; Shalev, et al. 1999). Moreover, a comparative study of the effects of the two chelators (deferiprone and deferoxamine) on survival and cardiac complications, showed greater activity of deferiprone on deposits of iron in the heart, thus improving the conditions of the organ. This action was attributed both to the ability of this molecule to enter into the cells and to its longer half-life and the opportunity to make an uninterrupted therapy (seven days a week), with more frequent doses (three *per* day) than that achieved with deferoxamine (Piga et al, 2003). It's possible to do combined therapy between deferoxamine and deferiprone, because they act synergistically respectively at cardiac and hepatic levels. The last generation (2007) of iron chelator drug is the Deferasirox (ICL670, Exjade®). It is taken orally with a single daily administration (Cappellini 2005) and has a high effectiveness reducing side effects with a relative long circulating half-life of 8 to 16 hours and documented cellular permeability.

Another method tolimit the amount of iron transfused is the "therapeutic erythrocytapheresis". A certain volume of blood containing a mix of both young and senescent erythrocytes is picked up to the patient. This is replaced with the same volume of blood from the donor, in which they were selected only the younger cells. In this manner, the cells survive for longer in the body of the transfused patient and there will be no direct transfer of senescent cells preventing a further iron accumulation in the spleen.

(iii) Drug therapies for the increase the erythroid differentiation and HbF induction.

Since a long time the properties of different molecules, natural or synthetic, are investigated about their ability to simulate the erythroid differentiation. Some of these molecules are already widely employed for the β-thalassemia and sickle cell anemia treatment, others there are in clinical trials. Furthermore, as reported in previous chapters, we have described as the switch from HbF to HbA ends in the sixth month of the child's life, but traces of HbF persist during adulthood (Rodgers and Sauntharajah 2001). Studies on patients with HPFH phenotype (High Persistence of Fetal Hemoglobin) has led to suggest an hypothetical new therapeutic approach based on the possibility to increase the γ-globin chains and HbF synthesis to compensate the deficit of adult hemoglobin in the anemic patients. An HPFH phenotype has been demonstrated that could make thalassemia patients less dependent by blood transfusion therapy and improves the overall clinical picture (Zuccato, Breda et al. 2012). In the **Table 4,** are

reported, the main molecules employed for induction of the erythroid differentiation and the expression of HbF, classified on the basis of the mechanism of action. We reported some examples in the following list of compounds.

Table 4 Principal HbF inducers and their mechanisms of action

INDUCERS	MECHANISM OF ACTION
Hydroxyurea	Ribonucleotide-reductase inhibitors
5-azacytidine	DNA hypomethylation
Cytosine-arabinoside (ara-C)	DNA hypomethylation
Butyrate	Histone deacetylase inhibitor
Tricostatina A (TSA)	Histone deacetylase inhibitor
Apicidine	Histone deacetylase inhibitor
Rapamycin, Everolimus	Protein-binding inhibitor
Mithramycin	DNA-binding inhibitor
Cisplatin and derivates	DNA-binding inhibitor
Chromomycin, Tallimustine, Distamycin	DNA-binding inhibitor
Angelicin	DNA-binding inhibitor
Resveratrol	Antioxidants
Erythropoietin	Hormone

a) Hypomethylating agents

Methyl-transferase inhibitors act on DNA hypomethylation, this mechanism cancels the downregulation of the synthesis of γ-globin chains. Already in 1982 it was observed that the 5- azacytidine, a cytotoxic drug, administered in mice stimulated an increase in HbF production. The drug was in clinical trials, but its use in therapy it is somewhat difficult due to possible carcinogenic effects. The 5-azacytidine and its less toxic harmful analogue 5-aza-2'-deoxycytidine (decitabine) were administered to β-thalassemia patients and the results were an increase of HbF (Curtin, Pirastu et al. 1985).

Another hypomethylating molecule with effect erythro-differentiating widely used in various cancer therapies is cytosine-arabinoside (ara-C). This drug works has a short plasma half-life but also to suboptimal concentrations it is able to induce a robust erythroid differentiation (Kufe DW 1985; Cortesi, Gui et al. 1998). To reduce the toxic effect it is often associated with retinoids (Cortesi, Gui et al. 1998).

b) Histone deacetylase inhibitors

It is now known as the histone deacetylase enzymes are responsible of the DNA wrapping and gene silencing. Studies on some molecules able to inhibit histone deacetylases have been shown to induce gene transcription. Butyrate and other compounds with small chains of fatty acids induce HbF inhibiting through histone deacetylase (Little, Dempsey et al. 1995). Butyrate-responsive elements (BRES) have been identified not only in the γ-globin promoter, but also in other genes (Glauber, Wandersee et al. 1991; Pace, Li et al. 1996). The effect of butyrate in the treatment of β-thalassemia patients seems to be optimal when the drug is not administered consistently (Perrine, Ginder et al. 1993; Sher, Ginder et al. 1995).

Other histone deacetylase inhibitors (HDAC) molecules are the Trichostatin A (TSA), an organic compound that serves as an antifungal antibiotic and Apicidin, a drug with antiproliferative action adopted in different cancer therapies. For these molecules are reported an ability to induce the HbF expression (Pufahl, Katryniok et al.; Witt, Monkemeyer et al. 2003).

c) Ribonucleotide-reductase inhibitors

Hydroxyurea is a myelosuppressive drug used for the treatment of various leukemias. This drug used in hemoglobin disease, has been shown to increase levels of HbF in a fairly satisfactory (Bradai, Abad et al. 2003; Alebouyeh, Moussavi et al. 2004; Watanapokasin, et al. 2006). The Hydroxyurea is used for its power to modify enzymes HDACs, which allows bone marrow cells to express again the γ-chain of HbF, however this drug has exhibited some cytotoxicity and transient effects and rather unpredictable.

d) DNA-binding molecules

Several drugs binding DNA in a sequence-specific manner have been identified. These molecules alter the binding of transcription factors to the promoter region silencing or activating the gene located downstream. Experiments *in vitro* on K562 erythroleukemia cells and on erythroid precursors have demonstrated how the chromomycin (Bianchi, Osti et al. 1999), tallimustine (Bianchi, Chiarabelli et al. 2001), the cisplatin and derivatives (Bianchi, Ongaro et al. 2000), analogs of

distamycin are able to stimulate an induction of erythroid differentiation and an increased HbF production (Bianchi, Chiarabelli et al. 2001). Additionally, other *in vitro* experiments have highlighted an increases of γ-globin mRNA accumulation and fetal hemoglobin (HbF) production following treatment with angelicin, an organic molecule of natural origin belonging to the furocoumarins family (Borgatti, Chilin et al.; Lampronti, Bianchi et al. 2003). In the category of drugs which bind DNA we find also mithramycin (Bianchi, Osti et al. 1999; Fibach, Bianchi et al. 2003) a very toxic antibiotic initially used exclusively as antineoplastic, but appears to be a a putative interesting molecules in the treatment of β–thalassemia, if use at low concentrations(Lozzio and Lozzio 1975; Fibach, Bianchi et al. 2003).

e) Protein-binding drugs

Rapamycin is an antibiotic its targeting a serine threonine kinase (mTOR, mammalian Target Of Rapamycin), which regulates the growth, proliferation, motility and cell survival. Several new cancer drugs are targeted to inhibit this enzyme. In particular, rapamycin binds the intracellular receptor FKBP12, inhibiting the mTOR signaling pathway. This mechanism induces a powerful effect on erythroid differentiation without inhibitis the proliferative activity of the cells. (Lozzio and Lozzio 1975; Rutherford and Harrison 1979; Gambari, del Senno et al. 1984; Mischiati, Sereni et al. 2004). On the same signaling pathway also acts Everolimus, a structural analogue of rapamycin with very low toxicity (Zuccato, Bianchi et al. 2007).

f) Antioxidants

We have seen in previous chapters as in thalassemia patients there is a extremely harmful iron accumulation. The overload of this metal in the body induces a strong oxidative stress. Resveratrol is an antibiotic naturally produced by several plants able to fight oxidative stress by reducing free radicals. It's been still conducted several studies on the numerous properties of this molecule, but it has already been demonstrated its ability to prolong the red blood cells life and to induce HbF production (Bianchi, Zuccato et al. 2009; Fibach, Prus et al. 2012), because the Resveratrol is easy available on the market it is necessary to play attention to the care do it yourself.

g) Hormones

Erythropoietin or EPO is a glycoprotein hormone produced by the kidneys in humans and to a lesser extent by the liver and the brain, which has as its primary function the regulation of erythropoiesis (production of red blood cells by the bone

marrow). The EPO was also produced in the laboratory and used as a drug to treat anemia in patients with kidney disease, blood diseases, and to allow a faster recovery after the administration of chemotherapy in cancer patients. The administration of EPO to β-thalassemia patients induces the synthesis of γ-globin during the maturation of erythroid precursor cells and, then the increase of HbF (Breymann, Fibach et al. 1999).

4.3 Gene therapy

Gene therapy has as its aim the care of a genetic defect, by using a DNA fragment containing the nucleotide sequence of the "correct" gene as a therapeutic agent. This concept has been applied in humans for the treatment of various genetic diseases, using molecular and cellular conbination therapies to "correct" DNA in the target cell by restoring the expression of the defective gene. In particular, the genetic strategies for the treatment of hemoglobinopathies, especially β-thalassemia and sickle cell anemia provide for the restore of the correct adult globin chains. For the achievement of this purpose could be proposed different experimental approaches.

a) *The gene transfer*

The gene transfer into hematopoietic stem cell is a technique widely used in animal models and, also, is present in clinical trials for several genetic diseases. It's possible use cells taken to the patient in order to eliminate all the problems related to the HLA compatibility. This strategy has four strengths: 1) the transfer of the single gene in specific hemopoietic stem cell, 2) the endogenous expression of the transgene at high levels, 3) the maintenance of the expression over time and 4- the use of not pathogenic vectors.

Initially to avoid the problems associated with the pathogenicity of the virus have been also tested non-viral vectors. Examples could be naked DNA, cationic lipid binding DNA through electrostatic interaction, and particles of condensed DNA. All these methods have proved ineffective for *in vivo* transfection; furthermore, the gene transfer results in transient expression of genes of interest.

The virus-mediated gene transfer seems to be more effective. After several approaches (eg. adenovirus, oncoviruses (Stathopulos 2003) was reached to the use of lentiviruses. The lentiviral vectors derived from HIV-1 and other lentiviruses have attracted great interest for their ability to transduce cells not actively dividing. They can transduce a broad spectrum of target cells, including neurons, retinal

photoreceptors, dendritic cells, macrophages, hepatocytes, and hematopoietic stem cells (HSCs). Another very important element is to be attributed to the lentivirus genome stability and to the good packaging capacity (Sadelain, Lisowski et al. 2005; Sadelain, Boulad et al. 2008). Has been also shown that incorporating in the lentiviral vector some LCR elements, it could be optimized the transgene expression (Sadelain, Lisowski et al. 2005). Some researchers have managed to obtain a new lentiviral construct containing the β-globin gene, the promoter sequences, enhancers and a proximal region comprising the elements LCR, including HS2, HS3 and HS4 sites. One of this vector is called TNS9 (Rivella, May et al. 2003). Using the TNS9 vector, the Sadelain's group have first demonstrated efficient gene transfer in bone marrow hematopoietic stem cells, tissue-specific transgene expression and long-term correction in a mouse model of β-thalassemia intermedia (Sadelain, Riviere et al.). Numerous studies concerned with identifying the incorporeted components into the lentiviral vector to guarantee a high, stable and robust expression. The element that has proved most effective is the insulator named cHS-4 from the chicken β-globin LCR, thanks to its ability to isolate the coding sequence by the influences of the surrounding chromatin. Moreover, the insulators have the additional aim to avoid the insertional oncogenesis, depending on the random integration of foreign genetic material, whether of viral or non-viral origin. This effect has been demonstrated in mouse models. A more recent study shown that a lentiviral vector expressing the human β-globin gene under control of its regulatory elements and flanked by chromatin insulator elements upon integration into host hematopoietic cells, has produced: (1) transduction at hight level on bone marrow progenitor cells from four patients, with transfusion-dependent thalassemia major, (2) complete phenotypic and functional correction of the in *vitro* model of human thalassemia erythropoiesis, (3) levels of β-globin similar to that derived from normal bone marrow progenitors, and (4) effective human erythropoiesis with circulating β-globin-producing human erythroid cells in genetically corrected xenografts, at levels comparable to normal bone marrow xenograft controls (Malik and Arumugam 2005). The successes achieved in mouse models in the treatment of sickle cell anemia and β-thalassemia intermedia have prompted researchers to further improve lentiviral vectors for gene therapy. It was constructed a new Lenti-Globin vector containing a β-globin gene nemed βA - T87Q, that produces HbβA-T87Q, which can be easily discriminated from normal hemoglobin as it has a mutation in codon 87 (Thr→Gln) of β-globin gene. This

vector contains a deletion in region 3'LTR and can function as SIN vector (self-inactivating); β-LCR elements; chicken cHS4 insulator elements which prevent the activation of genes adjacent the insertion (Rivella and Sadelain 1998).This vector is in Phase II clinical trial.

Ten patients (5 with β thalassemia and 5 with sickle cell anemia) were treated with this system by the Dr.Leboulch team in Paris (Bank, Dorazio et al. 2005; Bank 2006). A further lentiviral vector has been developed on the basis of the vector TNS9. This construct called T9W contains: a promoter of cytomegalovirus (CMV) in *5'*, a self-inactivating deletion in *3'-LCR*, post-transcriptional regulatory elements of the hepatitis virus (WPRE) located in cis. Some years ago, starting from the structure of the vector T9W it was built a new lentiviral vector, named AnkT9W, which carries the human β-globin gene both erythroid-specific ankyrin 5-'hyper-sensitive (HS) barrier insulator. (Imren, Fabry et al. 2004; Breda, Casu et al. 2012). The ankyrin elements allow stable integration of the transgene avoiding post-transcriptional changes. The vector AnkT9W in $CD34^+$ cells produced a significant increase of the HbA synthesis also, it was highlighted a correlation between increase of the HbA and integrated VCN (vector copy number). In the animal model AnkT9W showed a good improvement of β-thalassemia phenotype (Breda, Casu et al. 2012).

b) *The antisense strategies*

This is another type of gene therapy which allows the correction of the mRNA processing and can be used only with a particular set of mutations, referred top splicing sites. The antisense strategy is using oligonucleotides to modulate the transfer of informations from gene to protein altering RNA intermediate metabolism . It is possible to produce two different results depending on the type of oligonucleotide used and the target sequence recognized: gene silencing or through mRNA processing alteration. One key point of the mRNA molecules metabolism is the excision of introns, this reactions are specific for junction sequence and require the concerted action of many proteins, forming the splicesoma complex. Antisense oligonucleotides binding to the regions necessary for the junction can prevent the binding of factors required or physically prevent the cut reaction. Therefore, this implies, inhibition of mature mRNA production. Many researchers try to correct the effect of mutations using oligonucleotides, causing aberrant splicing to restore the normal gene function The oligonucleotides used to modify splicing are different from those used in silencing. In particular, they

should not activate RNase H, in fact this destroys the pre-mRNA target before being spliced, and should be able to compete effectively with splicing factors of access to the primary messenger, contained in the cell nucleus (Sazani and Kole 2003). Early studies, in which antisense oligonucleotides have been used to modulate splicing have provided for the use of these molecules to correct aberrant splicing of pre-mRNA of β-globin, caused by mutations in thalassemic disease (Dominski and Kole 1993). Another possible use of oligonucleotides can be the regulation of alternative splicing, stimulating or inhibiting the production of a variant protein (Mercatante, Sazani et al. 2001) and also the stimulation of the exon skipping, where the oligonucleotides can be used for promoting a process of alternative splicing, which avoids the inclusion of an exon defective in a mature mRNA.

c) *Gene targeting strategies*

The oligonucleotides used in the gene targeting strategy able to correct point mutations, include those that use triplex-forming oligonucleotides, RNA/DNA hybrid oligonucleotides (chimeraplasty) and small DNA fragments (SDFs), which are used in the small fragments homologous replacement (SFHR) strategy. The SDFs are composed of either single-strand (ssDNA) and double-strand DNA (dsDNA). These SDFs effect homologous exchange between incoming SDF sequences and endogenous (genomic or episomal) sequences resulting in phenotypic changes (Gruenert 2003). Gene targeting is the only procedure that can produce predefined alterations in the genome of eukaryotic cells. As such, gene targeting is an attractive approach to gene therapy of genetic diseases, because it can lead to the accurate correction of the defect in the target locus of interest. Also, this type of gene therapy it is not restricted by the size of the mutated gene, but the damaged portion need to be considered. Real correction of the defective gene should permanently block the genetic disease by restoring the normal production of the protein product; the corrected gene is under the control of its own regulatory sequences, and this avoids the problems related to a gene expression. Another element in favor of gene targeting is the limitation of the risk associated with an insertion mutation in proximity to an oncogene (Yanez and Porter 1998).

However in some cases have been observed some appeared mutations resulting from gene targeting. A potential disadvantage of gene correction using this method is the need to know the location and type of mutation to be corrected, and may

require the use of different therapeutic DNA for different patients presenting the same disease. While presenting numerous advantageous conditions, the biggest problem related to the use of gene targeting is its low transfection efficiency (Yanez and Porter 1998).

d) *Read-through strategy.*

This therapy approach is based on the continuation of mRNA translation to ribosomal level. Some patients are affected by a nonsense mutation leading to the production of a premature stop codon. This event produces a truncated protein (PTC) (Dominski and Kole 1993; Rivella and Sadelain 1998) (Salvatori, Breveglieri et al. 2009) (Salvatori, Cantale et al. 2009). Good results have been achieved in the treatment of mouse models and cell diseases such cystic fibrosis, Duchenne muscular dystrophy and hemophilia, doing hypothesize its use in the treatment of β-thalassemia, particularly in relation to mutation $\beta^0$39 (Altamura, Castaldo et al. 2013). Several molecules have been tested to verify if they could induce the PTC suppression, but those that were found to be the best have low molecular weight, in particular they belong to aminoglycoside antibiotic class. There are several human trials that are giving a positive outcome following treatment with aminoglycosides. These molecules have been an increase in the translation of mRNA containing PCT. Among the different aminoglycosides, appears to be the most used geneticin named G418. The general mechanism of PCT suppression by aminoglycosides is not completely clear, but it's known that their bactericidal effects as antibiotics, are due to inhibition or alteration of translation during the protein synthesis process. To date, the G418 is used both to conduct the *in vivo* experiments (Yang, Feng et al. 2007) and *in vitro*, for example as a reference molecule to test the read-through activity of other similar compounds (McElroy, Nomura et al.) and taken as model for the study and development of new aminoglycoside compounds exhibiting less side effects (Jung, Ku et al. 2011). For induction of the read-through will still use other aminoglycosides such as gentamicin and tobramycin. About the β-thalassemia, it must be considered that the most prevalent mutation is in Italy, a nonsense mutation, with a percentage of 70%, among affected persons, it's the β°39 mutation (Rosatelli, Dozy et al. 1992). In addition, there are many other stop mutations causing β-thalassemia in the world, So it's important to identifymolecules ever more effective and able to produce a lasting effect of correction with the stop mutation.

e) *Ribozyme*

A little hint talking about gene therapy should be made regarding the use of ribozymes. These molecules bind and degrade a specific gene transcription product. It has managed to achieve specific ribozymes for suppression of α-globin chains mRNA. The RNA degradation of this kind of globin-chain is a strategy to reduce the precipitation of α-globin tetramers thatcause serious damage to the erythroid precursors and could be very dangerous worsening for the clinical picture of thalassemia (Shen, Ikonomi et al. 1999).

f) *Using of iPSC* in *cellular therapy* Embryonic stem cells (hESCs) are pluripotent cells derived from ICM (Inner Cell Mass), can differentiate into several cellular types and, as result of injection into blastocysts, they can give rise to all tissues obtained from the three germ layers(mesoderm, endoderm and ectoderm) (Arora and Daley; Maekawa and Yamanaka; Fischbach and Fischbach 2004).

Embryonic stem cells could be used for the treatment of several serious diseases (congenital or acquired) such as Parkinson's disease, leukemia, hemoglobinopathies and spinal cord injury (Fischbach and Fischbach 2004). However, there are a number of ethical issues on the use of embryonic cells. One way around these problems is the generation of pluripotent cells directly from adult somatic cells, (which are cell differentiated) of the patient and executing one trans-transplant. These cells are called iPSC (induced pluripotent stem cells) and are pluripotent stem cells artificially generated and derived from a non-pluripotent cell (Arora and Daley; Okita, Yamakawa et al.). To date, several studies suggest that embryonic stem cells (hESCs) and induced pluripotent stem cells are very similar in many aspects, such as the expression of certain genes, the framework of promoter methylation and expression of specific markers for stem cells, the potential of differentiation (Arora and Daley 2012).

The first induced pluripotent stem cells (iPSCs) were generated in 2006 by Yamanaka et al, who used viral vectors (such as retrovirus and lentivirus) to transduce mouse fibroblasts using genes, which was found to be associated with stem cells and their pluripotency. Using different genes combinations Yamanaka concluded that the four key genes for the production of pluripotent stem cells were Otc-3/4, Sox-2, c-Myc and Klf-4. In fact, the crucial point is the amount of transcription that these genes are able to "produce" (Arora and Daley 2012; Maekawa and Yamanaka 2011).

Before you can use the iPSC such as cell therapy, it is necessary to differentiate them in the type of cells that we want to be able to perform a transplant. In the contest to develope a therapy for hemoglobinopathies, the usable cells could be the hematopoietic stem cells.To try this therapeutic approch it must be considered several issue:

- the way to obtain a sufficient number of hematopoietic stem cells to be transplanted starting from iPSC from (Watanabe, Kawano et al. 1999; Prelle, Zink et al. 2002).
- the difficulty of maintaining these cells in culture (Amit and Itskovitz-Eldor 2006).
- must be pay very attention to crop timing, because hematopoietic stem cells are not an immortalized line, but they undergo hemopoietic

These cells are really a great therapeutic resource, but the only limiting factor derived by the lack of the absolute Certainly that they generated the desired differentiated cells; in fact the iPSC could differentiate the back leading to generation of other specific kind of cell To the need to be absolutely Certain That These cells, once injected into the patient, give rise only to the Desired cell type.

g) *Gene editing strategies*

In recent years, is catching on an other kind of therapeutic genetic approach: the genomic engineering. The genome engineering exploits the natural mechanisms that the cell has for DNA repair in order to induce permanent changes in the target genome. So, the researchers must try to mimic the creation of a damage in the DNA double helix at the position where they want to introduce a change. When the genomic DNA is damaged, the cell tries to repair the damage by activating mechanisms that ensure the integrity of the genetic heritage of the cell. Usually, two mechanisms are used: *non-homologous end joining* (Lieber 2008) and *homologous recombination* (Kass and Jasin 2010). The first mechanism is active throughout the cell cycle, but it can induce mutations. The second is only active in the S and G2 phases when the chromatid brother is used as a mold to correct the DNA damage in a very precise way (SanFilippo J 2008). The mechanism non-homologous end joining can create mutations, adding or removing nucleotides, in the moment in which the two DNA ends are welded. Therefore, the researcher can use this mechanism to the targeted inactivation of a gene or by entering two points of damage in DNA can induce a deletion (Sollu, Pars et al.). However, some studies have shown that creating a lesion in the DNA double helix at a precise point and, simultaneously, providing exogenous DNA homologous to the target

site, the frequency of homologous recombination is far preferred over the non-homologous end joining (Rouet, Smih et al. 1994). This mechanism can be used to introduce desired DNA modification (Lombardo, Cesana et al.). To introduce the DNA lesion in a site-specific manner it should be used enzymes to cut the DNA site and exact dates. These enzymes, known as artificial nuclease, are dimers in which each monomer is composed of a portion able to bind DNA in a specific site and of a portion ale to cut the binded DNA. In particular, three are the most studied nuclease: the zinc finger nucleases (ZFNs), which have the cutting domain derived by FokI restriction enzyme and the DNA binding domain formed by 3-4 modules 'zinc finger' each capable of recognizing a triplet of DNA; nucleases with structure 'TALE', instead, have cutting domain linked to DNA binding modules, where a single module binds a single nucleotide and in general can also be present 18 modules in series; finally, we find the MEGA-nuclease having a very complex protein structure, in which the cutting domain is fused to the DNA binding domain. This property makes these nucleases inflexible and therefore not used to induce site-specific mutations for gene therapy. The nucleases most commonly used until today were the ZFNs. They are been numerous zinc finger modules able to recognize almost all the 64 possible nucleotide triplets allowing to have a lot of freedom in the choice of cutting site. To date, numerous studies have been conducted to limit the possible cutting errors in genomic regions. The cuts in the wrong regions, defined off-target, can cause deleterious effects in the target cell, such as gene inactivation, chromosomal translocations and oncogenes activation. To evaluate the more precise and safe artificial nucleases for use in gene therapy, many experiments have been carried out (Pattanayak, Ramirez et al.) and *in vivo* (Gabriel, Lombardo et al.) and there have been two very important elements: TALE nuclease are found to be much more specific in recognizing and cutting DNA than ZNFs and especially TALE were much less cytotoxic *in vitro* and in murine models (Mussolino, Morbitzer et al. 2011 (Tesson, Usal et al. 2011).

Recently the nuclease TALE have been engineered to create the site-specific cuts in the β-globin gene near a site of pathological mutation. The correction of the damage was later stimulated by inserting therapeutic DNA as a template, and we obtained a β-globin locus correction in 20% of the cells. (Lin, Fine et al.). TALE technology has been applied to iPSC of thalassemia patients, with stunning results; the correction of the mutation occurred in a specific manner without produces cases of off-target (Wang, Ye et al. 2012). In addition to the correction of

the β-globin gene, several other research teams are paying special attention to gene therapy directed to the γ-globin gene. The use of synthetic zinc-finger transcriptional activators (ZF-TF) (Honors in 2013, Ji Q 2014) and transcriptional activator-like effector (TALE-TF) can be very interesting for binding the γ-globin gene promoter. Some *in vitro* studies using $CD34^+$ cells have shown that the binding of these artificial nuclease at specific sites of the A γ-globin promoter produced an increase in γ-globin expression and HbF prodution (Wilber, Hargrove et al. (Graslund, Li et al. 2005) Costa, Fedosyuk et al.). The ZF-TF BCL11A was recently shown to function as a repressor of HbF expression (Roosjen 2014, Zhou 2010, Sankaran 2008). As has been shown in previous chapters, the process of globin switch from HbF to HbA is very complex. An elaborate network of researchers studying molecular activators and repressors must cooperate for developing strategies by engineering these mechanisms (ZFN or TALE) in order to control the various elements of these network, it could be activate transcription and correction of the globin genes eliminating or reducing the genetic cause of this disease.

h) *MicroRNA and thalassemia*

MicroRNAs (miRNAs) are a class of small non-coding RNAs that can regulate gene expression binding in a sequence-specific manner the target mRNA. Depending on the degree of compatibility between miRNAs and mRNAs occurs silencing of mRNA and the complete its degradation. Studies on the possible application of these small molecules in the treatment of β-thalassemia have been underway since some years. In 2001 Sankar et al. brought the first evidence that the over expression of two miRNAs, miR-15a and miR-16-1 (act as repressors of Myb) were directly correlated with increased of HbF levels. Even the over expression of another microRNA, miR-486-3p binding BCL11A, is able to produce an increased of HbF. Likewise, inhibiting the expression of miR-486-3 it occurs an activation of BCL11A and down regulation of the γ-globin expression (Lulli, Romania et al.). Other microRNAs resulting upregulate in association with γ-globin gene expression are: miR-210 (Sarakul, Vattanaviboon et al.; Bianchi, Zuccato et al. 2009), miR-26b (Alijani, Alizadeh et al.) and miR-451(Kouhkan, Soleimani et al.). In contrast, miR-96 has been identified as an inhibitor of the mRNA of the gene array globin (Azzouzi 2011). In the context of β-thalassemia, to control the accumulation of α-chainscould be interesting use microRNAs to regulate the

mRNA α-chain content. Studies have shown that miR-144 negatively regulates the alpha globin (Fu, Du et al. 2009).

Since all therapies so far described, innovative and not, we can see how the efforts of many researchers, in all examined areas, never stop to produce not only new therapeutic approaches, but also hope for all the patients affected by rare diseases. You can not define what is the safest and the most effective treatment, particularly the researchers have not yet reached at a summit of results, but certainly every day a lot of scientists are working to improve and optimize the treatments available to ensure to the patients a life style as much dignity as possible.

5. Collection of biological samples in Biobank and Biobanks around the world

In the last decades, researches have made very important goals in various medicine and pharmacology field. All knowledge, on the human genome and on the mechanisms involved in different diseases, have allowed to rebuild completely medical practices and to review classic treatment approaches. More and more frequently we talk about personalised medicine, which starts from specific characteristics of a patient in order to deliver the most appropriate therapy. Pharmacogenomics is a branch of personalised medicine which ideally allows each patient to have ‘tailor-made‘ treatments. However, until now, the use of pharmacology in clinical practice has rarely applied depending on many factors (Guttmacher and Collins 2003; Guttmacher, Porteous et al. 2007).

In particular, it would be very useful to use electronic health records (EHR) systems that integrate genomic data of each patient on a large scale (Kohane 2011). It would be possible to identify the best therapy for each patient by integrating all obtained data from an individual patient, and comparing them with the clinical information of other patients reported. There are several difficulties to create a truly personalized therapy (Bielinski, Olson et al.; Burke and Psaty 2007). Two major barriers are the availability of high quality samples with phenotype data and the validation in a timely manner of predictive genetic markers, in particular in the clinic context. The resolution of these problems could be represented by the establishment of a biobank (for example serum, tissues, DNA, RNA, cells) to have available biological samples of patients on which will be possible to carry out personalised clinical assay (Khleif, Doroshow et al.; Fiore and D'Avolio 2011). A

biobank is a systematic and organised collection of biological samples and all the information about the state of partecipant health (HEWITT 2011; RE 2011). In addition, it is essential to do genetic investigation doing DNA collection extracted from peripheral blood or buccal cells, for example. Detailed data regarding participants lifestyle (i.e. smoking, sporting etc.) can be very useful to evaluate all the influences and phenotype variations of the subjects (Kohane 2011). More informations are contained in the Biobank more scientists can evaluate how a small genetic variant could condition the phenotype. All informations and the biological samples contained in a biobank are strictly controlled by an Ethical Review Committee. All the people involved in the biobank data base necessarily have to sign an informed consent (McCarty CA 2005; Olson JE 2013).

Although the first forms of biobank date back to the 70s, the real increase in development occurred at the beginning of the new millennium. In fact, the need to have a large number of patients for epidemiological studies concerning different diseases has led to pool a lot of samples collections relatively to different contexts (eg. Universities, hospitals, pharmaceutical companies) and countries around the world.

There are different types of Biobanks now we describe some cases Biobank which are addressed to the study of specific variables and genetic disease-specific Biobanks (Riegman PH 2008; Terry SF 2011). In both cases, it would be very useful to monitor patients for a long period of time so as to could correlate the genetic variations to different prognostic factors. This can be very useful for prognostic patients stratification for the identification of therapeutic targets and for the best treatment choice.

Others Biobanks can also be defined as 'general'. This type is much less common than the disease-specific Biobanks. In this case participants are not selected on the basis of a specific disease and risk factors, but they are recruited on the basis of different mechanisms, type of work, the place where they live for example (i.e. UK Biobank) (Ollier W 2005). These general Biobanks are very flexible and can support several studies in relation to investigate the correlation among genotype, phenotype, and geographical area, or they can represent 'reference standards' for other studies (Davey Smith G and PR. 2005).

Independently of the typology, all Biobanks must respect very specific, and often restrictive, rules.

After signing the informed consent for a specific project, many participants decide to give consent to participate to future projects of which they do not know the details during recruitment. Different supervisory entities are involved to ensure the use of biological samples of participants for experimental purposes and always in compliance with the rules established by an ethical review committee. Also, ethics committees have to consider the researchers requests, protecting the participants privacy and establish the arrangements for treatment of all data derived from a clinical trial (O'Doherty KC 2011).

From an institutional point of view a Biobank requires some specific entities. The institutional review board (IRB) is the essential government entity of a Biobank. However, in most cases are used formal committees to approve the use of both biological samples and data (McCarty CA 2011; Olson JE 2013). These committees known as community advisory boards (CABs) are composed of expert members in the scientific, ethical and clinical areas. CABs revise scientific protocols, control the number of samples in the Biobank and the assessment of the results in the different trials (McCarty CA 2011; Haga SB 2013). Additionally, they interface with the participants and manage disclosures in scientific, academic and industrial partners. Essentially CABs are at the centre of communication between the different Biobank levels (participants, researchers, funders, etc.). An other essential component of a biobank is the scientific aspect.

CABs, as we said, control the scientific procedures, to standardize the experimental methods and evaluate the results obtained in laboratories. However, the results obtained must be validated in a Clinical Laboratory Improvement Amendments (CLIA), certified clinical laboratory. CLIA will validate all data obtained and will assess scientific methods to use (Wolf SM 2012). Only after verifying the experimental data, supervised by CLIA, it is possible to move forward to the scientific dissemination step controlled by CAB.

About 80% of Biobanks are actually based on public universities or national/regional authorities and have been set for both population-based studies and for research purposes specific to the disease. Most collections are small or medium and mainly consist of DNA, serum and whole blood and/or cellular tissue samples stored at different conditions, and different types of data associated including medical, demographic, genetic and environmental informations (Global Directory of Biobanks 2015). Is important to note that most of Biobanks are individual collections (ie not forming part of a network or partnership) with

database systems, which, in many cases, are closed. They are still very few Biobanks that are implementing policies for sharing samples both within the EU and all around the world (Laage-Hellman, Hansson et al. 2001; Cambon-Thomsen 2003). In this context, it is important to have a well-organized legislation regarding privacy and the needs of data protection between all the Biobanks involved. An important aim is to promote the network of Biobanks and maximize the benefits for public health, so at least a remarkable level of harmonization must be achieved. It would be appropriate to follow standard rules and scientific approaches (methods of sample collection, preservation, storage and data management). Experts have suggested the creation of a international (or at least European) organization network which establishes common operating procedures.

Biological samples correctly stored and easily identified through barcodes provide to the entire scientific community the opportunity to make enormous advances in the study of various diseases. Furthermore, considering the large number of patients participating Biobanks give the real possibility to find new therapies significantly reducing the time required for clinical trials of new molecules.

Existing Biobanks are very diversified and collect different types of samples: whole blood, plasma, serum, RBC(red blood cells), WBC (white blood cells), buffy coat, stem cells, bone and different cell lines. And yet buccal cells, DNA, RNA, proteins, different biological fluids such as urine, cerebrospinal fluid and amniotic fluid, solid tissues and samples from biopsies. In addition, these samples are collected from patients suffering from rare diseases or cancers such as basal cell carcinoma, bladder cancer, bone cancer, brain cancer, breast cancer, colorectal cancer, kidney cancer, ovarian cancer, pancreatic cancer, prostate cancer, lung cancer, melanoma, neuroblastoma, cancer liver and different types of leukemias and lymphomas. In addiction, several hospitals instituted with the aim of assisted fertilization provide a collection and storage service of embryos, oocyte and sperm. There are also Biobanks dedicated to patients with Alzheimer's, multiple sclerosis and other degenerative diseases. Should also mention the existence of Biobanks dedicated to plants and animals (Global Directory of Biobanks 2015)(**Appendix1**).

In the years this list is going to increase and we hope that Biobanks could help the researchers for developing new therapies and be able to improve the living conditions of the patients.

To date the scientific comunity has understood the importance of these structures so the member of Biobank are increasing considerably in a lot of country, because they have chosen to focus energies and funding for the development of new Biobanks.

The **Table 5** shows the amount of the principal Biobanks in the different countries. Unfortunately, you do not manage to have data sure 100% because the census of Biobanks in the world is complicated by the high variability of the samples collected in different parts of the world and by the lack of an official register. Moreover some Biobanks started initially as a little reality of research are subsequently developed as regular Biobanks.

Table 5 Distribution of main biobanks spread around the world.

Country	Number of Biobanks
Europe	126
Canada	18
USA	158
Mexico	1
Asia	25
Australia	13
Middle East	4

The situation in Italy does not differ so much from the European and global condition. In fact in the absence of a national registry is not easy to know the number of Biobanks (small and large) operating in Italy. There are eleven Biobanks well established, but we must add to these some realities, which are gradually consolidating its existence. In May 2014 the document of the National Committee for Bioethics was published on the website of the Office of the Prime Ministers (www.governo.it/bioetica), in which was approved the opening of "Pediatric Biobanks". They will deal with of the collection of the biological children samples for the purpose of scientific research. At present there isn’t a register in which shows locations of pediatric Biobanks.

As regards the thalassemias there are some Biobanks spread over several structures but which convey in a single entity, the Telethon Biobank (TNGB).

Within this network the different Biobanks perform different tasks. Some of these are concerned with collecting and storing DNA in order to conduct genetic investigations; others collect cell samples such as T-lymphocytes, fibroblasts and lymphoblasts, but with regard to their use and storage detailed information are not suitable. Specimen collection is implemented by the gathering of patient informations. The services offered by the Biobanks are various and available on the website http://www.biobanknetwork.org.

Other biobanks operating in thalassemia context might exist, but data and information are not reported, therefore it could be small companies not certified.

It's difficult to find funds to invest in research, the regulatory fragmentation and the lack of coordination at the central level are slowing down development of the biobanks.

If a European Biobank Networking Platform were successful in facilitating cooperation and creating a European biobanking logistics system, *"then it would, de facto, create or increase the long term security of existing national biobanks and biobanking infrastructures (the distinction is between an investigative project and a pure logistics operation). Such long term security is essential if the full value to molecular epidemiology of population-based and case-based studies is to be realised. If the....concept is developed through cooperation between existing biobanks and biobanking infrastructures, then that cooperation will itself help to lay the basis for a successful union that also can engage successfully with others in the biomedical community and will strengthen (rather than detract from) those existing organizations"* (Flyer 2006)

Now Biobanks can be considered a real cultural/scientific phenomenon of our decade. There are several areas involved, science, law, economics, and each must cooperate in order to allow the growth and the consolidation of these structures/organizations. Biobanks can provide a huge opportunity for development, research and knowledge and the beneficiaries will be all human beings and all future generations (An 2006).

It is now clear the decisive role of Biobanks to accelerate scientific discoveries. To maximize results it is important to dedicate attention to the quality of numerous samples collected in the Biobanks, the creation of a partnerships network and financial infrastructures. For the future it would be desirable to create a single European/national Biobank having a centralized single coordination center and in which all the methods of collecting clinical data, specimen collection and storage

are standardized. Finally, to facilitate the circulation of specimens and scientific data obtained it would be appropriate to create a single model of informed consent adaptable to different types of studies.

Aim of the thesis

The β-thalassemias are autosomal recessive genetic disorders caused by the absence or reduction of beta globin chains. Originally described in the Mediterranean area, to date β-thalassemias should be consisdered a worldwide pathology with more than 100,000 new cases annually with a high incidence especially in countries Africa and Central and Southeast Asia. The frequent and significant migration flows that have occurred over the decades have contributed irreversibly to the wide distribution of the disease. The β-thalassemia patients are treated with periodic blood transfusions associated to the assumption of iron chelating drugs and, when possible, with bone marrow transplantation (BMT). Despite scientific advances aimed looking for targeted and definitive treatments of the disease, to date a cure of β-thalassemia is not available.

My PhD research activity has been conducted in the context of a multicenter project called THALAMOSS (THalassemia MOdular Stratification System) for the determination of a personalized therapy of β-thalassemia. This project, which has as coordinator Prof. Roberto Gambari of the Ferrara University, was developed with the approval of the ethics committees of Verona-Rovigo and Ferrara Hospitals. THALAMOSS is aimed at the development of a computerized system that enables β-thalassemia patient stratification according to clinical, genetic and molecular features in order to implement a personalized therapy

In my study we have set the follows main steps:

a) Genotype analysis of the base β-globin mutation all patients enrolled in the trial.

b) Analysis of HbF-associate polymorphisms.

c) Production of cellular 'biobank' consisting of $CD34^+$ cells isolated from the peripheral blood from the β-thalassemia patients. This Biobank could offer many advantages for the patient, since multiple blood sampling are not further required and for research, since parallel comparison are possible.

d) Design and performance in-vitro experiments aimed at identifying molecules that can stimulate HbF production, already been demonstrated to be a very effective therapeutic strategy. This is not a resolutive approach of the disease but greatly improves the clinical status of the thalassemia patient.

e) Collection of information derived from all analyzes and experiments conducted in vitro to be combined with the clinical information for each patient.

This research acting will allow as a final goal patients stratification taking into account all the phenotypic/genotypic characteristics of the single individual and making it really possible the hypothesis of a personalized therapeutic approach.

Materials and Methods

1. Biological samples collection

For the biological samples collection has become necessary the approval of the Ethics Committee of Ferrara, project n°306201 approved on 20/06/2013 and the Ethics Committee of Verona-Rovigo district, project n°306201 approved on 30/07/2014.

The of peripheral blood samples collected from the patients from β thalassemia patients (before routine transfusion) and healthy subjects were obtained after informed consent with the collaboration of the 'Thalassemic Day Hospital' (DHT) of the Hospital and University Sant'Anna, Cona, Ferrara and of the DHT of Hospital Santa Maria of the Misericordia, Rovigo. Copy of each informed consent released to the patient at the time of blood sampling, in each of the two hospital structures is stored

2. ErPCs Isolation and culture

Peripheral blood (about 25 mL) were collect in Vacutainer LH treated tubes (BD Vacutainers, Becton Dickinson, UK)

The 25 mL total blood received has been divided into three parts:1 mL necessary for the genomic DNA extraction, 100 uL required for the HPLC analysis and remaining were used in immediate to $CD34^+$ cells isolation and purification.

The PBMCs isolation were obtained from whole blood by Ficoll-Hypaque density gradient centrifugation (Lympholyte®-H Cell Separation Media, Cedarlane, Euroclone, Italy). Spin at 2000 rpm for 30 min, at RT, max acceleration without brake. After the separation of the various blood components, the ring was harvested and washed with 1X DPBS W/O CA-MG (GIBCO, Invitrogen, Life Technologies Carlsbad, CA, USA)

At this point we have applied two different methods.

Two-phase liquid culture (protocol A): the cellular pellet obtained by Ficoll Hypaque separation was resuspended in the medium containing α-minimal essential medium (α-MEM, Sigma-Genosys, Saint Louis, Missouri, USA), prepared from a powder and diluted with water; a solution of PEN-STREP (PEN-STREP 10000 U/mL, Lonza, Verviers, Belgium); 10% FBS (Celbio, Milano, Italy); 10%

conditioned medium (CM), obtained from cell cultures of bladder cancer cells (5637); 1 ug/mL of cyclosporin A (Sigma-Aldrich), prepared from cyclosporine absolute ethanol and diluted in 1X DPBS (GIBCO), in the ratio 1:1. After 7 days in phase I culture, the nonadherent cells were washed once with 1XDPBS (GIBCO), and then cultured in phase II medium. This medium contains: α-MEM (Sigma Genosys), 30% FBS (Celbio), 1% deionized bovine serum albumin (BSA, Sigma Genosys), 10^{-5}M β-mercaptoethanol (Sigma Genosys), 2mM L-glutamine (Sigma Genosys), 10^{-6}M dexamethasone (Sigma Genosys), and 1 U/mL human recombinant erythropoietin (EPO Tebu-bio, Magenta, Milano, Italy), and stem cell factor (SCF, BioSource International, Camarillo, CA, USA) at the final concentration of 10 ng/mL.

Biobank protocol for lhe $CD34^+$ isolation and purification (Protocol C): cells Isolation of hematopoietic progenitor cells by $CD34^+$ MicroBead Kit using LS MiniMACS column placed in the magnetic field of the autoMACS Separator (both from Miltenyi Biotec, Bergisch Gladbach, Germany) according to manifacture's protocol. All steps from this moment onwards must be conducted strictly in ice. Briefly: Add 100 uL of $CD34^+$ MicroBead at the cells PBMCs obtained from separation by concentration gradient. The cellular pellet was resuspended in 600 uL of PBS 1% BSA (bovine serum albumin) solution. Subsequently were added 100 uL of $CD34^+$ microbeads. The tube containing the cell suspension and the microbeads should be kept in the dark and incubate with gentle agitation at 120 rpm for 15 min at 4°C. It's necessary equilibrate the columns for MACS separation before use. Work in sterile conditions in biohazard cabine, where you will use the unit magnet autoMACS. Apply 500 ul of $CD34^+$ MicroBead binded cells onto the column until exhaustion of the sample by alternating of washing with Beading Buffer

To increase the purity of $CD34^+$ cells, the eluted fraction can be enriched over a second LS Column. Repeat the magnetic separation procedure as described by using a new column (Wang, Hsu et al. 2001). Centrifuge the cells at 1200 rpm, at room temperature for 5 min, remove the supernatant and resuspend the cells in 5 mL of growth medium and transfer in a T25 flask.

The $CD34^+$ cells were maintained in an expansion medium containing: 5 mL of medium StemSpan Serum-Free Medium Expansion (VODEN, Vancouver, Canada); 50 microliters StemSpan® CC100 Cytokine Cocktail for Expansion of Human Hematopoietic Cells Stem Cell Technologies (VODEN); erythropoietin

(EPO, Tebu-bio) 2U/mL final concentration; dexamethasone (Dexamethasone 21-phosphate disodium salt, Sigma-Aldrich) 10^{-6} M final concentration, 50 microliters PEN-STREP (Lonza). During the expansion phase, the cells were diluted to maintain an ideal concentration of $1x10^6$cells/mL.

3. Freezing

Achieved the maximum expansion, the $CD34^+$ cells were frozen following the Naldini method's: IMDM (Iscove's modified Dulbecco's Medium, Life Tecnologies) with 50% FBS (Celbio, Milan, Italy) and 10% DMSO (Dimethyl Sulfoxide RPE-ACS, Carlo Erba, Italy). The cells in freezing medium preferably about $5x10^6$ cells in 1mL) were immediately placed at -80°C and subsequently stored in liquid nitrogen (-147°)

4. Thawing

The cells were thawed in 5 mL of IMDM (Life Tecnologies) 10x 5% FBS medium and incubated at 37°C with controlled humidity with 5% CO_2 in expansion medium in according to the protocol C.

5. Benzidine staining

The benzidine assay allows to evaluate erythroid differentiation. 5uL of 0.1mM benzidine, resuspended in 2.86 % glacial acetic acid and activated with 33% H_2O_2, were added to the same volume of the cells. The percentage of resulting blue cells indicates the level of erythroid differentiation.

6. Treatment with HbF inducers*.*

About four days after thawing the $CD34^+$cells have been treated with HbF inducers using a concentration previously tested on erythro-leukemic K562 cells. We used $2/3x10^6$ cells for RNA extraction to T^0. We have tested the cells with different types of HbF inducers: Mithramycin (Sigma-Aldrich), Hydroxyurea (Sigma-Aldrich), Rapamycin (Calbiochem, Merk Millipore, San Diego, CA, USA), Resveratrol (Sigma-Aldrich). At the end of the treatment we have performed another benzidine assayto test erythroid differentiation after treatment. Then the cells were washed twice with1X DPS (GIBCO). From the pellet obtained we have done haemoglobin analysis by HPLC and transcript analysis by RNA extractionand RT-qPCR

7. RNA extraction

All reagents and materials used were RNase–free. The cells (5-10x10^6) were centrifuged at 1,200 rpm for 5 minutes at 4°C, from the pellet obtained the total cellular RNA was extracted by TRI Reagent® (Sigma-Aldrich). After incubating for 3 minutes at room temperature, 200 uL of chloroform were added, stirring well, and incubating on ice for 5 minutes. The samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C. To the collected aqueous phase an equal volume of isopropanol was added. The samples were then placed at -80°C for at least 1 hour. The RNA precipitate was centrifuged at 12,000 rpm for 20 minutes at 4°C, washed with 500uL of cold 70% ethanol, re-centrifuged, freeze-dried and resuspended in 20uL of water RNase–free. The extracted RNA was analyzed by electrophoresis on 0.8% agarose gel.

8. Synthetic oligonucleotides

The oligonucleotides used as primers in the chain polimerization and sequencing reactions were synthesized by Sigma-Genosys (Cambridge, UK) (**Table 6**), while those used for the Real Time quantitative PCR were purchased from Applied Biosystems (Applera Italia, Monza, Italy). All the oligonucleotides were designed using the software Primer ExpressTM, version 2.2 (Perkin-Elmer Applied Biosystems) and the sequences are reported on **Table 7**.

9. RT-PCR and RT qPCR analysis

For gene expression analysis 1 µg of total RNA was reverse transcribed by using the TaqMan® Reverse Transcription Reagents and random hexamers (both Applera Italia, Applied Biosystems, Life Tecnolgies).

Quantitative real-time PCR assay, to quantify the expression of the globin genes, was carried out using using gene-specific double-quenched probes. Probes are labeled in 5’ with different fluorochromes, whereas in 3’ the BHQ (Black Hole Quencher™) is present.Reaction mixture contained 1X iQTM Multiplex Powermix (Bio-Rad, Hercules, California, USA), 300 nM forward and reverse primers and the 200 nM probe. The assays were carried out in iCycler IQ5 (Bio-Rad). After an initial denaturation at 95°C for 30 sec, the reactions were performed for 50 cycles (95°C for 10 s, 60°C for 45 s). To compare gene expression of each template amplified was used ΔΔCt method employing software IQ5 (Bio-Rad).

Table 6 Primers used in the polymerase chain reactions and in the sequencing reactions. For each primer the nucleotide sequence, Melting temperature (Tm) and the orientation of the primer have been reported.

Primer	Sequence (5'-3')	Tm (°C)	Orientation	Use
BGF	5'-GTGCCAGAAGAGCCAAGGACAGG-3'	72,1	forward	PCR and sequencing reactions.
T12F	5'-AGACCTCACCCTGTGGAGCC-3'	67,9	forward	sequencing reaction
T3F	5'-ACAATCCAGCTACCATTCTGCTTT-3'	65,7	forward	PCR and sequencing reactions.
BG6F	5'-CGCTTTCTTGCTGTCCAATTTC-3'	66,7	forward	sequencing reaction
BG5SF	5'-GCCTGGCTCACCTGGACA-3'	66,7	forward	PCR and sequencing reactions.
BG4	5'-TCAGGAGTGGACAGATCCCC-3'	66,5	reverse	sequencing reaction
T12R	5'-AGTTCTCAGGATCCACGTGCA-3'	67,1	reverse	PCR and sequencing reactions.
BGR	5'-CACTGACCTCCCACATTCCCTTTT-3'	69,8	reverse	PCR and sequencing reactions.
BGi2R	5-'GTTGCCCAGGAGCTGTGG-3'	67,1	reverse	PCR and sequencing reactions.

Table 7 Primers and probes employed in the multiplex quantitative real-time PCR. The 5' and 3' chromogenic molecules are also reported in the sequence.

Primer	Sequence (5'-3')
forward primer α-globin	5'-CGACAAGACCAACGTCAAGG-3'
reverse primer α-globin	5'-GGTCTTGGTGGTGGGGAAG-3'
α-globin *probe*	5'-HEX-ACATCCTCTCCAGGGCCTCCG-BHQ-3'
forward primer β-globin	5'-GGGCACCTTTGCCACAC-3'
reverse primer β-globin	5'-GGTGAATTCTTTGCCAAAGTGAT-3'
β-globin *probe*	5'-Texas Red-ACGTTGCCCAGGAGCCTGAAG-BHQ-3'
forward primer γ-globin	5'-TGACAAGCTGCATGTGGATC-3'
reverse primer γ-globin	5'-TTCTTTGCCGAAATGGATTGC-3'
γ-globin *probe*	5'-FAM-TCACCAGCACATTTCCCAGGAGC-BHQ-3'
forward primer RPL13A	5'-GGCAATTTCTACAGAAACAAGTTG-3'
reverse primer RPL13A	5'-GTTTTGTGGGGCAGCATACC-3'
RPL13A *probe*	5'-CY5-CGCACGGTCCGCCAGAAGAT-BHQ-3'

10. High Performance Liquid Chromatography (HPLC)

The cells pelleted and washed once with PBS1X after the treatment (Gibco, Invitrogen, Life Technologies) were lysed in water with cycles of freezing-thawing in dry ice. Hb proteins present in the lysates were separated by cation-exchange HPLC, utilizing a Beckman Coulter instrument System Gold 126 Solvent Module-166 Detector. Hemoglobins were separated using a Poly CAT-A column, samples were eluted in a solvent gradient utilizing aqueous sodium chloride–BisTris–KCN buffers and detection was performed at 415 nm. The standard controls were the purified HbA (Sigma-Aldrich) and HbF (Alpha Wassermann, Milano, Italy).

11. Genomic DNA Extraction

The DNA was extracted from 200-300 mL of whole blood using QiAmp DNA Mini Kit & QiAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to manifacture's protocol. Two DNA elutions were carried out, each with 50uL of AE buffer followed each by a incubation of 5 minutes at room temperature to increase the yield of DNA and centrifugation for 1 minute at 8,000 rpm always at room temperature. The DNA obtained was stored at -20 ° C

12. Agarose gel electrophoresis

The genomic DNA extracts were analyzed by electrophoresis on agarose gel 0.8%. The agarose powder was dissolved in 100 mL of 1X TAE buffer (0.04 M Tris-Acetato, 0.001 M EDTA pH 8), boiling until the agarose melt completely. The solution were cooled and 0.5 µg/mL ethidium bromide was added. The gel was done solidify for about 30 minutes in a clean casting plate with an appropriate comb and then immersed into 1X TAE buffer. 1 uL of loading buffer (0.25% Orange G, 50% glycerol in TE) was added to each sample before being loaded. On both the sides of the gel a molecular weight marker was added. 1 uL of 1kb DNA Ladder (10,000, 8,000, 6,000, 5,000, 4,000, 3,500, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500, 250 bp) (MBI Fermentas) or pUC Mix Marker 8 (1,116, 883, 692, 501, 489, 404, 331, 242, 190, 147, 111, 110, 67 bp) (MBI Fermentas) was up to the loading volume with water. The electrophoresis was performed at 80 Volt. The DNA was visualised using UV rays and then photographed with a Polaroid camera.

13. Quantification of DNA spectrophotometer

The spectrophotometric quantification was performed using the spectrophotometer SmartSpecTM Plus (BIORAD, Hercules, California, USA). Each quantification required from 0.5 to 1 uL of genomic DNA, in order to determine an optical density at 260 nm (OD260 nm) of 0.1 to 1.0: after deducting the value of optical density of the blank, the concentration of the extracted DNA was calculated considering that 1 OD260 nm corresponds to the concentration of 50 ng/uL. For each sample was also measured the optical density at 280 nm to determine the possible contamination of protein molecules, taking into account that the ratio OD260nm/OD280nm of the pure DNA is between 1.8 and 2.0.

14. Polymerase Chain Reaction (PCR)

The polymerization reaction chain is used to amplify a specific genomic sequence, in our case a specific region of the β globin gene. Each reaction was carried out in a final volume of 100 uL, in the presence of 300 ng of genomic DNA, 1X buffer (10 mM Tris-HCl pH 8.8, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100), 33uM dNTPs, 2U/uL of DyNAzyme^{TM}II DNA Polymerase (Finnzymes, Espoo, Finland), 0.25uM forward and reverse primers and ultra-pure water. Each reaction was subjected to an initial denaturation step at 96°C 3 minutes, followed by a second phase of 35 thermal cycles of amplification, each consisting of three steps: 30 seconds at 95°C for denaturation of DNA, 20 seconds at 65°C for the pairing with the filaments denaturing of the template DNA (the temperature varies according to the Melting Temperature of the primers used), 1 minute at 72°C for the elongation of DNA strands on the copied mold on the part of 'enzyme. The third and final stage consists of 10 minutes at 72°C to complete the process of elongation of all the filaments obtained.

15. Purification of PCR products with MicroCLEAN (Microzone Limited)

PCR products purification was carried out to remove the unincorporated PCR primers before sequencing. An equal volume of MicroCLEAN (Microzone Limited) reagent was added to each PCR product, then, after carefully mixing and incubating for 5 minutes at room temperature to allow DNA precipitation, this was collected by a first centrifugation at 13000xg for 10 minutes and then a second one for 1 minute to be able to remove all the supernatant. Finally, the desired volume of ultrapure water was added, before incubating at room temperature for 10

minutes and then mixing to allow the complete DNA dissolution. The purified samples were stored at -20°C until sequencing.

16. Sequencing Reaction: "Sanger"method

The reaction sequencing allows us to analyze the nucleotide sequence of the DNA and then to be able to evaluate the presence of β-globin gene mutation.

The PCR products purified obtained were sequenced according to Sanger's method (Sanger et al. 1977) with primers reported in **Table6** order to identify a possible pathogenic mutation. Sequence reactions were performed in a final volume of 20 uL, containing 60 ng of PCR template, 3.2 pmol of sequencing primer and ultrapure water to a 12uL volume. Then 8 uL of Terminator Ready Reaction Mix of ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies), was added, containing the four differently-labeled dideoxyribonucleotides (ddNTPs), the AmpliTaq® DNA polimerase, MgCl2 and Tris-HCl buffer at pH 9.0. A total of 45 amplification cycles were performed, as follows: denaturation, 96°C, 10s; annealing, 65°C, 5s; elongation, 65°C, 3min.

The reaction products were then purified from unincorporated ddNTPs by using a 96-well MultiScreen™ (Millipore Corporation) plate: after loading on pre-hydrated wells filled with Sephadex™ G-50 Superfine (Amersham Biosciences), samples were recovered by centrifugation at 900xg for 6 minutes and dried under vacuum.

Sequencing was finally performed by BMR Genomics (Padua), while the obtained sequence data were analyzed by the Sequence Scanner, version 1.0 (Applied Biosystems), software.

Results

The cellular Biobank is a collection of cryopreserved erythroid progenitor cells isolated from β-thalassemia patients. These patients can be stratified on the basis of molecular, genetic and clinical informations. The patients stratification offers the possibility of implementing a new therapeutic approach in which, on the basis of the specific characteristics of each one, we develop the formulation of 'ad personam' treatment.

1. Sample collection

All the subjects, who have contributed to the establishment of the cellular Biobank, gave their positive consent in total freedom and without any remuneration. The collection of the biological samples was necessary approved by the Ethics Committee of Ferrara, project n°306201 on 20/06/2013 and by the Ethics Committee of Verona-Rovigo district, project n°306201 on 30/07/2014.

The blood sampling were done before the routine blood transfusion of the β-thalassemia patients and healthy subjects, in collaboration with the 'Thalassemic Day Hospital' (DHT) of the Hospital and University Sant'Anna, Cona, Ferrara and of the DHT of Hospital Santa Maria della Misericordia, Rovigo. Copy of each informed consent released to the patient at the time of blood sampling, in each of the two hospital structures was stored.

The cellular Biobank that we set up to date is composed of 75 subjects **(Appendix2).** Most of them are β-thalassemia patients and only a small fraction (10 subjects) is composed of healthy subjects. The participants recruited by these two health center come from different areas of the Veneto, from the Ferrara Province and in sporadic cases from non-EU areas (individuals immigrants). The average age of the subjects is about 40 years with a fairly heterogeneous distribution ranging from 14 years to 65 years. Approximately 54.2% of the participants are women.

As it is clearly apparent from the graph in **Figure 6** the genotypes mainly present are those most common in the geographic areas of the individuals. In particular, the genotype $\beta^0$39/$\beta^0$39, β^+IVSI-110/$\beta^0$39 and β^+IVSI-110/β^+IVSI-110 are present with a percentage of 30, 22% and 11% respectively. On the contrary, other genotypes are poorly represented, demonstrating the high distribution of $\beta^0$39 and

β^{+}IVSI-110 mutations in the Emilia Romagna and Veneto areas from which the subjects are originating.

At the moment of the blood sample collection necessary for the isolation, expansion and the following freezing of $CD34^{+}$ cells, for the implantation of the Biobank, two other small volumes of blood are stored in parallel that will be used for DNA extraction and HPLC analysis conducted directly by lysing the blood sample.

A

Genotype	Subjects number	Vials containing precursor cells
$\beta^0$39/$\beta^0$39	22	240
β^+IVSI-110/$\beta^0$39	16	191
β^+IVSI-110/β^+IVSI-110	8	81
β^+IVSI-6/$\beta^0$39	4	67
β^+IVSI-6/β^+IVSI-6	2	15
β^+IVSI-110/β^0IVSI-1	1	7
$\beta^0$39/$\beta^0$44	1	10
β^+IVSI-110/β^+IVSI-6	1	11
$\beta^0$39/$\beta^0$28(+C)	1	10
$\beta^0$39/β^+5'UTR+20nt	1	27
HbS/$\beta^0$39	1	7
HbS/β^+IVSI-110	1	5
HbS/β^+IVSI-6	1	7
HbS/β^0IVSI-1	2	20
HbS/HbS	2	13
wt/wt	11	48
Genotype	**Subjects number**	**Vials containing precursor cells**
16	**75**	**749**

B

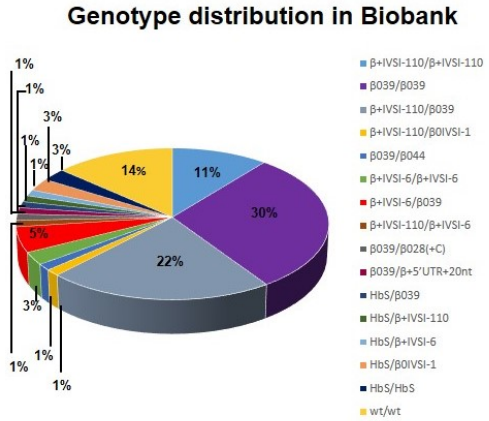

Figure 6 Genotype distribution. A) Here it's reported a table containing the genotype, the subject number having the specific indicated genotype and the vial number respectively stored in the Biobank. B) It's graphicated the pie chart reproducing the distribution (expressed as percentage) of the patient genotype represented in the cellular Biobank.

Regarding the genotyping of each patient, our laboratory at the Ferrara University was full responsible for sequencing all the β-globin gene **(Figure7)**, while the analysis of some HbF related polymorphisms were carried out at the Regional Hospital for Microcythemia in Cagliari.

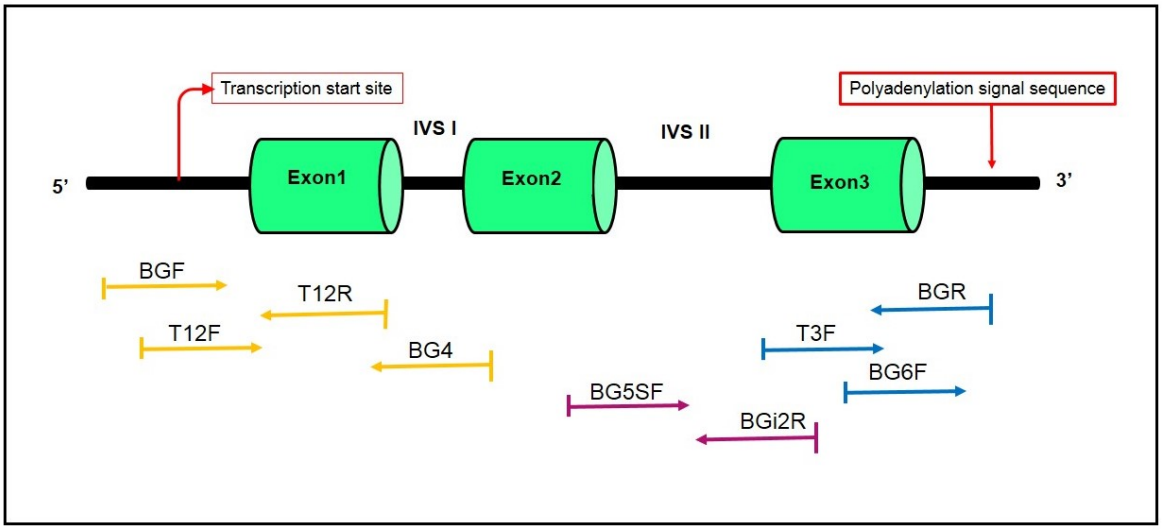

Figure 7 Primers used for genotye characterization of β-globin gene of the patients involved in the Biobank production. A schematic representation of the primers located in the β-globin gene and used in PCR and sequencing reactions. Primers of the same color can be used in pairs.

In particular polymorphisms *rs*1427407 (G/T), *rs*10189857 (A/G) were investigated for BCL11A. Also the XmnI polymorphism *rs*74482144 (+/-) in position -158 G-γ promoter and polymorphism HBS1L-Myb rs9399137 (T/C) located on chromosome 6q23.3 were analysed **(Figure8)**.

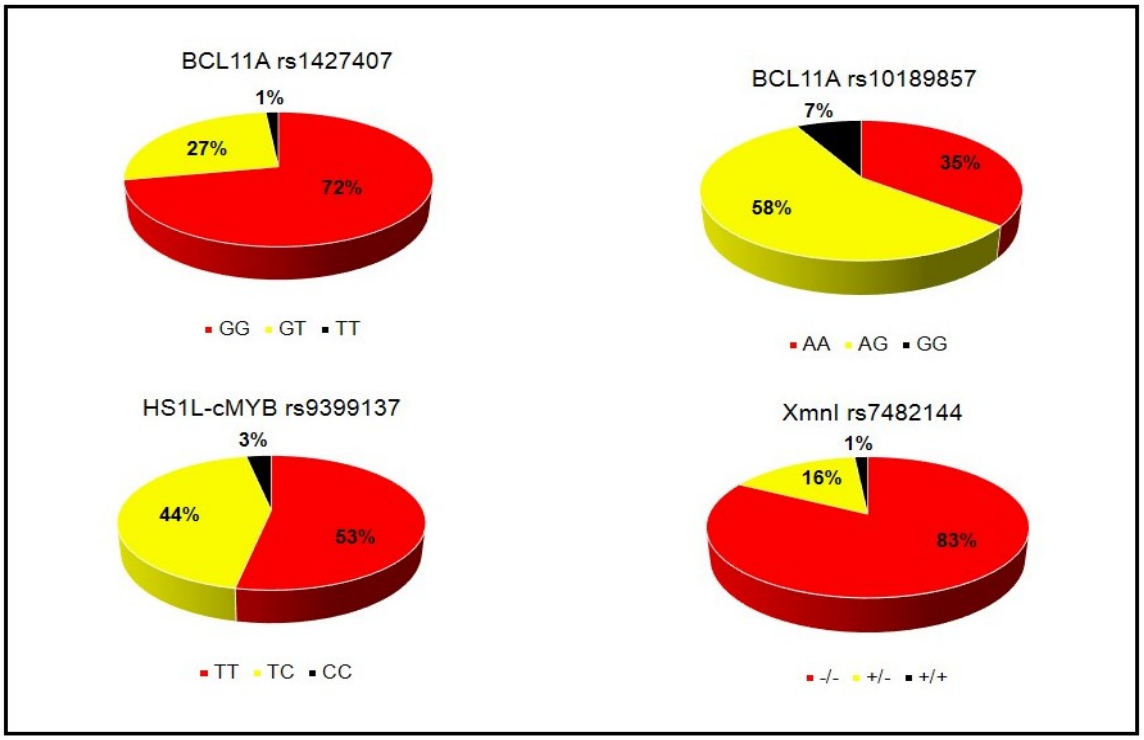

Figure 8 Polymorphisms associated with high HbF levels. The graphs shows incidence percentage for each polymorphism investigated in the β-thalassemia patients involved in the cellular Biobank. The homozygous phenotype not associated with high HbF levels (red), heterozygous (yellow), and homozygous phenotype associated with high HbF levels (black) are shown.

In particular about BCL11A *rs*1427407 (G/T), the 28% of the Biobank patients adherents are HbF related polymorphism carriers and only 1% have an homozygosity "T/T" condition. The *rs*10189857 polymorphism, instead, is present in 65% with 7% homozygous in the investigated patients. So *rs*10189857 (A/G) is BCL11A polymorphism more prevalent among the patients who joined the Biobank production. The HBS1L-Myb *rs*9399137 (T/C) polymorphism was found in 47% of cases analyzed with a 3% of C/C homozygous patients Finally, 17% of patients collected in Biobank are carrier of the XmnI *rs*74482144 (+/-) polymorphism and only 1% is homozygous T/T; thus this polymorphism is less frequent in the geographical area where the Biobank samples are representative.

2. $CD34^+$ cells: isolation, expansion and freezing

Each person who participated in the project was expected to donate about 20-25 ml of peripheral blood. At this point, we applied two complementary techniques in succession to obtain a selection of $CD34^+$ cells.

-Ficoll Hipaque. This phase allows to separate the different components of whole blood according to the density gradient. In particular, we isolated lymphocytes, macrophages, fibroblasts and erythroid precursor cells from the remaining cellular blood components.

-MACS Method (Magnetic Assay Cell Sorting): this method uses magnetic microbeads having a surface structure that can recognize and selectively bind $CD34^+$ cells. This step allows to set up very pure cell culture.

For the development of the expansion medium for optimal growth of $CD34^+$ cells a protocol described for the first time by Leberbauer was employed. This medium was later modified by Breda and Rivella, who have employed it in studies for β-thalassemia treatment with the gene therapy method. This medium contains StemSpan Serum-Free Medium Expansion, StemSpan® CC100 Cytokine Cocktail for expansion of human hematopoietic cells stem cell, erythropoietin (EPO), dexamethasone 21-phosphate disodium salt and PEN-STREP.

We have firstly evaluated the ability of this medium to stimulate the cell growth without influencing their natural differentiation state; then we decided to use it during $CD34^+$ cells expansion phase and after thawing.**(Figure9)**

The number of cryovials that can be obtained for each subject with this method starting from a simple peripheral blood collection was found to be very variable.

We have noticed that in the various stages of the Biobank setting up some parameters considerably influenced the successful of the cellular expansion.

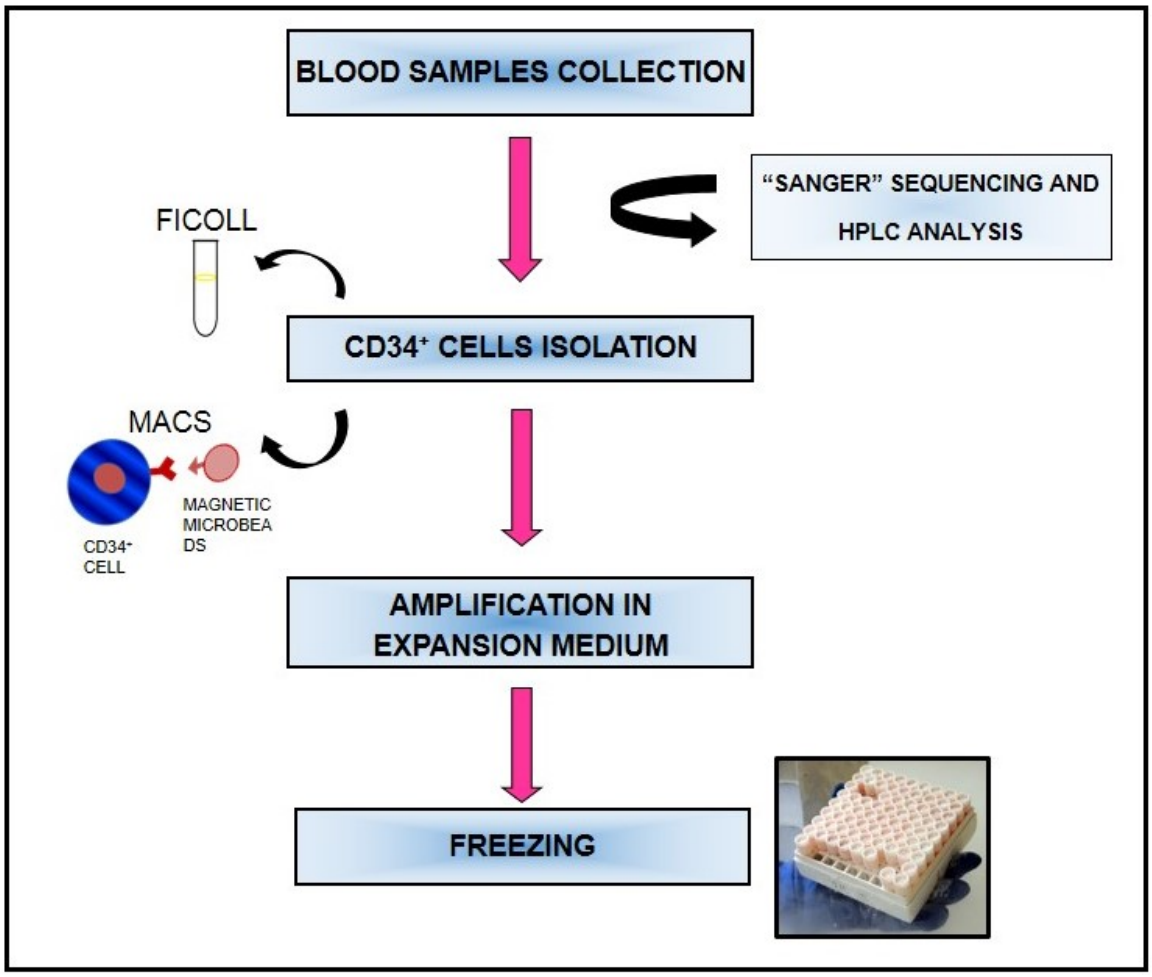

Figure 9 Purification and production of the cell collections. 20-25 mL of blood from each subject are used to purify the $CD34^+$ cells by ficoll and MACS separation. Than, the cells are amplified in expansion medium. Finally, the cells are frozen and stored in the Biobank when they reach the maximum progenitor cell expansion. Also, with a little amount of material we make sequencing reaction and HPLC analysis.

The clinical condition of the patient and the time that elapses from blood withdrawal until the start of the blood processing are two very important factors. We also have noticed that it is very difficult to determine the timing and standardize the experimental approach in order to obtain same responses from all samples employed. The cells of each patient, in fact, grow following its own timeline. The ability and experience of the operator are essential to understand what will be the trend of the culture and stimulate the growth of cells at the time and in an appropriate way.

Overall we can say that after the purification step from the blood we obtain only a few hundred of $CD34^+$ cells. To maintain a good level of vitality and to stimulate their growth we have noticed that it is appropriate to maintain a concentration of the culture of about $1x10^6$ cells/mL. When the culture has reached the peak of its growth potential, that is, after about 11 days, the cells are ready to be stored in the cellular Biobank. Each cellular cryopreserved aliquot will be formed by about $5x10^6$ cells. This is the optimal minimum number of cells to ensure a good recovery of the cryopreserved cultures after thawing.

3. Kinetics of EPO-induced erythroid differentiation following subculturing of cryopreserved ErPCs from β-thalassemia patients.

In order to assess what impact could have the freezing step on our cellular cultures we have thawed cells from 36 of the 75 subjects included in the cellular Biobank. We have performed on cultures isolated selected subjects with four different genotypes (β^+=16 subjects, β^0=6 subjects, β^+/β^0=12 subjects and HbS/β^0=2 subjects) in order to verify the behavior of the cells having different thalassemia mutations.

The degree of erythroid differentiation was evaluated by a benzidine assay 4 days and 9 days after thawing; the percentage of resulting blue cells indicates the level of erythroid differentiation within the cellular population. We have selected four thalassemia genotypes in order to obtain a more heterogeneous samples present in cellular Biobank. As we can see in **Figure 10**, in all cellular cultures analyzed, the 4th to 9th day after thawing, an increase of the percentage of B^+, demonstrating the ability of the cells to resume their viability after freezing, was observed.

The data obtained allowed us to conclude that the shock of thawing step performed on cryopreserved samples does not affect the natural differentiation process. The cell viability and the resumption of the differentiation process are two key elements to determine how the cells of the can be useful and reliable to conduct experiments on hemoglobin induction after storage in liquid nitrogen.

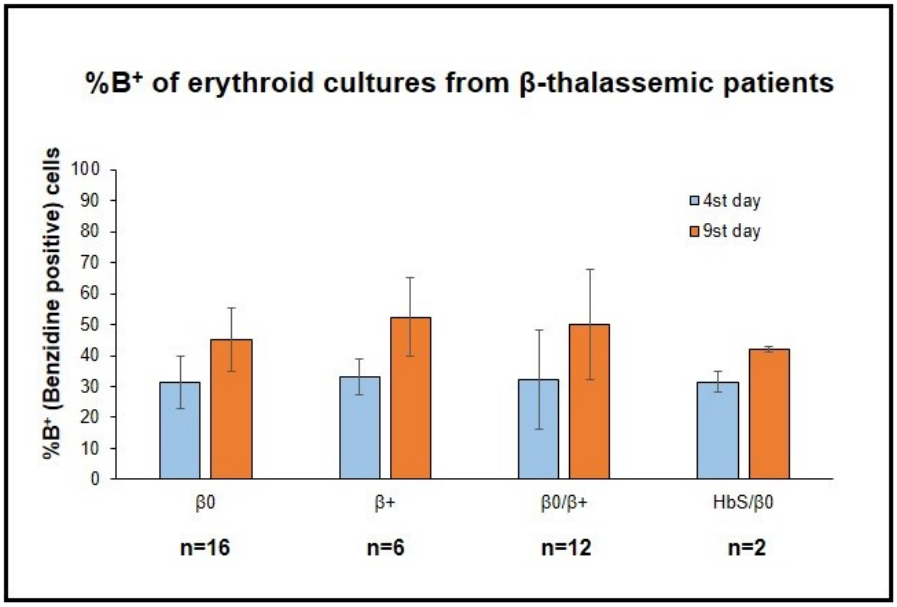

Figure 10 Erythroid differentiation of ErPcs from patients with different genotype during the culture. In the graph are reported the mean ± SD (as percentage) of B^+ obtained by benzidine assay at 4^{th} day (orange) and 9^{th} day (blue) of culture. For each genotype is specified the number of patient analyzed.

To go deeper into this aspect, we have selected 10 patients and we have compared the B^+ value obtained by the benzidine assay in three different stages of the culture: before freezing and 4 days and 9 days after thawing.

As shown in **Figure 11** a progressive increase of erythroid differentiation in function of time was recorded in all 10 patients in a very clear manner.

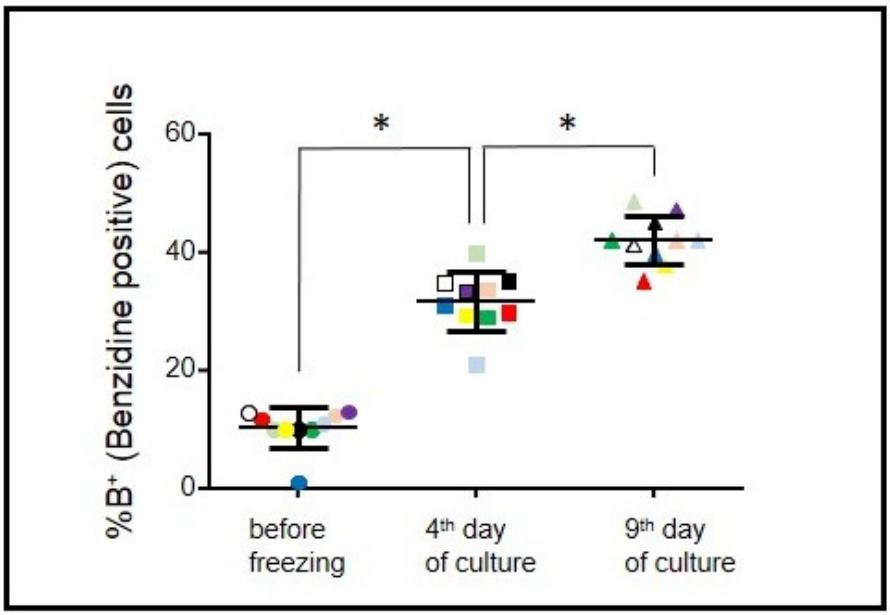

Figure 11 Erythroid differentiation evaluated at different days of cell culture. The graph shows with different colors the $\%B^+$ value from 10 patients at three time of cell culture: before freezing, $4t^h$ and 9^{th} day from thawing cells (*=P<0,05).

The confirmation of a good cell viability after thawing in all samples analyzed is critical to the validation of the cellular Biobank.

A further confirmation of the cell samples stability stored in the biobank is represented in **Figure 12.** We have reported an example of chromatograms obtained by an HPLC analysis concerning three cell samples derived from β-thalassemia patients with different genotypes. The hemoglobins, represented in the form of easily discriminable peaks, are recognizable because each elutes by the HPLC column at a specific 'retention time'.

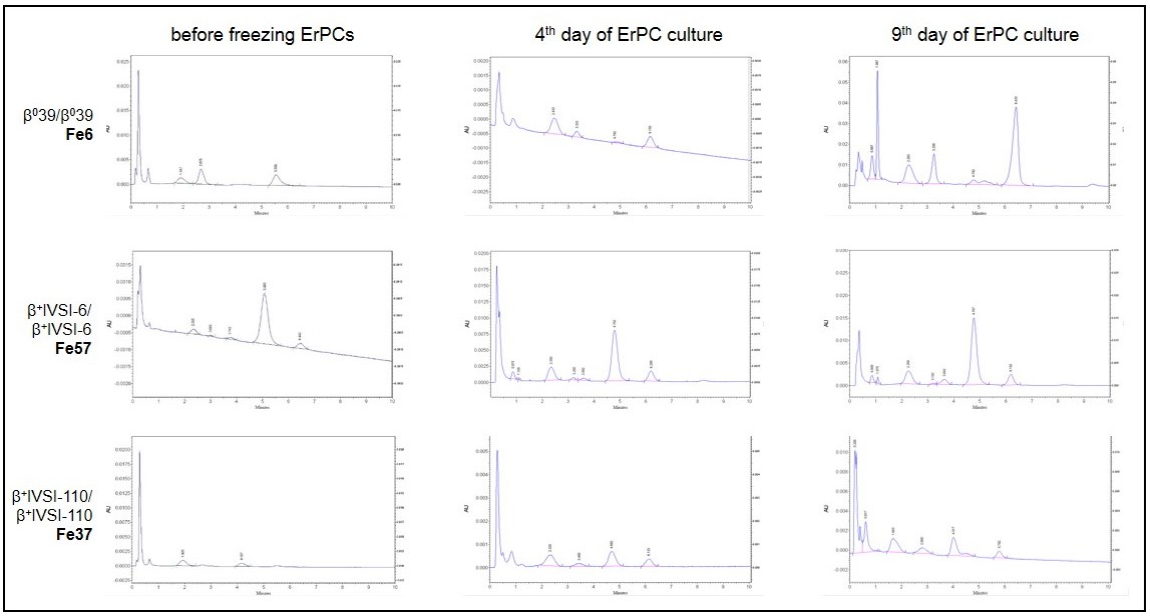

Figure 12 HPLC analysis of ErPCs obtained at three time of the cell culture. The chromatograms represent the hemoglobin profile obtained at three time of cell culture: before freezing, 4t^{h} and 9th day from thawing cells. The chromatograms of three patients affected by β-thalassemia (Fe6, Fe57, Fe37) presenting different genotypes are reported as example.

For each patient different chromatograms, corresponding to three different moments of the cultures, are reported. We can see that hemoglobins retain a similar trend in the various moments of the culture, comparing the chromatograms of each patient.

4. Validation of cell samples stored in the Biobank

The experiments conducted to date have shown that $CD34^+$ cells isolated and cultured with this new protocol and then cryopreserved in liquid nitrogen are still able to undergo a natural differentiation process. We also investigated how the genotypic characterization associated with analysis of specific polymorphisms would be useful in assessing the differentiation of cells of each patient in a more predictive manner.

4.1. Biobanked samples of a same β-thalassemia patient frozen and subcultured at different time maintain the same hemoglobin pattern.

To assess how the cellular samples collected and stored in the Biobank can really be used to conduct several experiments in an indeterminate period of time, we have investigated whether cells cryopreserved for increasing length of time maintain the same pattern of hemoglobin production. For this purpose, two cellular aliquots belonging to the same patient and derived from the same blood collection were thawed after several months one from the other and the hemoglobin pattern was evaluated by HPLC analysis. A representative example of the HPLC chromatogram is shown in **Figure 13**. As we can see the trend of hemoglobin is nearly perfectly superimposed showing that cryopreservation does not alter the hemoglobin gene expression of the cells stored in Biobank.

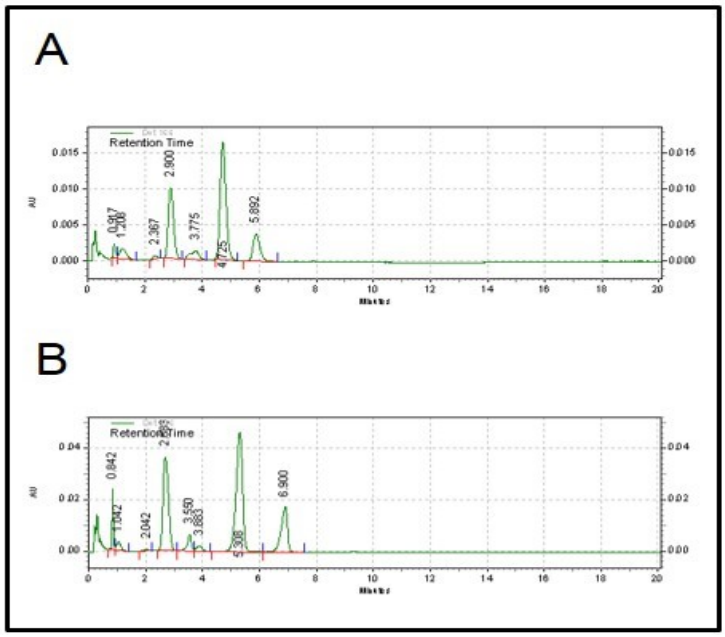

Figure 13 Erythroid independent cultures of the same blood collection from a patient. HPLC analysis (chromatograms in A and B) are carried out on the thawed cells purchased by two different vials and obtained from the same blood collection. Each hemoglobin peak is eluted from the column with a defined 'retention time'.

4.2. Biobanked samples originated from different blood sampling of a same β-thalassemia patient maintain the same hemoglobin pattern.

A furher validation of the cellular Biobank was performed on several withdrawals done at different time points on a same patient. The aim of this experiment was to verify whether the hemoglobin pattern is reproducibly obtained starting from different withdrawals of a same patient. To investigate this issue, we selected three patients, AVLTF, AVLTA and AVLTQ, exhibiting three different mutations of the β-globin genes ($\beta^0$39/$\beta^0$39, β^+IVSI-6/$\beta^0$39, β^+IVSI-6/$\beta^0$39 respectively) and/or different endogenous levels of HbF. Cryopreserved cells originated from two different withdrawals were subcultured and the hemoglobin pattern analyzed by HPLC. The results presented in **Figure14** are expressed as a percentage of each hemoglobin peak obained following quantitative evaluation of the HPLC chromatograms. Interestingly, the result obtained demonstrated that the trend of hemoglobin was found to be the same in cryopreserved cells originating from different withdrawals.

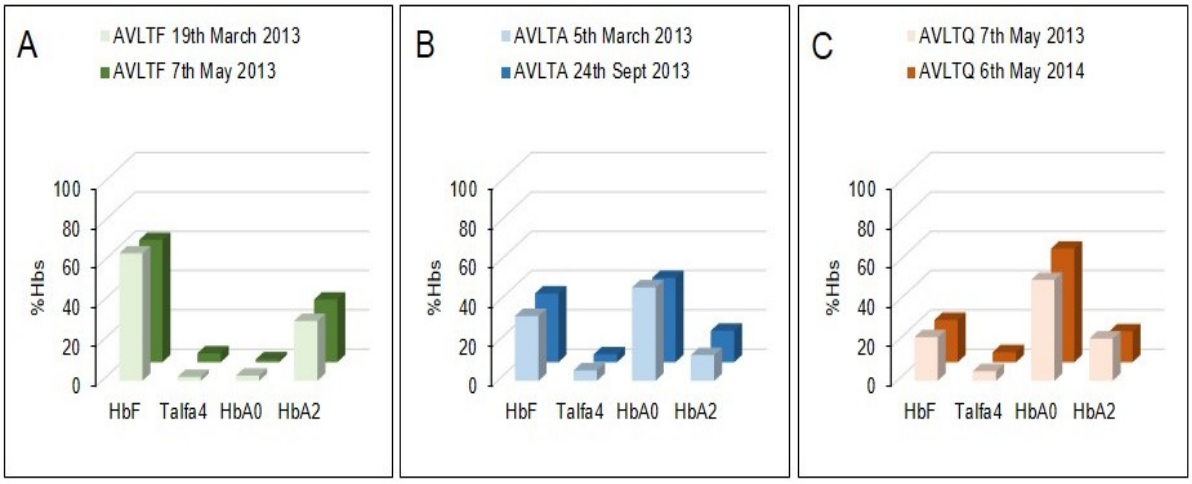

Figure 14 Erythroid cultures of blood collection from different patients. The panels correspond to three different patients (A) AVLTF, (B) AVLTA and (C) AVLTQ. The histograms indicate the mean of hemoglobins expressed as percentage. The HPLC analyzes in each panel were carried out on two cell cultures obtained from independent blood collection of the same patient.

4.3. Biobanked samples thawed and subcultured in different laboratories exhibit a similar pattern of hemoglobin production.

One of the most interesting applications of the developed cellular Biobank is based on the possibility of sending multiple cellular aliquots of the same patient in different laboratories of the world in order to conduct independent experiments

depending from the expertise of each laboratory involved. One possible use of this network-based project is the independent validation of novel HbF inducers. The confirmation of the HbF inducing capability by independent research group is of course very important to transfer basic/applied observations to pre-clinical and clinical appplications. A prerequisite of thios approach is the maintenance of the phenotype. To verify this issue, we performed experiments in parallel with Dr. Rivella at the Weil Medical College of Cornell University in New York. Aliquots of cells from different patients isolated and frozen at the Ferrara University have been sent to New York. The cell samples were thawed in parallel at Ferrara University and at Cornell University, the cells were allowed to differentiate using exactly the same culture protocol and the pattern of hemoglobin production analysed by HPLC using same experimental conditions. As we can see in the representative example given in **Figure 15** (panels **A** and **B),** the data obtained in the two laboratories were very similar.

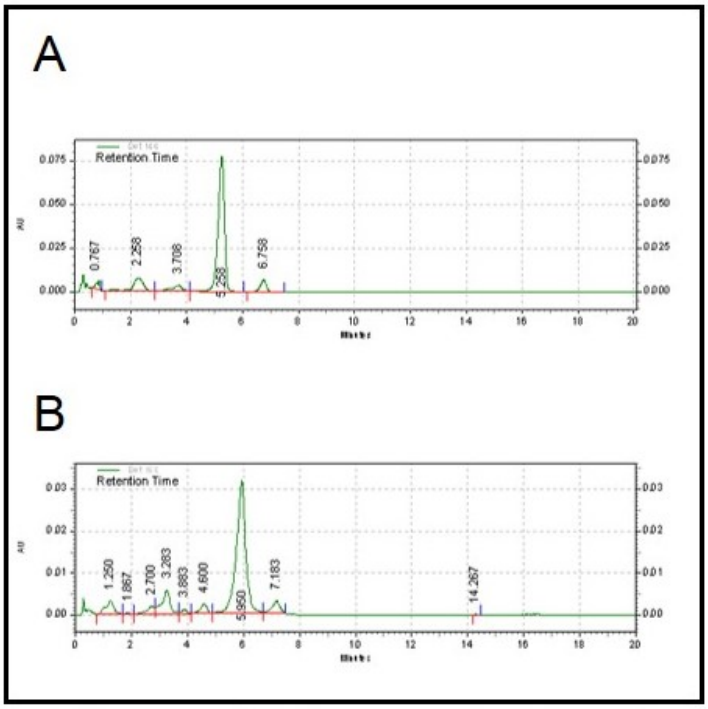

Figure 15 HPLC analyses performed on the ErPCs obtained from the same blood collection, but from cultures made in different laboratories. In the figure are reported the chromatograms of cellular lysates derivated from culrures performed in A, Ferrara's University and B, New York's University. Each hemoglobin peak is eluted from the column with a defined 'retention time'.

The experiment was conducted in parallel on the cellular samples derived from 12 patients of the Biobank each identified with a colored dot **(Figure16).**

The trend line defined by the correlation of induction in the two laboratories is R^2= 0.91 for HbF and R^2= 0.94 for HbA. in both experiments. These values indicate that HbF and HbA levels are, in all 12 patients, very similar when the analysis is conducted at Ferrara University and at Cornell University.

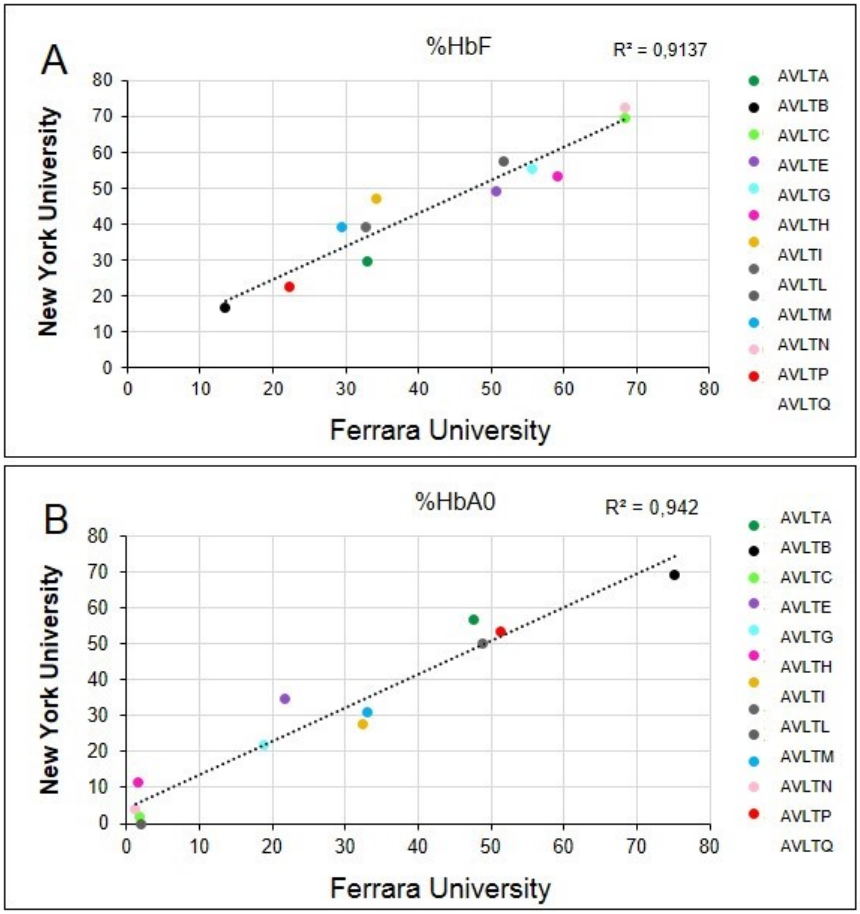

Figure 16 Comparison of HPLC analyses performed on the 12 different ErPCs obtained from the same blood collection, but from cultures made in different laboratories. The comparison regard the analyses made respectively at Ferrara's University and New York's University. The two panels show the correlation of percentage values of HbF (A) and HbA0 (B) on 12 patients picted using different colors.

4.4. Conclusions. All experiments conducted have validated the stability of the cells stored in the Biobank and confirmed the experimental repeatability and reliability of our cellular samples. Each of these points is crucial for the creation of a Biobank. The possibility of transferring the stored samples in the Biobank, according to the international Biobank laws, contributes to the formation of networks essential for progress in which several laboratories collaborate with each other to identify the best therapies and to progress in scientific research.

5. Induction of Biobanked CD34^{+} cells *versus* two liquid phases Fibach protocol.

After the initial validation of the Biobank allowing to conclude that the phenotype of the β-thalassemic cryopreserved cells is maintained, we were interested to verify whether the results obtained using crypreserved biobanked samples are in agreement with those obtained with the two liquid phase procedure originally developed by Fibach (Fibach, Manor et al. 1989). For this comparison, the response of cells after treatment with an HbF inducer was analyzed. The Fibach protocol is a method widely validated and appreciated by the entire scientific community and also used in several laboratories to verify the effects of HbF inducers.

We selected five patients with different genotypes representative for the patients recruited for the development of the Biobank. The cells of each patient were maintained in culture in parallel following the procedures and times provided in the Fibach protocol and those used for the preparation of the cellular Biobank. The two parallel cell cultures were treated with mithramycin (MTH) at the same concentration. For both methods, the hemoglobin production was evaluated by HPLC analysis and the results considering the HbF production and the level of the α-globin peak comparatively analyzed. It should be underline that usually the increase of HbF corresponds to a reduction of the α-globin peak. In **Figure17** all the data reported as treated/untreated ratios expressed in percentage. As it is evident, all the cellular cultures that have followed the protocol of expansion used for the generation of the Biobank exhibit basal HbF levels higher than those exhibited by the cells cultured with the Fibach method. However, in all the cellular cultures it was possible to appreciate an increase in the level of HbF hemoglobin correlated with a reduction of the α-globin peak. This experiment allowed us to demonstrate that the cells stored in the Biobank are sensitive to treatment with HbF inducers albeit starting from higher levels of HbF high levels. Furthermore, we observed a similar trend in the cell cultures when the two methods are considered. This results is crucial for our research interests because it demonstrates that the cells stored in the Biobank can really be used in experiments that include the use of molecules capable of inducing an increase of HbF. This will allow to set up experiments in parallel using cryopreserved cells from high number of patients in order to verify, among different HbF inducers, the best inducer for each patient,

using the cryovials stored in the Biobank without the need for repeated blood sampling.

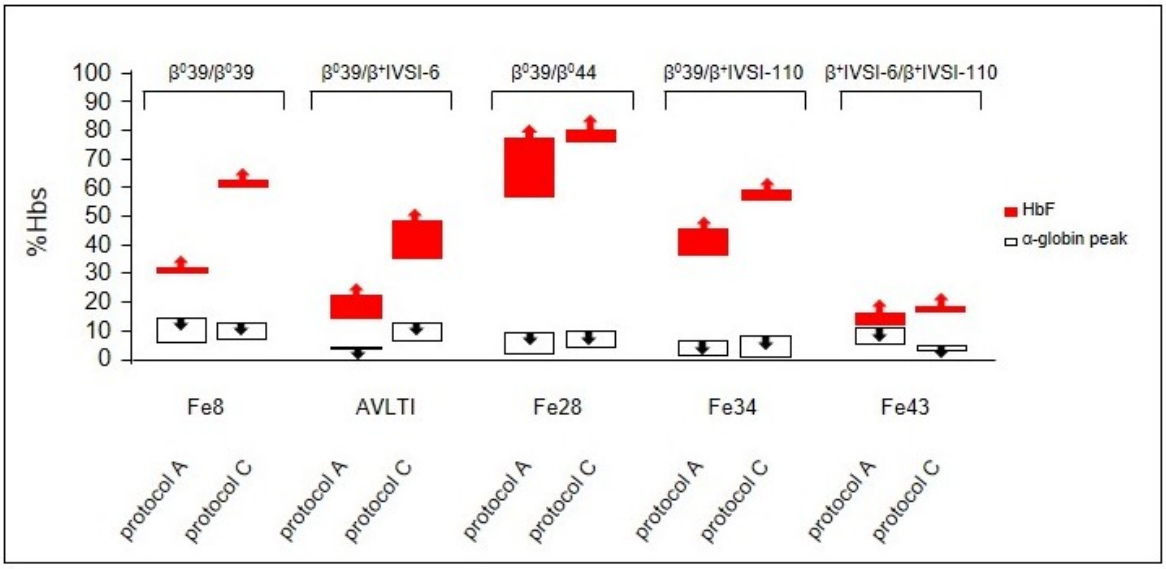

Figure 17 Comparison of the results obtained using protocol A and protocol C to culture the ErPCs for testing the MTH-erythroid induction. Cell samples were cultured and treated with MTH following two different methods: A= Fibach protocol, C= expansion protocol. The data of HbF (red) and α-globin peak (white) represent the increase, or the decrease respectively analyzed by HPLC in the untreated cells and in the treatedcells by MTH.

In conclusion, the results reported in **Figure 17** show that the method of cell expansion (protocol C) is effective to evaluate the increase of HbF levels following treatment with MTH. To further validate the data obtained by HPLC analysis, we decided to conduct a second set of analyses evaluating the increase of γ-globin mRNA in 9 patients by quantitative RT-qPCR. Also in this case, comparison between the two methods of cell culture (protocol A and protocol C) was performed. In the upper part of the panels displayed in **Figure 18** we have reported the data obtained from the samples treated with MTH (*T*) in relation to the untreated sample (*UNT*). Both values of *T* and *UNT* samples were normalized calculating the ratio γ-globin/α-globin mRNA. As we can see, MTH induces increase of γ-globin transcript occurs with both methods of culture. In conclusion, the biobanked samples, cultured with Protocol C, have produced excellent results on γ-globin increase following treatment with MTH, supporting the hypothesis of being able to use the cells stored in Biobank for the study of HbF hemoglobin inducers.

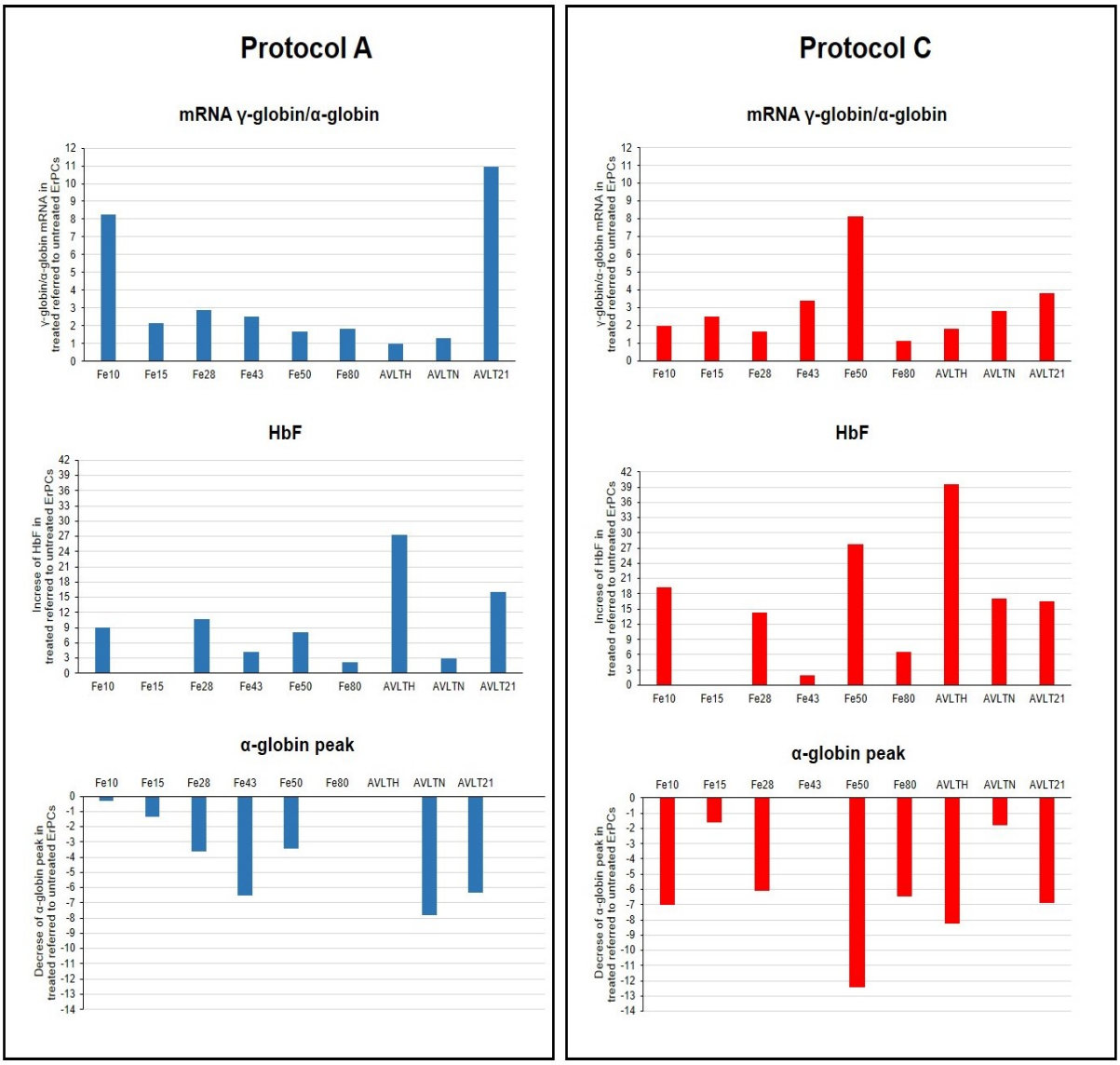

Figure 18 . Comparison of ErPcs coltured with protocol A and C induced to erythroid differentiation by MTH. The data resulted from the analyses carried out on the cells obtained from 9 different patients and cultuted with protocol A are picted in blue and with protocol C in red. In the upper part of the two panels, the increase of γ-globin/α-globin mRNAs is analyzed by RT-qPCR in MTH-treated cells with respect to untreated cell. In the middle and bottom, the normalized data of HbF and α-globin peak are reported and analyzed by HPLC on the same samples.

For each of the 9 treated cellular cultures, HPLC analysis was also carried out. In the central part of the two panels in **Figure 18** we have reported, in form of histograms, the percentage of HbF increase of the treated samples (*T*) compared to untreated (*UNT*) normalized according to the algorithm [(%HbF*T*-% HbF*UNT*)/(100-%HbF*UNT*)]. This data normalization allows us to evaluate how much the HbF increase is significant relative to its "potential maximum increase". As we can see when protocol C was used, the increase of HbF in the 9 cellular cultures treated with MTH results much more evident than the increase achieved with protocol A. Remarkably, however, cellular cultures derived from patient Fe15

did not show any increase of HbF when both protocols are used, suggeting that the precursor cells from this patient are not sensitive to MTH treatment.

Finally, at the bottom of the two panels results are reported demonstrating that the increase of HbF hemoglobin is inversely correlated with a decrease of the α-globin peak. Also, in this case, the data reported are represented as the ratio of *T*/*UNT* samples normalized as previously described for HbF.

In the graphs (the middle and bottom of the panels) shown in Figure 8, the inverse correlation between HbF and α-globin peak, it's very clear in the cell samples isolated and cultured with the expansion protocol (protocol C). In fact, we can easily see that in the cultures derived from patients Fe10, Fe28, Fe50, AVLTH, AVLT21 a substantial increase of HbF hemoglobin always corresponds to a substantial decrease of the α-globin peak.

In conclusion, the experiments described in this chapter have provided numerous confirmations about the possibility of using the cells stored in the Biobank for experiments on HbF hemoglobin induction.

6. Use of protocol A and protocol C with different HbF inducers.

In addition to mithramycin, we wanted to test other HbF inducers comparing the two protocols (protocol A and protocol C); also, in this case the results of the HPLC analysis were normalized using the previoulsly described algorithm [(%HbF*T*-%HbF*UNT*)/(100-%HbF*UNT*)] and all the treated samples were related to the untreated sample, originating from cultures performed in the absence of HbF inducers. In **Figure 19** the cell cultures performed using cells from different patients are indicated by different colors (each color correspond to cell cultures from a same patient). We comparatively used four different HbF inducers: MTH, Hydroxyurea, Resveratrol and Butyrate, represented in the figure with differernt symbols. After comparing the data obtained during the experiments of HbF induction performed using protocol A and protocol C, we obtain a relationship that deviates a little from the line trend (R^2=0,6). Some data obtained do not show an optimal correlation between the two protocols, but we have to consider that there are several variables, which can affect the treatment response.

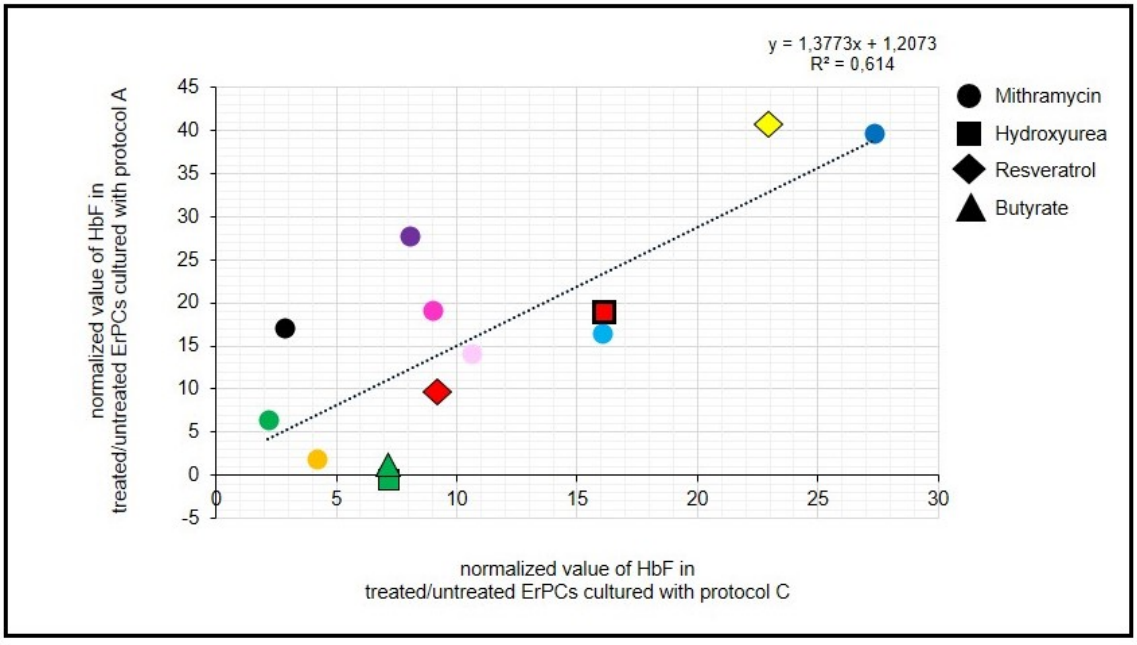

Figure 19 Comparison of the results obtained using protocol A and protocol C to culture the ErPCs and treated with HbF hemoglobin inducers. The HbF inducers Mithramycin (circle), Hydroxyurea (square), Resveratrol (rhombus) and Bytyrate (triangle) are indicated by different symbols and the patients are picted using different color. The comparison regard the HbF analyzed by HPLC made respectively using protocol C and protocol A to perform cell cultures (as reported in the axis of graph).

For example, patients with different genotypes can be sensitive to the HbF inducers action in a different manner, depending on the cell culture system employed; we must also consider that the HbF inducers used are acting with

different mechanisms of action. All these factors can contribute to the expected (although limited) variability in response.

7. Analysis with HbF hemoglobin inducers.

So far, we have shown that cell samples stored in the cellular Biobank, despite undergoing the step of freezing shock, are suitable for evaluating the HbF increase following treatment with HbF inducers in a fairly comparable manner when induction pattern obtained with protocol A and protocol C are compared.

At this point we wanted to investigate the extent to which the genotype of each patient could affect the HbF hemoglobin production after treatment with MTH. We have selected cells originating from some patients with β^+ genotypes and other originating from patients with β^0-thalassemia genotypes. All the cell samples used in the induction assay with MTH were from the cellular Biobank.

The upper part of **Figure 20** shows the data obtained by RT-qPCR. An increase in the γ-globin mRNA levels was obtained from the treated relative to untreated samples. All the data were normalized in relation to the α-globin.

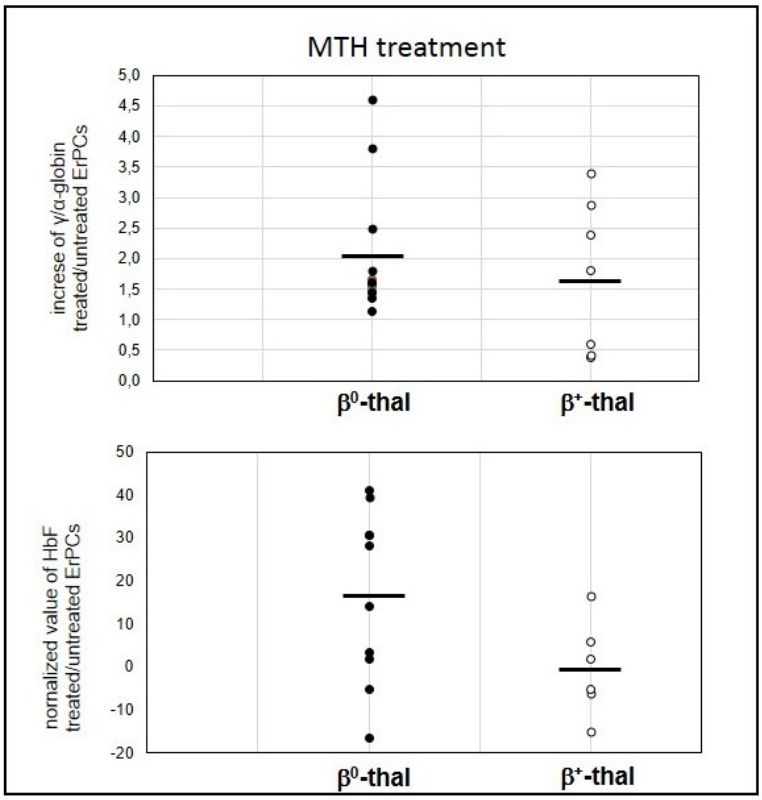

Figure 20 MTH induction of erythroid differentiation on ErPCs obtained from blood collection of patients with different genotype and cultured with protocol C. The ErPCs from patients with β+-thalassemia (n=7, white) and β^0-thalassemia (n=12, black) were treated with MTH used as HbF inducer. In the upper panel, the data of RT-qPCR are reported and in the lower panel, the normalized values of HbF obtained by HPLC analysis. The mean value for the tow groups are also reported.

We selected from the Biobank 12 thalassemia patients with β^0 genotype and 7 β^+-thalassemia genotype patients. As can be seen from the table, the average increase of γ-globin mRNA in the cells from β^0 patients is higher than the average increase in the cells obtained from β+ patients. In the same experiment was also conducted an HPLC analysis. In this case, we analyzed 10 β^0 patients, and 6 β+ patients.

The data obtained from HPLC analysis are reported, as it was done in the previous sections, comparing treated cells with untreated cells and were normalized using the previously decsribed algorithm [(%HbF*T*-%HbF*UNT*)/(100-%HbF*UNT*)]. The results obtained from this analysis confirm that cells from β^0-thalassemia patients show an increase of HbF greater than the cells from β^+-thalassemia genotype patients. In general, we can assert that these data are in line with what we have discussed in the previous paragraphs.

8. HbF hemoglobin inducers tested in cellular Biobank.

The HbF hemoglobin increase in cell samples treated with known inducers was validated by RT-qPCR and HPLC analysis using cells induced in protocols A and C. Accordingly, we decided to continue our investigation by focusing exclusively on the biobanked cells induced with protocol C.

We selected 13 cell samples from patients with different genotype. Each sample was treated with the HbF inducers MTH and Hydroxyurea in parallel **(Figure21)**.

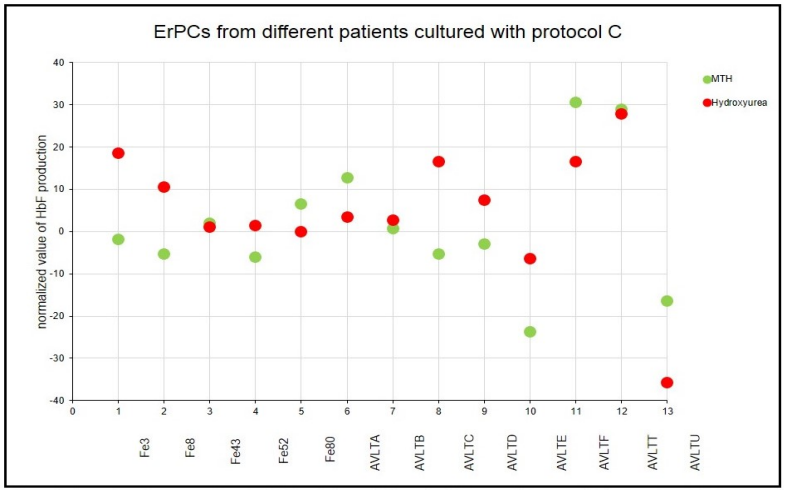

Figure 21 Comparison of induction of erythroid differentiation on ErPCs using MTH versus Hydroxyurea. Cell cultures from 13 patients were inducerd to erythroid differentiation both with MTH (green) and with Hydroxyurea (red). The data of HbF are reported and normalized with [(%HbF treated -%HbF untreated)/(100 - %HbF untreated)] algorithm.

The HPLC data were normalized as usual with the algorithm [(%HbFT-%HbFUNT)/(100-%HbFUNT)]. As we can see the best inducer was Hydroxyurea versus MTH respectively in 6 and 5 cellular samples. However, MTH reached very high levels of HbF induction in AVLTF and AVLTT cellular samples, both with β^0-thalassemia genotype. This confirms what we had previously obtained **(Figure20)** using β^0-thalassemia cellular samples that shown an HbF induction efficiency higher compared to β^+-thalassemia samples. Finally, two cell samples did not responde to both treatments. The same experiment was performed in 8 cellular samples in which we compared the HbF inducers MTH and Resveratrol. In **Figure 22** we can see that the cases in which we obtained an HbF induction were 4 samples treated with MTH against 3 samples treated with Resveratrol. A single sample showed an HbF increase level slightly lower than the baseline. In this experiment, the highest HbF increase was obtained again with MTH inducer in the Fe46 cellular sample having a β^0-thalassemia genotype.

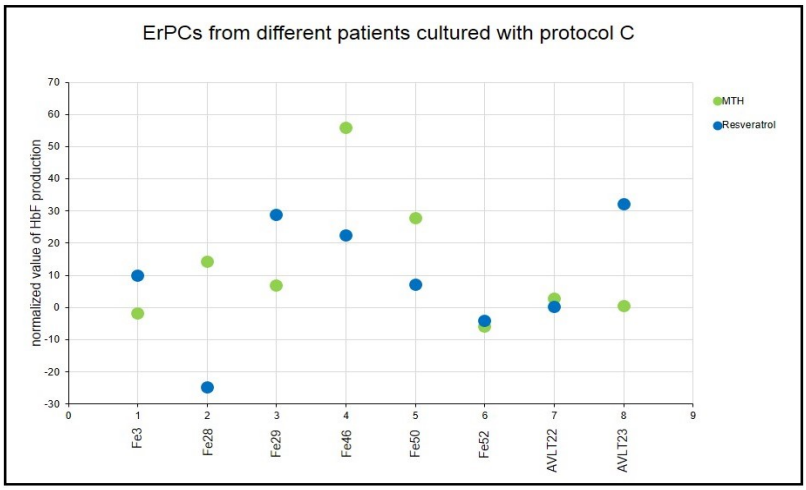

Figure 22 Comparison of induction of erythroid differentiation on ErPCs using MTH versus Resveratrol. Cell cultures from 8 patients were inducerd to erythroid differentiation both with MTH (green) and with Resveratrol (blue). The data of HbF are reported and normalized with [(%HbF treated -%HbF untreated)/(100 - %HbF untreated)] algorithm.

The conclusion of this set of experiments is that a great patient-to-patient variability is expected, as the cells from different patients are susceptible in different way to the HbF inducers. This can be associated to the patient's clinical status at the time of blood sampling and to the genetic characteristics of each patient.

The lack of response to some HbF inducers, when confirmed, strongly suggest that this apprach is needed to design personalized therapy.

Discussion

The knowledge on the human genome and of the molecular mechanisms involved in different diseases allows to completely rebuild medical practices and to review and futher develop classic treatment approaches. More and more frequently we talk about personalized medicine, which starts from specific molecular and genetic characteristics of a patient and ends in the deliver the most appropriate therapy. The general consensus on this specific issue is that it would be very useful to identify the best therapy for each patient by integrating all obtained data from an individual patient, and comparing them with the clinical information available for other patients (Kohane 2011). The need to gather information and biological material collected for patients to conduct studies of predictive nature, led to the establishment of new complex structures called Biobank (Khleif, Doroshow et al.; and Fiore D'Avolio 2011). A biobank is a systematic collection of biological samples (for instance genomic DNA, RNA, protein, cellular pellets) fully organized and containing also the information about the state of health of each participant (Hewitt 2011; King 2011)

It is in this context that the research conducted in this PhD Thesis took place. In detail this research activity has been conducted in the context of a multicenter FP7 European Project called THALAMOSS (THalassemia MOdular Stratification System), having as major objective the identification of molecular markers facilitating the development of personalized therapies for hemoglobinopathies, in particular β-thalassemia.

The β-thalassemias are autosomal recessive genetic disorders caused by the absence or reduction of β-globin chains (Silvestroni and Bianco 1948; Clegg and Weatherall 1999; Canali 2008). The β-thalassemia patients are treated with periodic blood transfusions associated to the assumption of iron chelating drugs and/or, when possible, with bone marrow transplantation (BMT) (Lucarelli, Galimberti et al. 1998). Despite scientific advances aimed at the development of targeted and definitive treatments of the disease, a cure of β-thalassemia is, at present, not available.

Originally described in the Mediterranean area (Haldane 1949), β-thalassemia should be at present considered a worldwide pathology with more than 100,000 new cases annually (Galanello and Origa 2010) and with a high incidence especially in countries belonging to Africa and Central and Southeast Asia. The

frequent and significant migration flows that have occurred over the decades have contributed irreversibly to the wide distribution of the disease (Flint, Harding et al. 1998). In Italy, the most affected areas are Sicily, the delta of the river Po and Sardinia, with an incidence of approximately 95.7% (Silvestroni, Bianco et al. 1950; Hill 1987; Rosatelli, Dozy et al. 1992; Canali 2008).

THALAMOSS is aimed at the development of a computerized system that enables β-thalassemia patient stratification according to clinical, genetic and molecular features in order to implement a personalized therapy.

Before the collection of biological samples, the approval by the Ethics Committee of Ferrara and Verona-Rovigo district was obtained. All the subjects, who have contributed to the establishment of the cellular Biobank, gave their positive consent.

The cellular Biobank that was established thanks to the experimental activities described in this PhD thesis, is at present composed of cells obtained from 75 subjects, most of them being β-thalassemia patients who do not present alterations of α-thalassemia. In addition, a small fraction (11 subjects) is composed by cells from healthy subjects.

The participants recruited originate in the province of Ferrara, some areas of Veneto; in sporadic cases, they originate from non-EU areas (individuals immigrants). The average age of the subjects was about 40 years and approximately 54.2% of the participants are women. Each subject adhering to the Biobank was genotyped in our laboratory at the Ferrara University for what refers to the β-globin gene, while the analysis of some HbF related polymorphisms were carried out at the Regional Hospital for Microcytemia in Cagliari. **(Figure 6-8).** From the β-globin gene characterization it can be concluded that the genotypes present mainly are those most common in the geographic areas of origin, i.e. $\beta^0$39/$\beta^0$39, β^+IVSI-110/$\beta^0$39 and β^+IVSI-110/ β^+IVSI-110, present with a percentage of 30%, 22% and 11%, respectively. Instead, analysis of HbF-associated polymorphisms revealed that *rs*10189857 (A/G) is the most prevalent BCL11A polymorphism and XmnI *rs*74482144 (+/-; +/+) polymorphism exhibit low frequency in the biobanked samples. Almost half of the patients (47%) is a carrier of a HBS1L-Myb *rs*9399137 (T/C) polymorphism.

To date, there are already biobanks operating, directly or indirectly, in the field of thalassemia. They take care of collecting and preserving DNA, in order to conduct genetic investigations, as well as and others cellular samples, such as T-

lymphocytes, fibroblasts and lymphoblasts. However, at present, at the best of our knowledge, no Biobanks are present composed of the hematopoietic stem cells isolated from peripheral blood and cryopreserved, in order to be ready any time for conducting studies on thalassemia.

In the construction of a cellular Biobank, several major problems can be encountered, such as the low number of circulating hematopoietic stem cells, and the possible inability of these cells to return viable after the freezing step and the cryopreservation period. This specific issues were the major objects of our research activities.

Several efforts were focused on overcoming a number of important issues related to the culture systems of these cells. First of all, we have selected the $CD34^+$ cells from a peripheral blood sample using two methods in succession. Ficoll Hipaque allows to separate the different components of whole blood according to the density gradient. In particular, we isolated lymphocytes, macrophages, fibroblasts and erythroid precursor cells from the remaining cellular blood components. Then we applied on this heterogenous cellular population the MACS Method (Magnetic Assay Cell Sorting). This method uses magnetic microbeads having a surface structure that can recognize and selectively bind $CD34^+$ cells (Watanabe, Kawano et al. 1999; Wang, Hsu et al. 2001; Zuccato, Breda et al 2012).. This step allows to set up very pure cell culture. At this point, we have tried to increase the cellular concentration by using an expansion culture medium. For the development of the expansion medium for optimal growth of $CD34^+$ cells a protocol described for the first time by Leberbauer was employed (Leberbauer, Boulme et al. 2005). This medium was later modified by Breda and Rivella, who have employed it in studies for β-thalassemia treatment with the gene therapy method (Breda, Casu et al 2012). This medium contains StemSpan Serum-Free Medium Expansion, StemSpan® CC100 Cytokine Cocktail for expansion of human hematopoietic cells stem cell, erythropoietin (EPO), dexamethasone 21-phosphate disodium salt and PEN-STREP.

In order to verify the suitability of this approach for studies on β-thalassemia, we have firstly evaluated the ability of this medium to stimulate the cell growth without influencing their natural differentiation state; then we decided to use this culture medium during $CD34^+$ cells expansion phase and after thawing cryopreserved packed cells.

From each blood sample, we can obtain an average of about 5 aliquots ($5x10^6$cells/mL) of $CD34^+$ cells for each patient. It is important to obtain this minimal quantity of cells in order to conduct functional studies (including proteomic and transcriptomic analyses) using cells stored in the cellular Biobank. This is of course of great interest not only for the scientific community, but also for the patients involved in this activity. In fact, this approach, unlike the two-phase culture system developed by Fibach, allows experimentation without the necessity of repeated sampling from the patients. After having developed protocols for isolation, expansion and storage of $CD34^+$ cells, we have decided to validate the experimental method by evaluating different cellular parameters important in the field of drug development for β-thalassemia.

In order to assess what impact the freezing step and the cryopreservation could have on our cellular cultures we have thawed cells from 36 of the 75 subjects of cellular Biobank with different thalassemia mutation/phenotype: β^+, β^0, β^+/β^0 and HbS/β^0 (**Figura10**) The degree of erythroid differentiation was evaluated by a benzidine assay 4 days and 9 days after thawing; in all cellular cultures analyzed an increase of the percentage of B^+ cells was observed. Confirmation of cell viability and cell differentiation efficiency was obtained by analyzing cellular samples for hemoglobins production by HPLC analysis (Huisman 1987) (**Figura 11**). The data obtained allowed us to conclude that freezing, crypreservation and thawing steps performed do not affect the natural erythroid differentiation process.

As a confirmation of the cell samples stability stored in the biobank, the chromatograms obtained by HPLC analyses of several cell samples derived from β-thalassemia patients with different genotypes, show that hemoglobins retain a similar trend in the various moments of the culture (**Figure12**).

To determine whether our cellular samples collected in the Biobank can really be used to conduct several experiments with different approaches in an indeterminate period of time, we have investigated whether the freezing of the cells for a long time could have significant effects on the hemoglobin pattern. Two cells aliquots from the same patient deriving from the same blood collection, were thawed after several months one from the other and we have evaluated the globin pattern in the two moments by HPLC analysis (**Figure13**). The hemoglobins trend in both experiments is perfectly superimposed showing that cryopreservation does not alter the erythroid differentiation potential of the cells stored in cellular Biobank.

In order to create a Biobank containing numerous cell samples for each subject suitable to conduct several experiments any time, we verified that the cells obtained with several samplings from the same patient exhibit a same pattern of hemoglobin production. To this aim, we selected some patients, we cultured cells isolated from two different withdrawals and finally we evaluated the hemoglobin pattern (**Figure14**). The results obtained confirmed that the pattern of hemoglobin poduction was found to be the same in all cellular samples analyzed and deriving from the same patient.

The conclusion of this first set of results is that the culture system employed for the generation of the cellular Biobank does not alter the kinetics and the type of hemoglobin produced by the cells from a same patient, after freezing, cryopresenvation and thawing.

However, an important feature of the cellular Biobanks involves the possibility of sending multiple cellular aliquots in different parts of the world in order to conduct predictive experiments to identify the best therapeutic approach for a given patient or patients subgroups. To verify this important issue, we performed experiments in parallel with the Weil Medical College of Cornell University in New York. Aliquots of cells from different patients isolated and frozen at the Ferrara University were sent to New York, and same experiments were done in duplicate with the same cellular cryopreserved samples. The results demonstrate that we have the real possibility of sending cellular aliquots of the Biobank to different laboratories of the world to conduct experiments on pre-validated samples (**Figure 15** and **16).**

Finally, an important goal of our experiments was to determine whether the cellular Biobank can be employed to identify and develop in vitro HbF inducers, already demonstrated to be a very effective therapeutic strategy for treatment of β-thalassemia. This is not a resolutive approach of the disease but greatly improves the clinical status of the β-thalassemia patients.

In the past, several erythroid cell lines have been used extensively as experimental model system to study β-thalassemia and to develop new therapeutic strategies. Until today, the erythroid cells were cultured either in semi-solid medium, where they develop into discrete colonies, or in liquid medium where they grow as single cells or clusters in suspension (Myers, Katz et al. 1984; Fibach, Manor et al. 1989; Leimberg, Konijn et al. 2003; Fibach and Prus 2005). In all these systems erythropoietin (EPO) is essential for full development of

hemoglobin containing erythroid cells (Box et al., 1996; Fibach, Bianchi et al. 2003).

In determining possible applications of the cellular Biobank we have developed, an important issue was to compare the cells stored in the biobank obtained with our expansion protocol (protocol C) with the cells cultures following the Fibach protocol (protocol A), with respect to the ability to increase HbF levels. We selected some cellular samples with different genotypes representative for the Biobank. The cells were maintained in culture in parallel following the procedures and times provided for the two culture methods and then were treated with different HbF inducers, including mithramycin (MTH) (Bianchi, Osti et al. 1999; Fibach, Bianchi et al. 2003), resveratrol (RSV) (Bianchi, Zuccato et al. 2009; Fibach, Prus et al. 2012), butyric acid (BA) (Perrine, Ginder et al. 1993; Sher, Ginder et al. 1995). and hydroxyurea (HU) (Bradai, Abad et al. 2003; Alebouyeh, Moussavi et al. 2004), considered at the reference compounds because it is already under clinical usage for β-thalassemia and sickle-cell disease.

The conclusion of these experiments, reported in detail in **Figures 17** suggest that when the cells have been cultured with Protoc C, they exhibit and “endogenous” starting HbF level higher than those obtained with the two-phase protocol A (the Fibach method). Despite this, in all cases it is possible to appreciate an increase in the HbF production with the different treatments and correlated with a reduction of the α-globin peak. The data obtained were then validated by RT-qPCR analysis (**Figure18**). This experiment allowed us to demonstrate that the cells stored in the biobank are responsive to treatment with HbF inducers albeit starting from higher HbF levels.

All data obtained confirm the potential use of the cellular biobank to conduct studies with HbF hemoglobin inducers. Considering that the cellular Biobank was set up starting from subjects genotypically different, we decided to investigate whether two different genotypes produce different induction effects following the mithramycin treatment. The experiments conducted by RT-qPCR and HPLC analysis showed that the cryopreserved cells obtained from β^0-patients display an increase of HbF greater than the cells obtained from β^+-thalassemia patients (**Figure20**).

The conclusion of the final set of experiments performed with different inducers on cryopreserved cells cultured with protocol C is that a great patient-to-patient variability is expected, as the cells from different patients are susceptible in

different way to different HbF inducers (**Figure 21-22**). This can be associated to the patient's clinical status at the time of blood sampling and to the genetic characteristics of each patient. Finally, the lack of response to some HbF inducers, when confirmed, strongly suggest that this apprach is needed to design personalized therapy.

The possibility to conduct pre-clinical testing on the cells of a patient stored in the cellular Biobank represents an immense resource for researchers and patients. Evaluate the response to innovative treatment in a timely manner provides essential information for researchers who in this way can evaluate the effects caused by the drug, and represents a protection for the patient by limiting the administration only for potentially curative molecules. This research activity will allow patients stratification taking into account all the phenotypic/genotypic characteristics of the single individual in association with *in vitro* HbF induction under treatment with effective inducers.

Further, the cellular Biobank can provide cell from different patients for the construction of induced pluripotent cells (iPS) for single patients. This is a great opportunity, when considered together the possibility of gene editing, leading to the correction of the genetic mutation by several approaches, including gene therapy and the use of strategies for homologous recombination.

We should point out that gene therapy is one of the most promising approaches for future management of the β-thalassemia patients. The most direct strategy of gene therapy aims at substituting the non-functioning endogenous β-globin genes with a normal β-globin gene carried by lentiviral vectors, thus obtaining de novo production of adult haemoglobin (HbA). In this case two gene therapy approaches have been demonstrated to be efficient, based on the use of lentiviral vectors carrying the human γ-globin gene sequences (in this case HbF is produced by an exogenously added γ-globin gene), or carrying sequences coding for artificial promoters of the γ-globin gene (in this case HbF is produced by inducing transcription of the silent endogenous γ-globin genes). In addition, novel therapeutic approaches have been recently validated, such as nuclease-mediated gene editing by homologous recombination of the human globin locus. In this case, correction of altered β-globin genes can be achieved. Endogenous genomic loci can be altered efficiently and specifically using engineered zinc finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs). Moreover, besides ZFN and TALEN, clustered regularly interspaced short

palindromic repeats (CRISPR) linked to Cas9 nuclease are now also being investigated for their utility in modifying β-globin.

As far as the cellular targets of gene therapy, human erythropoietic stem cells have been considered in most studies; however in the future we expect that induced pluripotent stem cells (iPSCs) from β-thalassemia patients will be also a useful cellular target of GT approaches. The cryopreserved biobanked cells can be a very important biological sources for these experiments.

As a final comment, Biobanks can provide a huge opportunity for development, research and knowledge and the beneficiaries will be all human beings and all future generations (An 2006). It is now clear the decisive role of Biobanks to accelerate scientific discoveries. To maximize results it is important to dedicate attention to the quality of numerous samples collected in the biobanks, to the creation of a partnerships network and to financial infrastructures.

Appendix

Appendix 1. Distribution of main biobanks spread around the world.

European Biobanks		
State	**City**	**Biobank name**
AT	Graz	Biobank Graz
AT	Graz	BioResurce-Med
AT	Graz	LifeCord Public Cord Blood Bank
AT	Krems an der Donau	Cells and Tissue Bank Austria
AT	Wels	European Cell and Tissue Bank
AT	Österreich	VITA 34 GmbH Österreich
AT	Vienna	MUW Biobank
AT	Vienna	Ludwig Boltzman Tumour Bank
AT	Vienna	Vitacord, Gesellschaft für Stammzellenlagerung m.b.H
BE	Brussels	SaintLuc Tumour Bank
BE	Brussels	Tissue Bank of the University Hospital Saint Luc
BE	Liege	GIGA-BIOBANK
BE	Liege	Belgian Cord Blood Bank
BE	Luxembourg	Integrated Biobank of Luxembourg
BG	Sofia	National DNA Bank of the Republic of Bulgaria
BG	Sofia	Tissue Bank Pirogov
CH	Bern	Biobank Suisse
CH	Pfaeffikon	CryoSave
CH	Bern	Tumor Bank Bern
CH	Geneva	East West Biopharma
CH	Monthey	Swiss Biobank
CY	Limassol	StemCure
DE	Berlin	Central Biomaterial Bank - German Heart Failure Network
DE	Berlin	Tissue Bank, Charité – Universitätsmedizin
DE	Berlin	SepNet Central Sample Bank
DE	Düsseldorf	Jose Carreras Cord Blood Bank
DE	Heidelberg	Patient DNA collection at Institute of Human Genetics
DE	Heidelberg	Human Melanoma Biobank
DE	Munich	BrainNet Europe
DE	Munich	KORAgen
DE	Regensburg	Danubian Biobank Consortium
DE	St. Ingbert	Kryoforschungs- & Demonstrationsbank der Fraunhofer Gesellschaft “Eurocroyo SAAR”
DE	Tübingen	European Searchable Tumour Line Database (ESTDAB)
DK	Copenhagen	The Danish National Birth Cohort
DK	Copenhagen	The National PKU Biobank
DK	Copenhagen	NUGENOB
DK	Copenhagen	The Danish Psychiatric Biobank
DK	Copenhagen	The Danish National biobank
EE	Tartu	Estonia Biobank
ES	Barcelona	Biobank of Hospital Clínic IDIBAPS

ES	Granada	Andalusian Regional Tumour Bank
ES	Madrid	HIV Biobank
ES	Madrid	Spanish National Tumour Bank Network
FI	Helsinki	National Biobank of Finland
FI	Helsinki	The Finnish Biaobank
FI	Tampere	Helsinki Sudden Death Study (Tampere University)
FI	Turku	Biobanking and Biomolecular Resources Research Infr astructure Institute BBMRI
FR	Amiens	Biobanque de Picardie
FR	Evry	Genethon DNA and Cell Bank
FR	Lyon	Biobank Resource Centre de France
FR	Lyon	European Sarcoma & Tumour Bank
FR	Marseille	Tumour Bank of Provence
FR	Toulouse	Southwest France Tumour Bank
GR	Athens	Hellenic Cord Blood Bank
GR	Marousi	StemHealth Hellas Stem Cell Bank
HU	Budapest	Hungarian NeurologicalPsychiatric Biobank
HU	Budapest	KRIO Institut
HU	Pecs	National Biobank Hungarian
IE	Dublin	Biobank Ireland Trust
IE	Dublin	Dublin Brain Bank
IE	Belfast	Northern Ireland Virtual Tissue Archive
IE	Wexford	Biostor Ireland
IE	Trinity	Trinity Biobank
IS	Reykjavik	Icelandic Biobank (deCode)
IS	Kópavogur	The Icelandic Cancer Society Biological Specimen Collection
IS	Reykjavik	Tissue Archives, University Hospital in Reykjavik
IT	Florence	daVinci European Biobank
IT	Genoa	Biological Resource Centre - National Institute for Cancer Research
IT	Genoa	Telethon Genetic Biobank Network
IT	Milan	BioRep
IT	Milan	Movement Disorders Biobank
IT	Milan	Istituto Nazionale Neurologico Carlo Besta
IT	Milan	The Neuromuscular biobank, Department of Neurological Science, University of Milano
IT	Milan	Bank of DNA cell line and nerve-musclecardiac disease
IT	Naples	Naples Human Mutation Bank of the Cardiomyology and Medical Genetics (NHGMB)
IT	Padua	EuroBioBank
IT	Siena	Cell lines and DNA bank of Rett syndrome, X-linked mental retardation and other genetic diseases
LV	Riga	Latvia Biobank
MT	Malta	Malta BioBank
NL	Groningen	ErasmusMC
NL	Groningen	Lifelines Biobank
NL	Rotterdam	European Human Frozen Tissue Bank
NL		TuBaFrost
NL	Maastricht	BioBank Maastricht

NL	Amsterdam	Blood bank of rare blood groups of the Council of Europe
NO	Levanger	Hunt Biobank
NO	Trondheim	Regional Biobank of Central Norway
NO	Rockville	JANUS Serumbank (Cancer Registry of Norway)
NO	Bergen	HUSK (Health Study of Hordaland)
NO	Oslo	MoBa (The Norwegian Mother and Child Cohort Study)
RO	Bucharest	Spitalul-clinic Colentina
RO	Bucharest	Medsana Bucharest Medical Center
RO	Bucharest	University Hospital “Panait Sarbu” Bucharest Assisted Reproduction Departmen, Sperm Bank,Embryo Bank
RO	Timisoara	IVF Center “Victor Babes” University of Medicine and Pharmacy
SE	Lund	Swedish Regional Biobank
SE	Malmo	European Cancer Biobank
SE	Stockholm	Karolisnka Biobank
SE	Stockholm	LifeGene
SE	Stockhom	Swedish National Biobank Program
SE	Umea	Biobank of Northern Sweden
SE	Birmingham	Central England HaematoOncology Research Biobank
SK	Bratislava	Eurocord Slovakia
UK	Cambridge	CamUroOncology Biobank
UK	Cambridge	Geneservice
UK	Cardiff	Wales Cancer Bank
UK	Dundee	Tayside Tissue Bank
UK	Edinburgh	Edinburg Brain & Tissue Banks
UK	Glasgow	Biopta
UK	Hertfordshire	onCore UK
UK	Hertfordshire	UK Stem Cell Bank
UK	Leicester	Children's Cancer and Leukaemia Tissue Bank
UK	Liverpool	Cancer Tissue Bank Research Centre
UK	London	Chernobyl Tissue Bank
UK	London	Confederation of Cancer Banks
UK	London	King's College Infectious Diseases BioBank
UK	London	Neuroendocrine Tumors Biobank
UK	London	UK Parkinson's Disease Tissue Bank
UK	London	UK Multiple Schlerosis Tissue Bank
UK	London	Virgin Health Bank
UK	Manchester	UK DNA Banking Network
UK	Midlothian	Roslin Wellcome Trust Tick Cell Biobank
UK	Newcastle Upon Tyne	HDBR Human Developmental Biology Resource
UK	Oxford	Autism Brain Bank
UK	Plymouth	BioVault
UK	Salisbury	European Collection of cell Cultures
UK	Southampton	Southampton Tumour Bank
UK	Stockport	UK Biobank

Canada Biobanks	
City	**Biobank name**
Edmonton	Canadian Biosample Repository
Edmonton	Alberta Research Tumor Bank
London	Lawson Brain Tumour Tissue Bank
Manitoba	Manitoba Breast Tumor Bank
Montreal	CARTaGENE
Montreal	Quebec Leukemia Cell Bank
Montreal	Tissue Bank of the Respiratory Health Network of the FRSQ
MontRoyal	PROCURE Quebec Prostate Cancer Biobank
Ottawa	Canadian Study on Health and Aging
Toronto	Canadian Virtual Brain Tumour Network
Toronto	Ontario Tumour Bank
Toronto	Blood Borne Pathogens Laboratory
Toronto	Sunnybrook Tumour Bank
Vancouver	British Columbia Breast Cancer Tumour Bank
Vancouver	British Columbia Gynaecologic Tissue Bank
Vancouver	British Columbia Tumour Tissue Repository
Winnipeg	Canadian Tumour Repository Network
Winnipeg	Manitoba Breast Tumour Bank

US Biobanks		
State	**City**	**Biobank name**
AL	Birmingham	University of Alabama Tissue Collection and Banking Facility
AL	Hunstville	Conversant Biologics, Inc.
AZ	Tucson	Arizona Cancer Center Tumor Bank
AZ	Sun City	Banner Health Brain & Tissue Bank
AZ	Phoenix	Arizona Biospecimen Locator
AZ	Phoenix	ScienceCare
CA	San Francisco	AIDS & Cancer Specimen Resource
CA	Los Angeles	Autism Tissue Program
CA	Monterey	Biobanc USA
CA	Fullerton	BioOptions
CA	Los Angeles	California Cryobank, Inc.
CA	Van Nuys	HemaCare Corp.
CA	Loma Linda	Loma Linda University Cancer Center Biospecimen Laboratory
CA	Los Angeles	National Neurological AIDS Bank
CA	San Francisco	PathServe
CA	South San Francisco	Cureline
CA	Solana Beach	PrecisionMed
CA	Romona	Protein Technologies
CA	Santa Monica	Sanguine BioSciences, Inc.
CA	Palo Alto	Stanford Tissue Bank
CA	Berkley	UC Berkley Biorepository
CA	Sacramento	UC Davis Biorepository

CA	San Diego	UC San Diego Biorepository
CA	Los Angeles	UCLA Brain and Spinal Fluid Resource Center
CA	San Francisco	UCSF AIDS Specimen Bank
CA	San Francisco	UCSF Brain Tumor Tissue Bank
CA	Mission Bay	UCSF DNA Bank
CA	San Francisco	UCSF Specimen Banking Core
CA	Westlake Village	Coreva Human Milk Bank
CO	Aurora	Beryllium BioBank
CO	Aurora	Rocky Mountain MS Brain Tissue Bank
CO	Nederland	Donor Sibling Registry
CT	Farmington	Research Tissue Repository - University of Connecticut
CT	Norwalk	Myeloma Tissue Bank
DC	Washington	Genetic Alliance Biobank
DC	Washington	PXE Internatonal Tissue Bank
FL	Boynton Beach	International Biobank, Inc.
FL	Gainesville	Barth Syndrome Biorepository
FL	Tampa	LifeLink TissueBank
FL	Tampa	M2Gen Biorepository
FL	Gainesville	University of Florida DNA & Tissue Bank
FL	Gainesville	University of Florida Molecular Tissue Bank
FL	Coral Gables	University of Miami Biorepository
FL	Coral Gables	University of Miami Brain & Tissue Bank for Devel opmental Disorders
FL	Coral Gables	University of Miami Tissue Bank
GA	Atlanta	Biorepository Alliance of Georgia for Oncology
GA	Augusta	Medical College of Georgia Tumor Bank
IL	Chicago	University of Chicago Human Tissue Center
IN	Indianapolis	BioStorage Technologies
IN	Indianapolis	National Gene Vector Biorepository
IN	Indianapolis	Susan G. Komen Tissue Bank
LA	New Orleans	Ochsner Tumor Tissue Bank
MA	Boston	Brighman & Women'sHarvard Biobank
MA	Boston	Dana Farber Tissue Bank
MA	Cambridge	Harvard Brain Tissue Resource Center
MA	Lexington	StoreaTooth Stem Cell Bank
MA	Pepperell	Masy Systems
MA	Waltham	Accelerated Cure Project MS Repository
MA	Boston	Crimson Biospecimen Core
MA	Boston	National Temporal Bone Pathology Resource Reg istry
MA	Milford	SeraCare Lifesciences
MA	Peabody	Progeria Cell & Tissue Bank
MA	Norton	ProMedDx
MA	Worcester	University of Massachussets Cancer Tissue Bank
MA	Shrewsbury	University of Massachussets Stem Cell Bank
MA	Waltham	Multiple Schlerosis (MS) Repository
MD	Rockville	BioReliance Biorepository
MD	Beltsville	Bioserve
MD	Rockville	Capital Biosciences

MD	Rockville	Fisher BioServices
MD	Gaithersburg	Genelogic
MD	Chestertown	ILSbio
MD	Gaithersburg	KamTek Biorepository
MD	Baltimore	Maryland Brain Collection
MD	Frederick	National Cancer Institute Biorepositories
MD	Bethesda	Cooperative Human Tissue Network (CHTN)
MD	Bethesda	NCI Office of Biospecimens
MD	Bethesda	NIDDK Central Biorepository
MD	Rockville	Origene
MD	Baltimore	Tissue Banks International
MD	Baltimore	University of Maryland Brain & Tissue Bank
MD	Rockville	US Biomax
MI	Detroit	Asterand
MI	Royal Oak	Beaumont Hospitals Biobank
MI	Detroit	JP McCarthy Cord Stem Cell Bank
MI	Detroit	Michigan Neonatal Biobank
MI	Ann Arbor	University of Michigan Biorepository
MI	Ann Arbor	University of Michigan Human Breast Cancer Cell Lines
MN	Rochester	Mayo Clinic Biobank
MN	Rochester	Mayo Validation Support Services Biobank
MN	Rochester	Mayo Mitochondrial Disease Biobank
MN	Minneapolis	Minnesota Liver Tissue Cell Distribution System
MN	Minneapolis	University of Minnesota Tissue Bank
MO	St Louis	Washington University Tissue Bank
NC	Durham	Duke Biospecimen Repository
NC	Durham	Duke Biobank
NC	Durham	Duke Cancer Tissue Bank
NC	Greensboro	Chordoma Foundation Biobank
NJ	Princeton	Autism Tissue Bank
NJ	Camden	Coriell Stem Cell Biobank
NJ	Hackensack	Hackensack University Tissue Bank
NJ	Edison	Musculoskeletal Transplant Foundation
NJ	Piscataway	Rutgers Cell & DNA Repository
NY	Syracuse	Central New York Eye & Tissue Bank
NY	New York	Columbia U Brain Bank
NY	New York	Mantle Cell Lymphoma Cell Bank
NY	New York	Manhattan HIV Brain Bank
NY	New York	Mount Sinai Biobank
NY	New York	New York University Specialty Tissue Banks
NY	New York	StemSave
NY	Manhasset	North Shore LIJ Biorepository
NY	Buffalo	Roswell Park Biorepository
NY	Vestal	CardioFacio- Cutaneous Syndrome International Biobank
OH	Cleveland	Case Western Biorepository
OH	Cincinnati	Cincinnati Biobank Core Facility
OH	Cleveland	Cleveland Clinic Genomic Medicine Institute
OH	Powell	Folio Biobank

OH	Columbus	Ohio State Biorepository
OH	Columbus	Ohio State Leukemia Tissue Bank
OH	Columbus	Ohio Tissue Resource Network
OH	Athens	Ohio U Diabetes Endocrine Biorepository
OH	Shaker Heights	Cellular Technology Ltd
OR	Portland	Oregon State Health Biolibrary
OR	Portland	National Psoriasis BioBank
OR	Portland	Oregon Brain Bank
PA	Philadelphia	CHTN Eastern Division - University of Pennsylvannia
PA	Philadelphia	Fox Chase Tumor Bank
PA	Danville	Geisinger Biobank
PA	Philadelphia	National Disease Research Interchange
PA	Pittsburgh	National Surgical Adjuvant Breast and Bowel Project Tissue Bank
PA	Philadelphia	University of Pennsylvannia Tumor Tissue Bank
PA	Windber	Windber Tisue Bank
RI	Providence	Rhode Island Biobank
SC	Greenville	South Carolina Biorepository System
SC	Greenville	Greenville Hospital System Cancer Biorepository
SC	Charleston	University of South Carolina Biorepository
TN	Memphis	World BioBank
TN	Nashville	Vanderbilt Biobank
TN	Nashville	Vanderbilt University (Cooperative Human Tissue Network)
TX	Austin	BioEden Tooth Cell Bank
TX	Houston	MD Anderson Clone Bank
TX	Houston	MD Anderson Gynecologic Cancer Tissue Bank
TX	Houston	MD Anderson Pancreas Tissue Bank
TX	Houston	SeqWright Biorepository
TX	Houston	Texas Medical Center Biobank
TX	San Antonio	South Texas Tumor Bank
TX	Galveston	University of Texas Tumor Bank
VA	Manassas	ATCC
VA	Charlottesville	University of Virginia Biorepository
VA	Fairfax	Fairfax Cryobank
VA	Richmond	UNOS Network for Organ Sharing
WA	Bainbridge Island	Inflammatory Breast Cancer Biobank
WA	Seattle	International Histocompatibility Cell and DNA Bank
WI	Milwaukee	Aurora Healthcare Biorepository
WI	Marshfield	Marshfield Clinic Biobank
WI	Madison	National Stem Cell Bank
WI	Madison	WiCell
WI	Milwaukee	Wisconsin Tissue Bank

Mexican Biobanks	
State	**Biobank name**
Mexico	Genomic variability and haplotype map of the Mexican population

Asian Biobanks	
State	**Biobank name**
China	Cancer Center Tissue Bank Fudan University
China	Origene China
China	Taizhou Biobank
India	Bangalore Brain Bank
India	Narayana Hrudayalaya Tissue Bank and Stem Cell Research Center
India	Tata Memorial Hospital Tissue Bank
India	TCG Life Sciences Biobank
India	Dhruv Dental Stem Cell Bank
Japan	Biobank Japan
Japan	Japanese Collection of Bioresource Materials
Japan	Riken BRC Cell Bank
Japan	Tokyo Cord Blood Bank (Japanese)
Korea	Korean National Tissue Bank
Korea	Seoul Cord Blood Bank
Malaysia	CryoCord Stem Cell Bank
Malaysia	Malaysian Tissue Bank
Malaysia	StemLife Stem Cell Bank
Singapore	Singapore Biobank
Singapore	National University Tissue Repository
Singapore	Singapore Cord Blood Bank
Singapore	Singapore Tissue Network
Singapore	StemCord Cord Blood Bank
Taiwan	Taiwan Biobank (Chinese)
Thailand	Asia Pacific Association of Tissue Banking
Thailand	Bankok Biomaterial Center

Australian Biobanks	
State	**Biobank name**
Adelaide	Australian Prostate Cancer Tissue Bank
Auchenflower	Ventyx Wesley Research Institute Biobank
Brisbane	Queensland Children's Tumour Bank
Carlton Victoria	Victorian Cancer Care Biobank
Chatswood	Multiple Sclerosis Research Australia Brain Bank
Clayton South	ASPREE Healthy Ageing Biobank

East Melbourne	Austrailasian Biospecimen Network
Queensland	Austrailasian Leukemia & Lymphoma Tissue Bank
Randwick	Australian Brain Bank
Sydney	Genetic Repositories Australia
Sydney	Westmead Paediatric Tumour Bank
Westmead	Australia Breast Cancer Tissue Bank
Westmead	CellBank Australia

Middle East Biobanks	
State	**Biobank name**
Iran	Iran National Tumor Bank
Israel	Sheba Medical Center Tissue Bank
Israel	Taburit Umbilical Cord Blood Bank
Israel	National Laboratory for the Genetics of Israeli Populations

Animal & Plant Biobanks	
State	**Biobank name**
Austria	VetBioBank
Canada	BC Fisheries Living Gene Bank
Ecudaor	Banco de datos de biodiversidad del Ecuador
Germany	EUPRIMNet Primate Biobank
Israel	Israel Plant Gene Bank
Italy	Global Crop Diversity Trust
Japan	Japanese Collection of Bioresource Materials
Japan	National Institute of Agrobiological Sciences Genebank
Malaysia	Renexus Group
New Zealand	Rare Breeds Gene Bank
New Zealand	NZ Rare Disease Biobank
Norway	Marbank National Marine BioBank
Norway	Nordic Genetic Resource Centre (Animals & Forestry)
Norway	Svalbard Global Seed Vault
Sweden	Nordic Genetic Resource Centre (Plants)
UK	Rare Breed Survival Trust
US	Aged Rodent Tissue Bank
US	Alpha Genesis
US	Bioreclamation
US	Folio Mouse Biobank
US	KBar Livestock
US	Magellan Bioscience Group
US	Marine Mamal Tissue Bank
US	Mouse ES Stem Cell Bank
US	NCI National Products Branch
US	SVF Foundation

Appendix 2. All subjects (patients and healthy subjects) collected in biobank in table are listed. For each of them an identification code and the genotype beta globin are given. Only in β-thalassemia patients polymorphisms HPFH related known were investigated

CODE	GENOTYPE	BCL11A *rs*1427407	BCL11A *rs*10189857	HS1L-cMYB *rs*9399137	XmnI *rs*7482144
Fe3	β^{+}IVSI-110/β^{+}IVSI-110	GG	GA	TT	-/+
Fe6	β^{0}39/β^{0}39	GG	AG	TT	-/-
Fe8	β^{0}39/β^{0}39	GT	AG	TT	+/-
Fe9	β^{0}39/β^{0}39	GG	GG	TT	-/+
Fe13	β^{0}39/β^{0}39	GT	AG	TC	-/-
Fe15	β^{0}39/β^{0}39	GT	GA	TC	-/-
Fe23	β^{+}IVSI-110/β^{0}IVSI-1	GG	AA	TC	-/-
Fe24	β^{+}IVSI-110/β^{+}IVSI-110	GG	GG	TT	-/-
Fe25=Fe49	β^{+}IVSI-110/β^{+}IVSI-110	GG	AA	TC	-/-
Fe28	β^{0}39/β^{0}44	GT	GA	TC	-/+
Fe34	β^{+}IVSI-110/β^{0}39	GT	GA	TT	-/+
Fe35	β^{+}IVSI-110/β^{+}IVSI-110	GG	AA	TC	-/-
Fe37	β^{+}IVSI-110/β^{+}IVSI-110	GG	AG	TT	+/-
Fe38	β^{+}IVSI-6/β^{+}IVSI-6	GG	AA	TC	-/-
Fe42	β^{+}IVSI-110/β^{0}39	GG	GG	TT	-/-
Fe43	β^{+}IVSI-6/β^{+}IVSI-110	GT	AG	TC	-/-
Fe46	β^{0}39/β^{0}39	GT	AG	TT	-/-
Fe52	β^{+}IVSI-110/β^{+}IVSI-110	GG	GA	TC	-/-
Fe57	β^{+}IVSI-6/β^{+}IVSI-6	GG	AA	TT	-/-
Fe64	HbS/β^{0}39	GG	AA	TC	-/-
Fe67	HbS/β^{+}IVSI-6	GG	GA	TT	-/-
Fe69	HbS/HbS	GG	GA	TT	-/-
Fe73	β^{0}39/β^{0}39	GG	GG	TC	+/-
Fe75	HbS/β^{+}IVSI-110	GG	AG	TT	-/-
Fe78	HbS/β^{0}IVSI-1	GG	AA	TC	-/-
Fe80	β^{+}IVSI-110/β^{0}39	GG	AG	TC	-/-
Fe81	HbS/HbS	GG	AA	TT	-/-
Fe82	HbS/β^{0}IVSI-1	GG	AG	TC	-/-
AVLTA	β^{+}IVSI-6/β^{0}39	GG	AA	TT	-/-
AVLTC	β^{0}39/β^{+}5'UTR+20nt	GT	AG	TT	-/-
AVLTD	β^{+}IVSI-110/β^{0}39	GG	AG	TT	-/-
AVLTE	β^{+}IVSI-110/β^{0}39	GT	AA	TC	-/-
AVLTF	β^{0}39/β^{0}39	GT	AA	TC	+/-
AVLTG	β^{+}IVSI-110/β^{0}39	GG	AG	TC	-/-
AVLTH	β^{0}39/β^{0}39	GG	AG	TT	-/-
AVLTI=Fe10	β^{+}IVSI-6/β^{0}39	GG	AA	TT	-/-
AVLTL	β^{+}IVSI-6/β^{0}39	GG	AA	TT	-/-
AVLTM	β^{0}39/β^{0}39	GG	AG	TT	+/+
AVLTN	β^{+}IVSI-110/β^{0}39	GT	AA	TC	-/-
AVLTO	β^{0}39/β^{0}28(+C)	GT	AA	TT	-/-
AVLTP	β^{0}39/β^{0}39	GT	AG	TC	-/-
AVLTQ	β^{+}IVSI-6/β^{0}39	GG	AG	TT	-/-
AVLTR	β^{0}39/β^{0}39	GG	AA	TC	-/-

AVLTS	$\beta^0$39/$\beta^0$39	GG	AG	TC	-/-
AVLTT	$\beta^0$39/$\beta^0$39	GG	AG	TC	-/-
AVLTU	$\beta^0$39/$\beta^0$39	GG	AA	TT	-/-
AVLTV	β^+IVSI-110/β^+IVSI-110	GG	AG	TC	-/-
AVLT21	$\beta^0$39/$\beta^0$39	GG	AA	CC	-/-
AVLT22	β^+IVSI-110/$\beta^0$39	GG	AG	TT	-/-
AVLT23	β^+IVSI-110/$\beta^0$39	GG	GA	CC	-/-
AVLT24=Fe29	$\beta^0$39/$\beta^0$39	TT	AA	TT	-/+
AVLT25	$\beta^0$39/$\beta^0$39	GG	AA	TT	-/-
AVLT27=Fe50	$\beta^0$39/$\beta^0$39	GG	GA	TT	-/-
AVLT28	β^+IVSI-110/$\beta^0$39	GG	GA	TT	-/-
AVLT29	β^+IVSI-110/$\beta^0$39	GT	AA	TT	-/-
AVLT31	β^+IVSI-110/$\beta^0$39	GT	GA	TC	-/-
AVLT32	$\beta^0$39/$\beta^0$39	GG	GA	TT	-/+
AVLT33	$\beta^0$39/$\beta^0$39	GT	AA	TC	-/-
AVLT34	β^+IVSI-110/$\beta^0$39	GG	AG	TC	-/-
AVLT35	β^+IVSI-110/$\beta^0$39	GG	GG	TC	-/-
AVLT36	β^+IVSI-110/$\beta^0$39	GG	AG	TT	-/-
AVLT37	$\beta^0$39/$\beta^0$39	GT	AA	TT	-/-
AVLT39	β^+IVSI-110/β^+IVSI-110	GG	AG	TT	-/-
AVLT40	β^+IVSI-110/$\beta^0$39	GG	AG	TC	-/-
dZC	wt/wt	nd	nd	nd	nd
dPL	wt/wt	nd	nd	nd	nd
dRoMuLu	wt/wt	nd	nd	nd	nd
dRoDeAA	wt/wt	nd	nd	nd	nd
dRoDaCr	wt/wt	nd	nd	nd	nd
dGiAg	wt/wt	nd	nd	nd	nd
dTG	wt/wt	nd	nd	nd	nd
dPF	wt/wt	nd	nd	nd	nd
dLePe	wt/wt	nd	nd	nd	nd
dNB	wt/wt	nd	nd	nd	nd
dLCC	wt/wt	nd	nd	nd	nd

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