International Journal of
HEMATOLOGY
Supplement II

Carrier Screening and Genetic Counselling in β-Thalassemia

Antonio Cao

Dipartimento di Scienze Biomediche e Biotecnologie, Università Studi Cagliari, Italy

Abstract

This paper review the most important aspects of carrier detection procedures, genetic counselling, population screening and prenatal diagnosis of β-thalassemias. Carrier detection can be made retrospectively, following the birth of an affected child or prospectively. Several programmes, with the aim of preventing homozygous β-thalassemia, based on carrier screening and counselling of couples at marriage; preconception or early pregnancy, are operating in several Mediterranean at-risk populations. These programmes have been very effective, as indicated by increasing knowledge on thalassemia and its prevention by the target population and by the marked decline of the incidence of thalassemia major. Carrier detection is carrierd out by haematological methods followed by mutation detection by DNA analysis. Prenatal diagnosis is accomplished by mutation analysis on PCR-amplified DNA from chorionic villi. Future prospects include automation of the process of mutation-detection, simplification of preconception and preimplantation diagnosis and fetal diagnosis by analysis of fetal cells in maternal circulation.

This paper review the most important aspects of carrier detection procedures, genetic counselling, population screening and prenatal diagnosis of β-thalassemias.

Carrier detection can be made retrospectively, following the birth of an affected child or prospectively. Several programmes, with the aim of preventing homozygous β thalassemia, based on carrier screening and counselling of couples at marriage; preconception or early pregnancy, are operating in several Mediterranean at-risk populations.

These programmes have been very effective, as indicated by increasing knowledge on thalassemia and its prevention by the target population and by the marked decline of the incidence of thalassemia major. Carrier detection is carrierd out by haematological methods followed by mutation detection by DNA analysis. Prenatal diagnosis is accomplished by mutation analysis on PCR-amplified DNA from chorionic villi.

Future prospects include automation of the process of mutation-detection, simplification of preconception and preimplantation diagnosis and fetal diagnosis by analysis of fetal cells in maternal circulation.

1. Introduction

The β-thalassemias are a markedly heterogeneous group of autosomal recessive disorders resulting from reduced (β^+) or absent (β^0) production of the β-globin chains, which together with the n-chains make up the hemoglobin tetramere ($\alpha_2\beta_2$) [1,2]. The shortage of β-chains results in an excess of unassembled α-chains, which precipitate damaging the membrane and determining premature apoptosis of the red blood cell precursors, thereby resulting in ineffective erythropoiesis.

Three different clinical and haematological conditions are recognized i.e. the β-thalassemia carrier state (heterozygous β-thalassemia), thalassemia intermedia, and thalassemia major. The latter two result from homozygosity or compound heterozygosity for -thalassemia alleles, Homozygosity refers to the presence of the same mutation in both copies of the gene. Compound heterozygosity refers to the concept that each copy of the gene is mutated, but the mutation is distinctly different.

The β-thalassemias have a remarkably high frequency in the Mediterranean area the Middle East, the Far East, and East Asia. A relatively high incidence is also observed in people of African origin. However, due to

population flow, the β-thalassemias are now widespread and also occur in Continental Europe, North and South America, and Australia. The best available estimate indicates that approximately 240 million people worldwide are heterozygous for β-thalassemia and at least 200,000 affected homozygotes are born every year [1-3].

The homozygous state for β-thalassemia usually result in transfusion-dependent thalassemia major and less frequently in milder forms referred to as thalassemia intermedia whose molecular mechanism and criteria for identification will be discussed later on. In thalassemia major life expectancy with a regular blood transfusion program and iron chelation with Desferrioxamina B extend into the 3rd decade.

An alternative to traditional management is bone marrow transplantation from HLA identical siblings which, in patients at low risk (absence of iron overload and iron mediated parenchimal damage) result in a disease-free survival of about 90-95%.

In the past decades, the molecular pathology of the β-thalassemias and the mutation-phenotype relationships has largely been elucidated. This knowledge has been applied to carrier identification and prenatal diagnosis in a number of Mediterranean populations and has resulted in a dramatic reduction in the homozygous state in several populations at risk.

Herein, we review the most important aspects of carrier detection procedures, genetic counseling population screening and prenatal diagnosis.

2. Carrier Detection

In preventive genetics, a carrier detection procedure should be designed that should be precise enough to avoid missing any couple at risk. If even a single carrier is missed, the entire preventive programme may be discredited.

Heterozygous β-thalassemia, either the β^0 or the β^+ type, is characterized by high red blood cell count, microcytosis, hypochromia, increased haemoglobin A_2 (HbA_2) levels and unbalanced u-globin/non-u-globin chain synthesis. However several environmental or genetics factors may modify this haematological phenotype, thereby causing problems in carrier identification (Table 1).

Although iron deficiency may decrease the high HbA_2 levels typical of heterozygous β-thalassemia, in our experience these levels remain within the β-thalassemia carrier range unless a severe anemia is present.[4] In any case, iron studies may lead to the ruling out of associated iron deficiency.

In many carrier detection procedures the preliminary selection of individuals at risk of being heterozygous for a form of thalassemia is based on MCV-MCH determination. However, it is worth noting that double heterozygotes for β-thalassemia and α-thalassemia could have normal MCV and MCH values and may therefore be missed by this approach [5.6]. In carriers of β-thalassemia the α-thalassemia determinants that can give this effect are the deletion of 2 of the 4 α-globin structural gene and the non-deletion mutations affecting the major α_2- globin gene.

It is therefore imperative that, at least in populations in which both α and β-thalassemia are prevalent; in addition to MCVand MCH determination, the first set of tests of the carrier detection procedure should include HbA_2 quantitation. In the Sardinian population, for instance, because of the high frequency of α-thalassemia approximately 3.5% of β-thalassemia heterozygotes have normal MCH and MCV values and could be missed by MCH and MCV basal screening procedures. A high frequency of α and β thalassemia is common in many populations at risk in the Mediterranean area, the Middle and the Far East.

Elevation of HbA_2 is the most important feature in identifying heterozygous β-thalassemia [2]. However, a number of heterozygotes for β-thalassemia may have normal or borderline HbA_2 levels [7] (Table 1). The first groups of these atypical carriers are heterozygotes for some mild β^+ thalassemia mutations, i.e. mutations associated with a consistent residual output of β-globin chains from the affected β locus. Typical examples in this category is that of the heterozygotes for the β^+ IVS1 nt6 T→C mutation. A normal HbA_2 level is also a feature of the β and α-thalassemia double heterozygotes who, nevertheless, maintain low MCV and MCH values. These double heterozygotes should be differentiated from α-thalassemia carrier by globin chain synthesis analysis and or gene analysis. γδβ and δβ-thalassemias also have normal HbA_2. However δβ-thalassemia is easily defined by the presence of thalassemia-like hematological features and by the marked increase of the HbF, which is heterogeneously distributed (Table 2).

Another major problem in carrier screening is the identification of the silent β-thalassemia or the triple α-globin gene arrangement, both of which may lead to the production of intermediate forms of thalassemia by

Table 1.
Heterozygous β-thalassemia: Phenotype Modifications.

Phenotype	Genotype
Normal red cell indices	α and β thalassemia interaction
Normal Hb A_2 level	• iron deficiency • coinheritance of δ and β thalassemia • some mild β thalassemia mutations • γδβ thalassemia
Normal red cell indices and Hb A_2 level (silent)	• silent β thalassemia mutations α globin gene triplication
Severe heterozygous β thalassemia	• hyperunstable haemoglobin • coinheritance of heterozygous β thalassemia and triple α globin gene

interacting with typical heterozygous β-thalassemia.

Silent β-thalassemias are characterized by having normal MCV and MCH values normal HbA_2 and HbF, and are solely defined by the slight imbalance in the α-globin/non α-globin synthesis [7,8]. However, on examining the hematological features of these carriers, it should be possible sometime to find some borderline HbA_2 or MCV and MCH values which may signal the presence of atypical β-thalassemia, thus calling for further studies. The most common silent β-thalassemia is the β^+- 101 C→T mutation, the other types being very rare [8]. Nevertheless, the phenotype resulting from the triple α- globin gene arrangement is at times completely silent, according to globin chain synthesis analysis. It is worth nothing, however, that compound heterozygotes for silent and typical β-thalassemia and double heterozygous for typical β-thalassemia and the triple α-globin gene arrangement result in attenuated form of thalassemia [9-11].

An extreme, although rare, instance of thalassemia gene combination, which may result in in carrier diagnosis pitfalls is the presence of α, δ, and β- thalassemia together which may lead to a complete silent phenotype.

On the basis of this analysis in carrier identification we suggest of the use the flow chart outlined in the Figure 1 [12].

The first group of tests includes MCV and MCH determination and Hb chromatography by HPLC. HPLC may lead to detection of the most common clinically relevant Hb variants, such as HbS, HbC, HbD Punjab, HbO Arab and HbE, all of which may result in a sickle disorder in homozygosity or compound heterozygosity.

HPLC may also be used to quantative HbA_2 and HbF [13]. It should be noted that HPLC is also capable to detecting Hb Knossos, a mild β-thalassemia allele, which is not defined by the commonly used electrophoretic procedures in Hb analysis. With this flow chart, only the silent β-thalassemia and the triple α-globin gene arrangement may be missed. In the presence of low MCH and MCV and elevated HbA_2 levels, a diagnosis of heterozygous β-thalassemia is made.

A phenotype characterized by microcytosis, hypochromia, normal/borderline HbA_2 and normal HbF may result from iron deficiency, α-thalassemia, γδβ-thalas-

Table 2.

β-thalassemia Heterozygotes with Normal or Borderline Hb A_2.

Reduced MCV	Normal MCV
IVS I-6 T→C	-101 C→T
δ-thalassemia+β thalassemia	-92 C→T IVS II -844 C→G Triplicated α gene

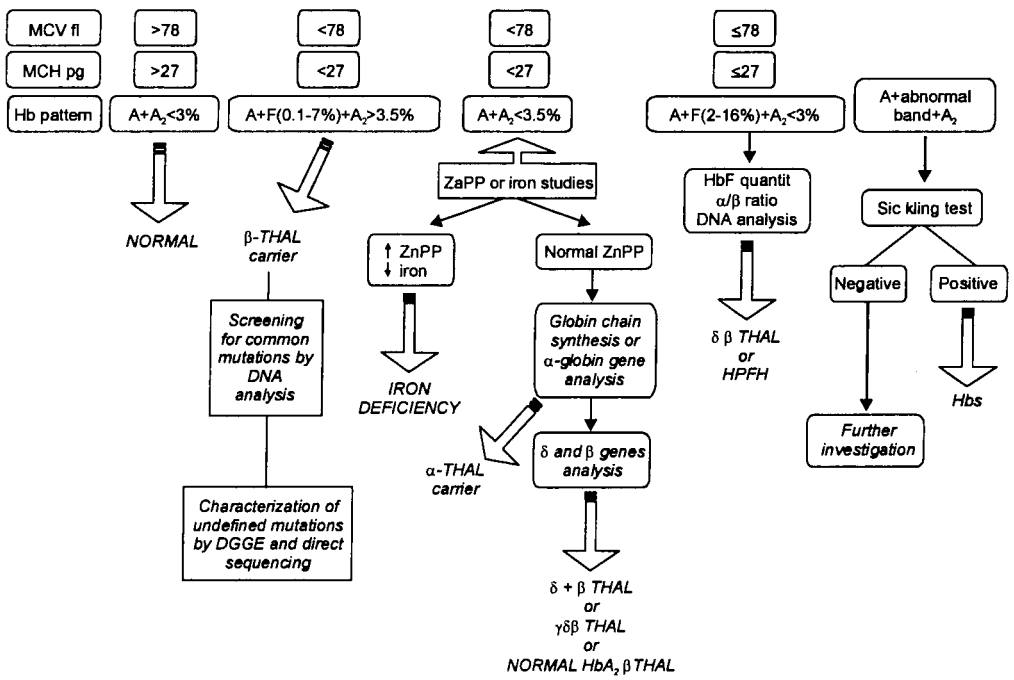

Figure 1. Flow chart used in the carrier detection program at the Ospedale Regionale per le Microcitemie in Cagliari.

semia, β+δ thalassemia or mild β-thalassemia. After exclusion of iron deficiency by erythrocyte ZnPP determination and evaluation of transferring saturation, the different thalassemia determinants leading to this phenotype are discriminated by globin chain synthesis analysis and eventually by α, δ and β-globin gene analysis.

In the presence of normal MCV and borderline HbA_2 levels, we suspect the presence of a silent mutation (for instance β^+-101 C→T, β^+ -92 C→T, or β^+IVS2 nt844 C→G) or the triple α-globin gene arrangement and proceed directly to α and β-globin gene analysis because in many of these cases the α/β ratio could be normal.

Definition of the type of thalassemia in these carriers is recommended solely when they mate with a someone with a typical high HbA_2/-thalassemia or with a carrier of an undetermined type of thalassemia.

In those rare cases showing normal or low MCH and MCV, normal or reduced HbA_2 levels and high HbF, we suspect the presence of δβ-thalassemia which should be differentiated from HPFH. The distinction between δβ-thalassemia and HPFH is accomplished by the analysis of the red blood cell distribution of HbF, which is heterogeneous in δβ-thalassemia and homogeneous in HPFH, by globin chain synthesis analysis (normal in HPFH at unbalanced in δβ-thalassemia) and/or β cluster gene analysis.

In population with a relatively low incidence of both β and α-thalassemia screening by MCV and MCH or osmotic fragility could be acceptable, because in this condition the number of false negative resulting from double heterozygosity for α and β thalassemia may be very low.

3. Awareness Raising and Population Education

Since the late 1970s, population screening programs of adults at child-bearing age, genetic counselling, and prenatal diagnosis have been introduced among the populations at risk in the Mediterranean area, including Sardinians, continental Italians, Greeks, and Cypriots. Although details regarding these programs have been described elsewhere, the most relevant characteristics are discussed herein, focusing on those that have been operating over a long period, and about which a larger amount of data is available (Sardinia, Greece, Cyprus) [1,14-17]. These programs were directed either to couples with previous affected children (retrospective diagnosis) or to childless couples (prospective diagnosis). Though very useful for individual families, retrospective diagnosis has a limited effect on the control of homozygous β-thalassemia at the population level. All these programs have been characterised by intensive education campaigns with the population. Education has been conducted mostly through mass media, including local newspapers, radio, television, and magazines (Figure 2). In some programs, especially in Sardinia at the beginning, lectures have been

Organized for the general public in factories, large stores, and shops. Family physicians, obstetricians, pediatricians, midwives, and nurses have all been trained in this new field of preventive genetics. Posters and information pamphlets have been made available at marriage registry offices, general practitioners and obstetricians offices, as well as family clinics. The pamphlets provide the following information: (a) that the β-thalassemia carrier state can be easily identified with the appropriate methodology; (b) who should get the test, where, and how carrier tests are available; (c) that heterozygotes are not at a disadvantage; (d) the natural history of the homozygous condition is described and it is stressed that this is a severe disease for which a continuous transfusion program combined with iron chelation therapy is needed for survival, and that cure may be achieved through bone marrow transplantation in the limited proportion of homozygotes that have an HLA identical donor sibling; and (e) that the homozygous state can be safely prevented by that fact that couples identified as being at risk, in the case of both members being carriers, have a number of options, including fetal diagnosis, to avoid giving birth to affected children. Every year in Cyprus, magazines and booklets are distributed. Seminars with parents' associations are held periodically with the purpose of planning proper means of information to educate the population. In addition, community-based parents' associations have played an invaluable role as an influential group, by increasing the financial support for public education and providing psychological assistance to patients and families. In Cyprus the Greek Orthodox Church has made a substantial contribution by requiring marriage candidates to produce a certificate stating that β-thalassemia carrier testing has been carrierd out. Educational videotapes have also been introduced in secondary schools to teach students about inherited anemias and especially β-thalassemias. In Sardinia, for the past three years this educational session has been followed by carrier testing at the end of secondary school (see later), This procedure has indirectly led to raise the awareness of β-thalassemia among the tested children's parents. Since 1980 the educational campaign has not been held at the population level in

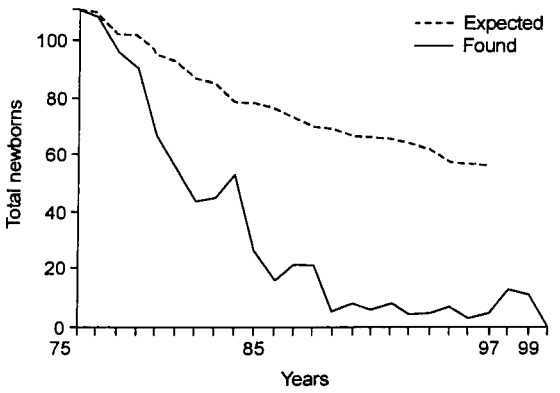

Figure 2. Prevention of β-thalassemia in Mediterranean at-risk population.

Sardinia. The only educational activity carrierd out at present is the teaching of β-thalassemia in secondary schools and the introduction of β-thalassemia as a topic in courses for doctors, nurses, and obstetricians.

A critical evaluation of the information channel through which the population at large has been informed has been carrierd out twice in Sardinia; once at the beginning of the program and again more recently [12]. At the start of the program most spouses were informed through the mass media (44%), general practitioners (31%), and obstetricians (23%). This trend has been modified recently, since the information is now given by physicians (family doctors, obstetricians, and genetic counsellors), and reaches more than 70% of the population.

4. Target Population

The target population for screening has been couples at marriage, preconception, or early pregnancy, Nevertheless, even nowadays a limited number of couples request testing where already pregnant, which may lead to marked emotional stress. In Cyprus and Sardinia the number of young unmarried people requesting screening is increasing steadily, this being a clear indication of improved awareness of the disease and related prevention methodology. As mentioned above, both in Cyprus and in Sardinia screening of adolescents and school children has been introduced recently [1,14].

In these populations, heterozygote screening has been carrierd out on a voluntary basis. Though informed consent was not requested in these programs, every effort has been made to inform the patient about the meaning of the carrier state and the potential adverse effect associated with its detection.

5. Efficacy of Carrier-Screening

At present, in Cyprus and Sardinia at least, the large majority of couples both before and after marriage present voluntarily at the screening centres for testing and counselling. In both countries adequate facilities for screening have been provided; in Sardinia for instance we have 13 centres spread all over the country (Figure 3). Screening of relatives, informed about the risks by He counselled carrier, has strengthened the efficacy of the screening process. In Sardinia, for instance we managed to detect most of the couples at risk, i.e. approximately 90% of those predicted on the basis of the carrier rate, by screening only a small proportion (~11%) of the population at child-bearing age [14].

6. Counselling

Counselling has been non-directive and generally based on a private interview with the individual or couple [1,14]. The provided information is aimed at giving an informed basis on which to make a reproductive choice, i.e. birth control, mate selection, adoption, fetal testing or artificial selection by a donor. Particular emphasis has been given to details of fetal analysis, i.e. sampling procedure, risk for the fetus, failure, and misdiagnosis. An explanatory booklet is usually provided. The predicted natural history of the disease based on the genotype at the α, β, and γ loci is usually discussed. In Sardinian families with previous normal or heterozygous children, we propose and eventually carry out HLA typing on fetal DNA, to assess whether a sibling is HLA-identical, and thus a suitable bone marrow donor. This information allows the parents to have further alternatives in the event of an affected fetus, i.e. bone marrow transplantation. Prenatal diagnosis uptake has been very high since first trimester diagnosis became available (see later) [18]. Finally, in the counselling session, we inform the carriers about the risks to their relatives and recommend that they should inform them so that they can choose to take the test as well. In Sardinia most relatives opted to be tested.

7. Molecular Diagnosis

In couples at risk identified by the above described carrier detection procedure, the specific β-thalassemia mutation is defined by one of the several available PCR-based methods [19]. The most widely used procedures are primer-specific amplification (ARMS) [20], and reverse dot blot analysis (RDB) [21] (Table 2) with a series of primers or probes complementary to the most common mutations in the specific population [14]. As mentioned above, in each population at risk, β-thalassemia results from a limited number (4-20) of common mutations and a variable number of rare mutations. Alternatively, the specific mutation may be defined by denaturing gradient gel electrophoresis (DGGE), which has the additional advantage of detecting also the unknown mutations [3,24]. Following localisation by DDGE, the mutation is defined through direct sequencing of the DNA contained in the abnormal migrating fragment. If the mutation is not detected by DDGE, we search for the presence of small deletions through polyacrylamide gel electrophoresis of the PCR-amplified products prepared for ARMS or RDB analysis, which may lead to the detection of small deletions of the -globin gene, whose presence may be suspected by finding very high HbA_2 levels. Larger deletions of the cluster may be identified with restriction fragment length polymorphism analysis carrierd out with PCR-based procedures. In a very limited number of cases (0.1% in our experience), direct sequencing from position -600 bp to 60 bp downstream from the β-globin gene failed to detect a disease- causing mutation which may lie elsewhere in the genome (locus control region or genes coding far transcription factors). Counselling and decision-making can be quite difficult in these cases (see later) [25]. Finally mutation detection by either DNA high pressure liquid chromatography (DHPLC) or oligonucleotide microchip array are very appealing, new approaches [26,27].

As previously mentioned, δ-globin gene analysis may be necessary to define double heterozygotes for δ and

β-thalassemia which may be mistaken for α-thalassemia heterozygotes. The suspicion of interacting δ-thalassemia may arise when borderline HbA_2 levels are found or when family studies show segregating δ-thalassemia (characterised by normal MCV-MCV and low HbA_2) and β-thalassemia. Identification of δ and β double heterozygotes, however, may be accomplished by globin chain synthesis analysis and/or α, β and δ-globin gene analysis.

Definition of the δ-thalassemia mutation may be carrierd out using one of the previously mentioned PCR-based methods. As in β-thalassemia, also in δ-thalassemia, each population at risk has its own spectrum of common δ-thalassemia mutations, that may be defined through a limited number of specific primers/probes. In Sardinians, for instance, only three δ-thalassemia mutations have been detected so far. The list of δ-thalassemia mutations is available at the repository of the human β and δ-globin gene mutation [28]. Though most of the δ-thalassemia determinants are in trans (on opposite chromosomes) to β-thalassemia, some have also been detected in cis (on the same chromosome) [29-34].

Definition of the α-globin gene arrangement may be carrierd out to discriminate between heterozygosity for α-thalassemia and double heterozygosity for δ and β-thalassemia or γδβ-thalassemia. This analysis could also be useful in defining co-inherited α-thalassemia in homozygous β-thalassemia, which may lead to the prediction of a mild clinical condition. Deletion α^0 or α^+-thalassemias are detected by PCR using two primers flanking the deletion breakpoint, which amplify a DNA segment only in presence of specific deletions. As a control, DNA from a normal chromosome is simultaneously amplified using one of the primers flanking the breakpoint and a primer homologous to a DNA region deleted by the mutations. Non-deletion α-thalassemia may be detected by restriction endonuclease analysis or allelic oligonucleotide specific probes on selectively amplified α_1 and α_2-globin genes [35-36].

Definition of co-inherited HPFH determinants can be useful in predicting the development of the phenotype of an affected fetus. As mentioned above in fact, on increasing the γ-chain output, co-inherited HPFH with homozygous β-thalassemia may lead to a milder phenotype.

The presence of high HbF in the parents may lead to the suspicion of double heterozygosity for -thalassemia and HPFH. At present, the molecular definition of HPFH is limited to two forms of non-deletion HPFH, namely -196 C→T Aγ and -158 C→T Gγ, which have been proved to be capable of ameliorating the clinical phenotype of homozygous β-thalassemia. These HPFH determinants may easily be detected through restriction endonuclease or dot blot analysis with oligonucleotide-specific probes on PCR-amplified DNA. In the future, the identification of the HPFH determinants as linked or unlinked to the -cluster may lead to improving the capability of predicting the phenotype.

8. Prediction of a Mild Phenotype

Informative and reliable genetic counselling requires an accurate description of the clinical phenotype. This is particularly important when evaluating carriers whose offspring are at risk for thalassemia intermedia [37-39]. Under the diagnosis of thalassemia intermedia we comprehend a spectrum of disorders of varying severity that lies between that seen for the β-thalassemia carrier state and thalassemia major. The large majority of patients with thalassemia intermedia are homozygotes or compound heterozygotes for β-thalassemia. By identifying those capable of molecular compensation, clinical disease severity can be predicted. Studies carrierd out in the past few years have delineated a number of molecular mechanisms capable of reducing the ration of α: non α (β+γ) haemoglobin chains. Importantly, this ratio correlates with the clinical phenotype. An example is the inheritance, in homozygosity or compound heterozygosity, of a mild or silent β-thalassemia mutation. These are associated with a substantial residual output of β-thalassemia from the affected locus (Table 3). Another example is homozygosity or compound heterozygosity for typical β-thalassemia and δβ-thalassemia. This is associated with high γ-chain production. An alteration of the α: non-α ratio may occur as a result of co-inheritance of α-thalassemia [37,40,41]. This leads to a reduction in α-globin gene output and hence to a reduced/non inbalance. Co-inheritance of a genetic determinant capable of sustaining continuous production of γ-chains in adult life (deletion and non-deletion hereditary persistence of fetal Hb [HPFH]), may also cause altered ratios of the α: non-α globin chains. finally, some cases of mild β-thalassemias result from the co-inheritance with homozygous β-thalassemia of heterocellular HPFH (alterations at loci other than the globin chain loci)). This condition is genetically heterogeneous and may be linked or unlinked to the α-globin gene cluster. To date, three loci have been mapped: one on the Xq 22.2-22.3 region, one on chromosome 6 (6q22.3-

Table 3.
PCR Based Procedure.

Known mutations
ARMS (amplification refractory mutation system)
RDB (reverse dot blot)
OLA (oligonucleotide ligation assay)
Enzyme restriction digestion
Primer specific restriction map modification
Unknown mutations
DGGE (denaturing gradient gel electrophoresis)
SSCP (single strand conformation polymorphism)
PTT (protein truncation test)
CCM (chemical cleavage method)
ECM (enzyme cleavage method)

23.1) and one on chromosome [8,42,43] Nevertheless, many others are likely to exist as well [44]. More rarely thalassemia intermedia results from double heterozygosity for the triple α-globin gene arrangement, which is associated with high α-chain output, and typical β-thalassemia, or also from the presence of highly hyper unstable β-globin (dominant β-thalassemia) [45,48].

We may conclude that, besides β-globin gene analysis, and γ globin gene analysis has the potential to better define the phenotype thus improving genetic counselling. However, it should be noted that only inheritance of homozygosity for mild/silent β-thalassemia is consistently associated with a mild phenotype.

9. Prenatal Dignosis

Prenatal diagnosis for the β-thalassemias was carrierd out successfully in the 1970s, through the use of globin chain synthesis analysis of foetal blood [49]. Thanks to the molecular characterisation of the β-thalassemias, the introduction of chorionic villous analysis, and the development of PCR-based methods for DNA analysis, it became possible to assess the fetal genotype within the first trimester of pregnancy by foetal trophoblast analysis [24,50-51].

10. Fetal Sampling

In the 1970s fetal blood for analysis was obtained by placentocentesis, later in the early 1980s by foetoscopy, and finally since 1984 by cordocentesis. With the introduction of the methodologies that allowed the direct detection of fetal DNA mutations, we used amniocentesis (1983-1984), transcervical chorionic villous sampling (TC-CVS) (1984-1986), and, since 1986 transabdominal chorionic villous sampling (TA-CVS) with the free hand technique [52-54]. In our experience TA-CVS appears to be the safest and most reliable procedure. Additional advantages are simplicity, speed, better patient acceptance [55], and lower risk of infection and bleeding. Moreover TA-CVS can be performed at any gestation period, which is particularly important for couples who present late in gestation. The use of prenatal diagnosis increased dramatically (99.4% versus 93%) after the introduction of CVS [55].

11. Fetal DNA Analysis

Nowadays fetal DNA is analysed by one of the above mentioned PCR-based methods [14,24,56]. In order to reduce the occurrence of misdiagnoses, in our centre we use two different PCR-based procedures for each case (e.g. RDB hybridisation and primer-specific amplification).

DNA analysis gave very accurate results. We observed misdiagnosis only by fetal blood analysis and by oligonucleotide hybridisation on electrophoretically separated non-amplified DNA fragments, but never by PCR-based methods. However, misdiagnosis may occur for several reasons: such as failure to amplify the target DNA fragment, false paternity, maternal contamination, and sample exchange. Misdiagnosis due to failure of DNA amplification may obviously be less frequent with the duplicate methodology used in our Centre. In order to limit the possibility of misdiagnosis due to false paternity or maternal contamination, besides mutation analysis, we also carry out studies of an appropriate polymorphic sequence. The effect of maternal contamination can also be limited by careful dissection of the maternal decidua from the foetal blood trophoblast under the inverted microscope.

12. Efficacy of Prevention Programs

All prevention programs have been very successful, since the large majority of populations at risk improved their knowledge of β-thalassemia and practised its prevention without any substantial adverse effect [39]. Following non-directive counselling the large majority of couples at risk opted in favour of prenatal diagnosis. Moreover, in all the populations in whom education and counselling was introduced in the Mediterranean area, we observed a substantial decline in the birth rate of thalassemia major. For example, in Sardinia, the incidence of thalassemia major declined from 1 in 250 live births to 1:4000, with an effective prevention of the large majority of cases who would have probably been born had a prevention program not been in place (Figure 2).

The most frequent reasons for the limited number of births affected with thalassemia major in our population today include the absence of information, misdiagnosis, false paternity, and adverse attitude towards prenatal diagnosis and/or unwillingness to terminate pregnancy. Very similar results have been obtained in all the other Mediterranean populations in whom screening and counselling have been introduced [1,14,16].

13. The Reason(s) for Success

If all these programs have been successful, it is first of all due to the educational campaign, that fully informed the large majority of adults about thalassemia at, or before, marriage, thus offering them the opportunity of making an informed decision about reproduction. This population education programme, at least in Sardinia, took advantage of the fact that most of the population was distributed in small villages of 2,000-3,000 inhabitants, where it was easier to spread the knowledge of thalassemia. As mentioned above, the efficacy of the screening program was magnified by the fact that the relatives of carriers and patients were also screened. Lately, the introduction of teaching on thalassemia in secondary schools may also have played a role in further reinforcing the knowledge of thalassemia. Since screening, counselling, and prenatal diagnosis were introduced through the Social Health Service, they are completely free of charge. This obviously removed potential economic access barriers to health facilities. An-

other very important prerequisite for success was the implementation of adequate facilities to meet the demand for screening, counselling, and prenatal diagnosis before the educational campaign. Finally we believe the program would not have been so successful without the collaboration of a very motivated staff, especially at critical times, such as for counselling and prenatal diagnosis.

14. Further Prospects

Technical speaking, the oligonucleotide microchip procedure [27] is very likely to be introduced. This may lead to direct mutation detection as a screening procedure and subsequent elimination of carrier detection based on haematological studies. Thanks to microchip analysis, moreover, other common disorders of our population, such as G6PD deficiency, Wilson's disease, cystic fibrosis and autoimmune polyendocrinopathy type I, may be included in the carrier screening process.

Chorionic-villous DNA analysis could be simplified by using an automated procedure, such as DNA high pressure liquid chromatography analysis (DHPLC) [26]. We have already set up the procedure of pre-implantation diagnosis through the analysis of a single blastomere from an eight cell embryo following in vitro fertilizaion [57]. The option for pre-implantation diagnosis will soon be discussed in the counselling session, especially with couples who have had several interrupted pregnancies due to affected fetuses and are therefore against further pregnancy termination. It is worth mentioning that in a study carrierd out at our Centre on preimplantation genetic diagnosis, the large majority of women who underwent CVS with subsequent pregnancy interruption prefer preimplantation diagnosis in future pregnancies [58].

As at other centres, we too are carrying out studies to make prenatal diagnosis feasible in clinical practice through the analysis of foetal cells in maternal circulation [59,60]. Promising and encouraging results have been obtained by isolation of nucleated-red blood cells by micro dissection under light microscopy and non-radioactive PCR analysis following density gradient separation of mononuclear cells from maternal blood, enrichment of fetal cells by magnetically activated cell sorting using the anti-transferrin receptor antibody, and immunostaining of fetal cells by anti-foetal or embryonic Hb antibodies.

Nevertheless, the most important challenge for the future is the organisation of this kind of program in populations where β-thalassemia is prevalent; such an enterprise is still not possible at the present state of development.

References

1. Angastiniotis M, Modell B, Boulinzhenkov V. Prevention and control of haemoglobinopathies. *Bul WHO.* 1995;73:375-386.
2. Weatherall DJ, Clegg JB. The Thalassemia Syndromes. Ed 4. Blackwell Scientific Publication, Oxford, 2001.
3. WHO Scientific Group: Control of hereditary diseases. WHO Technical Report Series 865, 1996.
4. Galanello R, Ruggeri R, Addis M, Paglietti E, Cao A. Hemoglobin A_2 in iron deficiency β-thalassemia heterozygotes. *Hemoglobin.* 1981;5:613-618.
5. Melis MA, Pirastu M, Galanello R, et al. Phenotypic effect of heterozygous α and β0-thalassemia interaction. *Blood.* 1983; 62:226-229.
6. Rosatelli C, Falchi AM, Scalas MT, et al. Hematological phenotype of the double heterozygous state for α and β-thalassemia. *Hemoglobin.* 1984;8:25-35.
7. Galanello R, Barella S, Ideo A, et al. Genotype of subjects with borderline Hb A2: Implication for β-thalassemia carrier screening. *Am J Hemat.* 1994;46:79-81.
8. Gonzales-Redondo JM, Stoming TA, Kutlar A, et al. A C-rT substitution at nt -iOl in a conserved DNA sequence of the promoter region of the β-globin gene is associated with "silent" β-thalassemia. *Blood.* 1989;73:1705-1711.
9. Galanello R, Ruggeri R, Paglietti E, et al. A family with segregating triplicated u-globin loci and β-thalassemia. *Blood.* 1983;62:1035-1040.
10. Thein SL. 51-Hakim I and Hoffbrand AB Thalassemia intermedia: a new molecular basis. *Br J Haemat.* 1984;56:333-337.
11. Kulozik AE, Thein SL, Wainscoat 15, et al. Thalassemia intermedia: interaction of the triple α-globin gene arrangement and heterozygous β-thalassemia. *Br J Haemat.* 1987;66:109-112.
12. Cao A, Rosatelli C. Screening and prenatal diagnosis of the haemoglobinopathies. In: Higgs DR & Weatherall DJ, eds. *Bailliere's Clinical Haematology. The Haemoglobinopathies.* 1993;6:263-286.
13. Galanello R, Barella S, Gasperini D, et al. Evaluation of a new automatic HPLC analyzer for thalassemia and hemoglobin variants screening. *J Autom Chem.* 1995;17:73-76.
14. Cao A, Galanello R, Rosatelli MC. Prenatal diagnosis and screening of the haemoglobinopathies. In Bailliere's Clinical Haematology. International Practice and Research: The Haemoglobinopathies (eds Higg D.R. & Weatherall D.J) p.215-238 (Bailliere Tindall, London,1998).
15. Cao A. 1993 William Allan Award Address. *Am J Hum Genet.* 1994;54:397-402.
16. Cao A, Saba L, Galanello R, Rosatelli MC. Molecular diagnosis and carrier screening for β-thalassemia. *JAMA.* 1997; 278:1273-1277.
17. Loukopoulos D. Current status of thalassemia and the sickle cell syndromes in Greece. *Sem Haematol.* 1996;33:76-86.
18. Cao A, Cossu P, Monni G, Rosatelli C. Chorionic villus sampling and acceptance rate of prenatal diagnosis. *Pren Diagn.* 1987;7:531-533.
19. Old JM. DNA-Based Diagnosis of the Hemoglobin disorders. In Steinberg MH, Forget BG, Higgs DR, Nagel RL. (eds): Disorders of Hemoglobin. Genetics, Pathophusiology, and Clinical Management. Cambridge University Press 2001, pp 941-957.
20. Newton CR, Graham A, Hepteinstall LE, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acid Res.* 1989;17:2503-2516.
21. Saiki RK, Walsh PS, Levenson CH, Erlich HA. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc Natl Acad Sci USA.* 1989;86: 6230.
22. Cai 5P, Kan YW. Identification of the multiple β- thalassemia mutations by denaturing gradient gel electrophoresis. *J Clin Invest.* 1990;85:550-553.
23. Rosatelli MC, Dozy A, Faa V, et al. Molecular characterisation of β-thalassemia in the Sardinian population. *Am J Hu Genet.* 1992;50:422-426.
24. Rosatelli C, Tuveri T, Scalas MT, et al. Molecular screening and fatal diagnosis of β-thalassemia in the Italian population. *Hum Genet.* 1992;89:585-589.

25. Murru S, Loudianos G, Porcu 5, et al. A P-thalassemia phenotype not linked to the P-globin cluster in an italian family. *Br J Haemat.* 1992;81:283-287.
26. Underhill PA, Jin L, Lin AA, et al. Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. *Genome Res.* 1997;7:947-949.
27. Yershov G, Barsky V, Belgovskiy A, et al. DNA analysis and diagnostics on oligonucleotide microchips. *Proc Natl Acad Sci USA.* 1996;93:4913-4918.
28. Huisman TH, Carver MF. The β- and 5-thalassemia repository (Ninth Edition). *Hemoglobin.* 1998;22:169-195.
29. Moi P, Paglietti E, Sanna A, et al. Delineation of the molecular basis of δ and normal HbA2 β-thalassemia. *Blood.* 1988;72:530-533.
30. Tzetis M, Traeger-Synodinos J, Kanavakis E, Metaxotou-Mavromati A, Kattamis C. The molecular basis of normal HbA2 (type 2) β-thalassemia in Greece. *Hematol Pathol.* 1994;8: 25-34.
31. Galanello R, Melis MA, Podda A, et al. Deletion S- thalassemia; the 7.2 kb deletion of Corf δβ-thalassemia in a non-β-thalassaemic chromosome [letter]. *Blood.* 1990;75:1447-1449.
32. Loudianos G, Cao A, Pirastu M, Vassilopoulos G, Kollia p, Loukopoulos D. Molecular basis of the δ-thalassemia in cis to hemoglobin Knossos variant. *Blood.* 1991;77:2087-2088.
33. Olds RJ, Sura T, Jackson B, et al. A novel δ^0 mutation in cis with Hb Knossos: A study of different genetic interactions in three Egyptian families. *Br J Haematol.* 1991;78:430.
34. Pirastu M, Ristaldi MS, Loudianos G, et al. Molecular analysis of atypical β-thalassemia heterozygotes. *Ann N Y Acad Sci.* 1990;612:90-97.
35. Miggs DR, Bowden DK. Clinical and Laboratory features of the α-thalassemia syndromes In Steinberg MH, Forget BG, Higgs DR, Nagel RL. (eds): Disorders of Hemoglobin. Genetics, Pathophysiology, and Clinical Management. Cambridge University Press 2001, pp 431-469.
36. Kattamis AC, Camaschella C, Sivera P, Surrey S, Fortina P. Human α-thalassemia syndromes: detection of molecular defects. *Am J Hematol.* 1996;53:81-91.
37. Wainscoat JS, Them SL, Weatherall DJ. Thalassemia intermedia. *Blood Reviews.* 1987;1:273-279.
38. Weatherall DJ. Phenotype/genotype relationships in monogenic disease: lessons from the thalassemias. *Nat Rev Genetics.* 2001; 2:245-255.
39. Cao A. 1993 William Allan Award Address. *Am J Hum Genet.* 1994;54:397-402.
40. Galanello R, Dessi E, Melis MA, et al. Molecular analysis of β^0-thalassemia intermedia in Sardinia. *Blood.* 1989;74:823-827.
41. Kan YW, Nathan DG. Mild thalassemia: the result of interactions of α and β-thalassemia genes. *J Clin Invest.* 1970;49: 635-642.
42. Chang YC, Smith KD, Moore RD, Serjeant GR, Dover GJ. An analysis of fetal hemoglobin variation in sickle cell disease: the relative contribution of the X-linked factor, β-globin haplotypes, α-globin gene number, gender and age. *Blood.* 1995;85:1111-1117.
43. Dover G, Smith KD, Chang YC, et al. Fetal hemoglobin levels in sickle cell disease and normal individuals are partially controlled by an X-linked gene located at Xp22.2. *Blood.* 1992;80:816-824.
44. Stamatoyannopoulos G, Grosveld F. Hemoglobin Switching. In Stamatoyannopoulos G, Majerus PW, Perlmutter RM, Varmus H (eds). The Molecular Basis of Blood Diseases, ed.3. W.B. Saunders Company, 2001, pp 135-182.
45. Galanello R, Ruggeri R, Paglietti E, Addis M, Melis MA, Cao A. A family with segregating triplicated α-globin loci and -thalassemia. *Blood.* 1983;62:1035-1040.
46. Kanavakis E, Metaxotou-Mavromati A, Kattamis C, Wainscoat JS, Wood WG. The triplicated α gene locus and β-thalassemia. *Br J Haematol.* 1983;54:201-207.
47. Kulozik AE, Them SL, Wainscoat JS, et al. Thalassemia intermedia: interaction of the triple α-globin gene arrangement and heterozygous β-thalassemia. *Br J Haematol.* 1987; 66:109-112.
48. Them SL, Hesketh C, Taylor P, et al. Molecular basis for dominantly inherited inclusion body β-thalassemia. *Proc Natl Acad Sci USA.* 1990;87:3924-3928.
49. Kan YW, Golbus MS, Klein P, Dozy AM. Successful application of prenatal diagnosis in a pregnancy at risk for homozygous β-thalassemia. *N E J Med.* 1975;292:1096-1099.
50. Kan YW, Lee KY, Furbetta M, Angius A, Cao h. Polymorphism of DNA sequence in the β-globin gene region: application to prenatal diagnosis of β-thalassemia in Sardinia. *N E J Med.* 1980;302:185-188.
51. Pirastu M, Kan YW, Cao A, Conner BJ, Teplitz RI, Wallace RB. Prenatal diagnosis of β-thalassemia. Detection of a single nucleotide mutation in DNA. *N E J Med.* 1983;309:284-287.
52. Monni G, Ibba RM, Olla G, Rosatelli C, Cao A. Chorionic villus sampling by rigid forceps: experience with 300 cases at risk for thalassemia major. *Am J Obstet Gynecol.* 1987;156: 912-914.
53. Monni G, Ibba RM, Olla G, Rosatelli C, Cao A. Prenatal diagnosis of β-thalassemia by second trimester chorionic villus sampling. *Pren Diagn.* 1988;8:447-451.
54. Monni G, Ibba RM, Lai R, et al. Early transabdominal chorionic villus sampling for couplet at high genetic risk. *Am J Obstet Gynecol.* 1993;168:170-173.
55. Monni G, Olla G, Cao A. Patient's choice between transcervical and transabdominal chorionic villus sampling. *Lancet.* 1988;i:1057.
56. Rosatelli MC, Dozy A, Faa V, et al. Molecular characterisation of β-thalassemia in the Sardinian population. *Am J Hum Genet.* 1992;50:422-426.
57. Handyside AM, Lesko JG, Tarin JJ, et al. Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. *N Engl J Med.* 1992;327:905-909.
58. Monni G, Lai R, Cau G, et al. Acceptability of preimplantation diagnosis. In From Gametes To Embryos. *Pren Diagn.* 1992;12S:S24.
59. Bianchi DW, Flint AF, Pizzimenti MF, Knoll JH, Latt SA. Isolation of fetal DNA from nucleated erythrocytes in maternal blood. *Proc Natl Acad Sci. (USA)* 1990;87:3279-3283.
60. Cheung M-C, Goldberg JD, Kan YW. Prenatal diagnosis of sickle cell anemia and thalassemia by analysis of fetal cells in maternal blood. *Nat Genet.* 1996;14:264-268.