

The background of the cover features a detailed, three-dimensional illustration of numerous red blood cells. These cells are depicted in various sizes and orientations, with a realistic biconcave shape and a textured surface. They are scattered across the white background, with some appearing in the foreground and others receding into the distance. A solid green horizontal band is positioned across the upper portion of the cover, serving as a backdrop for the title and subtitle.

PREVENTION AND DIAGNOSIS OF HAEMOGLOBINOPATHIES

A SHORT GUIDE FOR HEALTH PROFESSIONALS AND LABORATORY SCIENTISTS

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PREFACE

This booklet on the prevention and diagnosis of the haemoglobinopathies is intended to be a reference booklet serving as a reminder or a short guide directed towards health professionals and laboratory scientists undertaking haematological screening and molecular diagnosis of the thalassaemias and other haemoglobin disorders.

It is a simplified reference guide to the essential principles of prevention and diagnosis of the haemoglobinopathies and assumes a reasonable knowledge of the pathophysiology and genetics of the haemoglobinopathies, which is covered in several TIF publications on thalassaemias and sickle cell disorders. This information and the practical details of haematological screening and DNA analysis techniques can be found in the two updated TIF publications on prevention and diagnosis (principles in Volume 1 and the technical procedures in Volume 2).

The contents of this booklet have been formulated from the contributions to the above mentioned TIF publications on prevention and diagnosis and the published best practice guidelines for carrier identification and prenatal diagnosis of the haemoglobinopathies, and the author wishes to acknowledge all the authors of these publications for their expert contributions which have essentially formed the DNA of these guidelines.

John Old
Chief editor

FOREWARD

FOREWARD FROM TIF

This book is a short version of the two volume textbooks published by the Thalassaemia International Federation (TIF) in 2013 and 2014, under the title "Prevention of the thalassaemias and other haemoglobin disorders". The author has also updated some laboratory tests and procedures. The Federation regards prevention of these hereditary disorders as an important component of a comprehensive policy aiming to minimize the health burden and to provide services aiming to improve the survival and quality of life of existing patients.

Management of these disorders is costly in terms not only of health economics but also in their impact on the family and the community as a whole, which is requested to provide adequate supplies of blood and other social support to patients who in the absence of an effective prevention programme they are increasing both in age and numbers. If the supply of blood or drugs cannot satisfy the needs of the patient population, then increasing complications and premature death will be the result. The majority of children born with these disorders are born in low resource countries of Asia and Africa, which makes their impact even more significant and the need for prevention policies imperative. Prevention has been shown to be cost-effective and also successful in all countries where an approach which includes community education, population screening to identify carriers followed by counselling of at-risk individuals or couples, and services such as prenatal diagnosis, was applied at the national level. In applying such policies, ethical, legal and cultural issues in each community must be taken into consideration and the programme adapted to the local/national situation. In the original two volume text books, these considerations are described in more detail and the reader who wishes to learn more will need to refer to these. The authors are a group of experts in the field, with the author of this shorter version, Dr John Old as the chief editor. The other authors, in alphabetical order were: Michael Angastiniotis, Androulla Eleftheriou, Renzo Galanello, Cornelis L Harteveld, Mary Petrou and Joanne Traeger Synodinos. There were other contributions from scientific experts in the field including Piero Giordano, Eric Jauniaux, Bernadette Modell and Gamal Serour.

This shorter version was designed to be a more practical handbook for the health practitioner, whether medical or laboratory scientist or public health officer who is planning or contributing to the formulation of a national programme. For laboratory scientists and any professional interested in interpreting the laboratory results, including all possible pitfalls in diagnosis, this is an invaluable resource.

TIF hopes that this shortened version is a valuable edition to its educational programme and material, and as always any suggestions or comments towards their further improvement, will be most welcome and will be considered in their next updated versions. TIF is committed to providing updated and reliable information to its Health Care Professionals and patients/families communities, and hence regularly updates all its educational material.

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CHAPTER 1

HAEMOGLOBINOPATHY BASICS

TYPES OF HAEMOGLOBINOPATHIES

The haemoglobinopathies constitute the commonest recessive monogenic disorders worldwide. They are caused by mutations which affect the synthesis of globin chains which form haemoglobin (the thalassaemias) and mutations which alter the structure of globin chains (haemoglobin variants or abnormal haemoglobins).

In general terms, they are autosomal recessive disorders and the homozygous or genetic compound states result in clinically significant phenotypes of variable severity (i.e. thalassaemia major, thalassaemia intermedia, sickle cell syndromes, Hb E syndromes). Heterozygotes are symptom-free but present various haematological characteristics, which are often useful for their identification.

The complexity of the haemoglobinopathies arises from the numerous types of thalassaemia and variant haemoglobin genotypes which can interact when co-inherited, creating complex haematological phenotypes that are often difficult to interpret accurately, and require further investigation by family studies and DNA analysis to arrive at a correct diagnosis.

Table 1.1 The major types of haemoglobinopathies.

Thalassaemias	α -thalassaemia β -thalassaemia δ -thalassaemia $\delta\beta$ -thalassaemia $\epsilon\gamma\delta\beta$ -thalassaemia
Hereditary persistence of fetal haemoglobin (HPFH)	Pancellular types Heterocellular types
Thalassaemic Hb variants	α -variants, eg Hb Constant Spring β -variants, eg Hb Lepore, Hb E
Abnormal Hb variants	α -chain variants β -chain variants δ -chain variants γ -chain variants

THE THALASSAEMIAS

The thalassaemia syndromes are a heterogeneous group of disorders in which there is either a reduced synthesis or an absence of synthesis of a globin chain (1). This results in a deficit of one globin chain type and an altered synthesis ratio between the two globin chains comprising the affected haemoglobin. They are classified according which gene or genes are affected and also according to whether the mutation results in a reduced synthesis of globin from the affected gene, eg β^+ -thalassaemia for a reduced expression of the β -globin gene, or by a complete absence of gene expression, eg $(\delta\beta)^0$ -thalassaemia for no expression of the β - and δ -globin genes.

The thalassemias (and variant haemoglobins) are regionally specific, with each local population having its own characteristic spectrum of mutations. For most of these populations the range of mutations has been identified by molecular analysis and the frequency of each mutation relative to the others in heterozygous individuals has been determined and published (2). Complete lists of the different thalassaemia mutations are kept up to date on two databases: Hb VAR on the globin gene server (<http://globin.bx.psu.edu/hbvar>) (3) and IthaGenes on the ithanet web site (<http://www.ithanet.eu/db/ithagenes>) (4). This information is the first step required for the control of the thalassemias through an integrated program of carrier screening, genetic counseling, and prenatal diagnosis. Tables listing all the alpha and beta thalassaemia mutations, together with their gene frequency and distribution in 56 countries, have been published in the Annexe chapters of Vol 1 of the TIF prevention book (5).

α^+ -THALASSAEMIA:

The α^+ -thalassaemias are defined by either a reduced expression or a completely absent expression of one of the two α -globin genes ($\alpha 1$ and the $\alpha 2$ -globin gene, *HBA1* and *HBA2* respectively). Although most commonly caused by the deletion of one of the two α -globin genes ($-\alpha$), more rarely it can be caused by point mutations or small deletions/insertions of just a few nucleotides and these are described as non-deletional α^+ -thalassaemia mutations in the literature.

- **Deletional α^+ -thalassaemia:** Although eleven different deletion mutations have been identified, only two are commonly encountered in practice. These are the 3.7kb deletion ($-\alpha^{3.7}$), which has reached high frequencies in the populations of Africa, the Mediterranean area, the Middle East, the Indian subcontinent and Melanesia, and the 4.2kb deletion ($-\alpha^{4.2}$), which is commonly found in Southeast Asian and Pacific populations.
- **Non-deletional α^+ -thalassaemia:** More than 100 such mutations have now been described, with those located in the dominant $\alpha 2$ -globin gene resulting a more severe in clinical phenotype than those located in the $\alpha 1$ -globin gene. Those with the severest phenotype are α^+ -thalassaemia mutations which also result in an unstable α -chain variant. In the homozygous state some of these mutations may result in the production of Hb H and a form of Hb H disease, and in combination with an α^0 -thalassaemia allele, have sometimes been observed to result in a hydrops foetalis syndrome (6).

α^0 -THALASSAEMIA:

Characterised by a lack of gene expression of both the $\alpha 1$ and $\alpha 2$ globin genes on the same chromosome. Mostly results from large DNA deletions that remove both the $\alpha 1$ and $\alpha 2$ globin genes, with two Mediterranean alleles (--MED and - $\alpha^{20.5}$) and three Southeast Asian alleles (--SEA, --FIL and --Thai) being the most common types. No α^0 -thalassemia deletions have been reported in individuals from sub-Saharan Africa, although two very rare α^0 -thalassemia alleles involving the combination of two α^+ -thalassaemia mutations in cis have been described.

The carrier state for α^0 -thalassemia is asymptomatic. The two symptomatic forms of α^0 -thalassemia are Hb H disease and Hb Bart's hydrops foetalis.

- **Hb H disease:** The compound heterozygous state of α^0 -thalassemia trait with α^+ -thalassaemia trait results in the condition of Hb H disease. Hb H, a homotetramer of β -globin chains (β_4) is detected by electrophoresis, HPLC or CE. The clinical phenotype varies from a mild asymptomatic form to a severe anaemia requiring intermittent blood transfusions, and may include jaundice hepatosplenomegaly. Compared to patients with β -thassaemia major, they have relatively little ineffective erythropoiesis. However there are some very rare forms of unusually severe Hb H disease associated with hydrops fetalis, for which prenatal diagnosis may be indicated.
- **Hb Bart's hydrops foetalis:** The homozygous state for α^0 -thalassemia results in the condition called Hb Bart's hydrops foetalis syndrome. Hb Bart's Hydrops Foetalis, is usually fatal as infants either die in utero (23-38weeks) or shortly after birth (unless subjected to intrauterine blood transfusion therapy) and prenatal diagnosis is always indicated (see chapter 6).

β -THALASSAEMIA:

Approximately 300 mutations causing β -thalassaemia have been described in the HBB gene locus, the majority of which are point mutations, small deletions or insertions. Carriers of these mutations are characterised by a hypochromic microcytic anaemia and a raised Hb A_2 level. A small number of large gene deletion mutations have also been identified to cause β -thalassaemia, and these usually characterised by a higher than expected Hb A_2 level (6.5-8.5%) due to the deletion of the β -globin gene promoter region. There are several different classes of β -thalassaemia mutations, classified by their different severities of clinical phenotype which generally corresponds with the magnitude of the residual expression of the β -globin gene:

- **β^0 - and severe β^+ types:** These mutations give rise to a defective β -globin gene with either no or minimal gene expression. They result the transfusion-dependent condition of thalassaemia major in the homozygous or compound heterozygous states. This group forms the majority of the β -thalassaemia mutations.
- **Mild β^+ types:** These mutations result in the milder condition of thalassaemia intermedia in the homozygous state.

- **Normal HbA₂ and silent β -thalassaemia types:** These mutations are the mildest types, with some being labelled β^{++} in the literature. They result in the minimal deficit of β -globin production and carriers may have no detectable thalassaemic phenotype by haematological screening techniques.
- **Dominant β -thalassaemia:** The most severe type, resulting in a thalassaemia intermedia like condition in the heterozygous state. Most mutations are in exon 3 of the β -globin gene and produce a hyperunstable β -globin chain which precipitates in erythroid bone marrow precursors and results in ineffective erythropoiesis.

δ -THALASSAEMIA:

Classified as δ^0 and δ^+ types, similar to that for β -thalassaemia mutations. Approximately 30 different mutations have been described. This type of thalassaemia has no clinical significance, apart from the fact that the lowering of the Hb A₂ level in carriers may compromise the diagnosis of co-inherited β -thalassaemia trait.

$\delta\beta$ -THALASSAEMIA:

The $\delta\beta$ -thalassaemias can be divided into $(\delta\beta)^+$ and $(\delta\beta)^0$ types, based on the residual output of δ - and β -chains from the affected chromosome. $(\delta\beta)^+$ -Thalassaemia includes the Hb Lepore determinants and more complex disorders resulting from the presence of two different mutations within the same β -like gene cluster (Corfu and Chinese $\delta\beta$ -thalassaemia determinants). $(\delta\beta)^0$ -Thalassaemias are due to large deletions involving the $\epsilon\gamma\delta\beta$ -gene cluster, removing the δ - and β -genes, but leaving one or both γ -genes intact, ie $(\gamma\delta\beta)^0$ and $(\delta\beta)^0$ -thalassaemia respectively.

$\epsilon\gamma\delta\beta$ -THALASSAEMIA:

A rare disorder which results from approximately ten different large deletions that remove the ϵ , γ , and δ -genes, together with the β -gene or the β -gene promoter region of one chromosome. Only reported in heterozygotes, as the homozygous condition is thought to be lethal during fetal development. Infants heterozygous for this rare condition are born with a severe haemolytic, hypochromic anemia and microcytosis, but the condition improves at 3 to 6 months after birth.

HEREDITARY PERSISTENCE OF FETAL HAEMOGLOBIN (HPFH)

Hereditary persistence of fetal haemoglobin (HPFH) is used to define a group of conditions characterised by increased levels of Hb F in adults, due to a persistent synthesis of γ -globin chains after birth without any significant clinical or haematological manifestations. The disorder is caused by at least 25 different mutations, either large deletions in the globin gene cluster or point mutations in the γ -gene promoter regions.

ABNORMAL HAEMOGLOBINS

Abnormal haemoglobins (or haemoglobin variants) mostly result from globin gene mutations that change the sequence of the affected globin chain, causing a substitution, addition, or deletion of one or more amino acids. More rarely, they result from mispairing and crossover between two like genes during meiosis, creating a fusion protein of both gene sequences (eg Hb Lepore, Hb Kenya).

The first Hb variants to be discovered were described by letters of the alphabet (eg Hb E was the fourth abnormal haemoglobin to be identified in 1954), but as more were discovered, a convention arose to name them according to the location of the first description. More than 700 have now been characterised by protein sequencing and/or DNA sequencing, but many are clinically silent. They only result in haematological changes or a disease if the molecular defect results in a change of function, solubility, stability or a reduced synthesis of the haemoglobin molecule.

Table 1.2 Properties of haemoglobin variants

Property	Example	No (%)
Clinically silent	Hb G-Philadelphia	372 (55%)
Abnormal oxygen transport function: <ul style="list-style-type: none">• Low O₂ affinity (mild anaemia possible)• High O₂ affinity (familial polycythaemia)	Hb Kansas Hb Chesapeake	198 (29%)
Unstable (haemolytic anaemia)	Hb Köln	175 (26%)
Reduced Hb synthesis (thalassaemic phenotype)	Hb E	59 (5%)
Sickling	Hb S	12 (1%)
Methaemoglobinaemia (familial cyanosis)	Hb M-Boston	9 (1%)

HB VARIANT INTERACTIONS

A few haemoglobin variants interact with thalassaemia alleles or other abnormal haemoglobins, resulting in a wide range of clinical disorders of varying severity. For example Hb S interacts with β -thalassaemia alleles to produce a disorder similar to sickle cell disease, the severity of which is related to the phenotypical severity of the β -thalassaemia mutation. Hb S also interacts with a few other Hb variants: for example with Hb C to produce a mild form of sickle cell disease, and Hb O-Arab or D-Punjab to produce a severe form of sickle cell disease. Likewise, Hb E interacts with β -thalassaemia mutations to produce a disorder similar to either thalassaemia intermedia or thalassaemia major depending on phenotype of the β -thalassaemia mutation. There are many types of Hb E syndromes observed, due to various interactions with α -thalassaemia, β -thalassaemia and other variants. These interactions are summarised in chapter 5.

SCREENING AND PREVENTION

The aim of screening (or carrier testing) is to identify carriers of haemoglobin disorders (thalassaemias and interacting Hb variants) in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality. A knowledge of the frequency and heterogeneity of the haemoglobinopathies in a target population is a critical prerequisite in planning an adequate strategy of carrier identification and in selecting the most suitable laboratory methods. In addition, however, the technical facilities, infrastructure and financial resources available affect both the strategy and the choice of methods for carrier identification.

There are four main categories of severe disease states, for which carrier screening, genetic counselling, and possibly prenatal diagnosis is indicated, summarised here as follows. A more detailed list for each category is provided in chapter 5)

- **β -Thalassaemias:** thalassaemia major / intermedia (co-inheritance of two β -thalassaemia mutations including inheritance of $\delta\beta$ -thalassaemia mutations and Hb Lepore).
- **Sickle cell syndromes:** Sickle cell anaemia due homozygosity for Hb S, and sickle cell disorders resulting from Hb interactions: Hb S/C, Hb S/ β -thalassaemia, Hb S/D-Punjab, Hb S/O-Arab, Hb S/Lepore, Hb S/Hb E).
- **Hb E syndromes:** Hb E / β -thalassaemia (co-inheritance of β^0 and severe β^+ -thalassaemia mutations with Hb E).
- **α -Thalassaemias:** Hb Bart's hydrops foetalis syndrome (homozygous α^0 -thalassaemia, genotype: --/--), and more rarely, Hb H hydrops foetalis syndrome (genotypes: --/ $\alpha^0\alpha$ or $\alpha^0\alpha/\alpha^0\alpha$).














































In many populations the β -thalassaemia syndromes, sickle cell syndromes and Hb E syndromes are clinically more relevant than the α -thalassaemias, since the severe forms are more common and require life-long treatment and clinical management. However, in populations which have a high prevalence of α^0 -thalassaemia defects, such as the Chinese and Southeast Asians, or in countries with significant immigrant populations from these areas, α -thalassaemias are also relevant.

The severest form of α -thalassaemia, Hb Bart's hydrops foetalis has no effective treatment and is usually fatal as infants either die in utero (23-38 weeks) or shortly after birth (unless subjected to intrauterine blood transfusion therapy after early prenatal detection). Even with perinatal treatment it is a very severe condition with these patients requiring lifetime transfusion therapy and iron chelation and survivors experiencing a high prevalence of congenital malformations and some children also have long term neurological complications (7). Furthermore, hydropic pregnancies are frequently associated with serious complications in the mother, and most pregnancies in which the foetus is diagnosed as affected are terminated due to the increased risk of both foetal and maternal morbidity.


In the next chapters these guidelines focus on best practice in laboratory methods (8) and will summarise the various approaches to screening, the haematological laboratory methods required for carrier detection, an algorithm for differential diagnosis of the haemoglobinopathies and the pitfalls to avoid, and the DNA analysis methods required for the definitive identification of Hb variants and some thalassaemia alleles for both carrier screening and for prenatal diagnosis.


A table of parental carrier state combinations that give rise to the risk of a foetus with significant sickle cell disease or β -thalassaemia is shown in **Figure 1.1**.


Figure 1.1: Table of parental carrier state combinations that give rise to the risk of a foetus with significant sickle cell disease or β -thalassaemia


Carrier of:	α^+ thal	α^0 thal	Hb S	β thal	$\delta\beta$ thal	Hb Lepore	Hb E	Hb O-Arab	Hb C	Hb D-Punjab	HPFH
α^+ thal											
α^0 thal											
Hb S											
β thal											
$\delta\beta$ thal											
Hb Lepore											
Hb E											
Hb O-Arab											
Hb C											
Hb D-Punjab											
HPFH											

Key:


Serious risk: refer couple for genetic counselling - prenatal diagnosis is indicated.


Less serious risk: refer couple for genetic counselling - further investigation may be required


Possible hidden risk: co-inherited α^0 -thalassaemia trait should be investigated by molecular analysis.


Minimal risk

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CHAPTER 2

PREVENTION AND EPIDEMIOLOGY

The haemoglobin disorders are the commonest clinically serious single gene disorders. It is estimated that over 300,000 affected children are born every year globally. Around 60,000 of these will have a thalassaemia syndrome and the rest will suffer from sickle cell disease [1]. Because of the severity of the anaemia and other serious complications, many of these syndromes are incompatible with long survival, without adequate treatment, although treatment since the 1960's has steadily improved the outlook. If however treatment is inadequate due to lack of adequate resources or poor knowledge of the condition, then survival remains poor and the quality of life seriously compromised [2, 3]. As the patient grows a multi-organ involvement develops, which necessitates a series of interventions in order to counter the effects of iron toxicity on each organ. Comprehensive management results in survival that has now become open ended and the quality of life has improved allowing patients to fulfil their expectations in education, employment and marriage.

OPTIMUM MANAGEMENT OF PATIENTS

The elements of optimum thalassaemia management:

- Adherence to good practice guidelines and national standards
- Adequate and safe donor blood supply
- Monitoring and controlling iron overload
- Availability and affordability of iron chelating agents
- Adherence to all treatment modalities
- Free medical care
- Monitoring of complications and early response
- Adequate management of complications
- Multidisciplinary care in expert centres and networking with secondary centres
- Psychosocial support and holistic care
- Recognition of patients' rights.

Similarly for the sickle cell syndromes, long term management includes:

- Careful follow up to detect complications
- Neonatal screening to identify patients early and initiate infection prophylaxis
- Expertise in the management of vaso-occlusive crises

- Identification of patients at risk of serious complications with the aim of prevention or early intervention [4, 5]
- Stroke control, including monitoring children with transcranial doppler
- Education of patients/families: early recognition and response to symptoms and signs, hydration
- Pain management at home and in hospital
- Psychosocial support
- Blood transfusions and exchange transfusions according to criteria
- Monitoring and management of iron overload.

Haematopoietic stem cell transplantation (HSCT) is at present the only possible cure for both the thalassaemia syndromes and the sickle cell syndromes.

Only a very small fraction of the global patient population benefits from this level of care. Most affected children are born in low resource areas of the world [6].

There is great variability in the prevalence of haemoglobin disorders across the globe but also in their distribution within a country. The real burden of disease depends partly on prevalence, the number of affected individuals, but also on the overall health picture of each country and its socio-economic development. It can only be estimated by epidemiological studies.

EPIDEMIOLOGICAL DATA

Useful epidemiological data include:

- Prevalence data - accurate patient numbers
- Patient location within a country. Micro-mapping, which is particularly relevant to large populations with divergent ethnic groups
- Incidence, which is the number of new affected births which in turn depends on carrier frequency
- The frequency of consanguinity which may have an impact on birth frequency
- Changing epidemiology and the effect of migrations
- Outcome measures: these include complication rates, survival rates, causes of death, and quality of life studies
- Cost effectiveness studies: these are necessary public health components of service provision which should be coupled with Health Technology Assessment studies for the best possible use of resources.

Means for collecting data include:

- Patient registries which include demographics, basic health information and outcomes.
- Surveys
- Screening data.

Calculating the number of expected homozygote births from the carrier rate, is achieved by applying the Hardy-Weinberg rule:

The Hardy-Weinberg equation is as follows: $p^2 + 2pq + q^2 = 1$

- p = thalassaemia gene frequency ($\frac{1}{2}$ carrier frequency)
- q = Hb A gene frequency = $1 - p$
- p^2 = the frequency homozygotes at birth
- pq = the frequency of heterozygotes
- q^2 = the frequency for homozygote normal

PREVENTION POLICY

The reasons why prevention has been accepted and promoted lies in the nature of these hereditary conditions and their natural history, which has been significantly modified by a difficult, demanding and expensive treatment, which has been applied by few countries, with only a fraction of the affected patients benefitting across the globe. The remaining majority has a poor quality of life and faces the possibility of premature death. Any country considering the support of patient care has to consider the increasing burden on resources that the annual increase in the number of patients will inevitably bring. It is the possible inability to cope with such an increase in patients, and thus the inability to continue providing optimum care, that has led to the development of prevention programmes in high prevalence regions of the world. The policy of prevention was strongly supported by the WHO through resolutions on haemoglobin disorders. In resolution EB118.R1, of May 2006 member states are urged:

“to design, implement and reinforce in a systematic, equitable and effective manner, comprehensive national, integrated programme for prevention and management of thalassaemia and other haemoglobinopathies, including surveillance, dissemination of information, awareness-raising and screening, such programmes being tailored to specific socioeconomics and cultural contexts and aimed at reducing the incidence, morbidity and mortality associated with these diseases”.

Where prevention has been adopted, experience has shown that the strategies that are required to properly implement the policy are the following:

- Public awareness and education
- Screening to identify carriers
- Provision of genetic counselling to individuals and at-risk couples, respecting the autonomy of the couples
- The availability of choices for prevention, including prenatal diagnosis and pre-implantation genetic diagnosis
- Adequate dialogue with legal, ethical and religious leaders in order to establish acceptability of the policy according to cultural environment of the country and the population.

Any comprehensive health policy for the haemoglobin disorders, will include prevention as an adjuvant to keeping up with advances in patient management.

PREVENTION PROGRAMMES

Several countries have set up comprehensive national prevention programmes, which include public awareness and education, carrier screening, and counseling, as well as information on prenatal diagnosis and preimplantation diagnosis. These countries are Italy, Greece, Cyprus, UK, France, Iran, Thailand, Australia, Singapore, Taiwan, Hong Kong, and Cuba. Partial programmes including antenatal screening according to ethnic origin are available in several countries in Northern Europe (Netherlands, Belgium, and Germany) and in many developing countries, a prenatal diagnosis service has also been introduced (7).

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CHAPTER 3.

SCREENING METHODOLOGY

TYPES OF SCREENING

The aim of screening (or carrier testing) is to identify carriers of haemoglobin disorders in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality. Haemoglobinopathies are possibly unique amongst all genetic diseases in that detection of carriers is possible (in fact essential) by haematological and biochemical tests rather than DNA analysis. Thus, carrier detection is best carried out using haematological and biochemical tests. DNA analysis is employed for the determination of carrier status in complex cases or when the haematological/biochemical results are unclear, such as the differentiation of homozygous α^+ -thalassaemia from α^0 -thalassaemia trait or the definitive identification of an abnormal haemoglobin. Depending on acceptable practices in each society, at-risk couples can then be offered the reproductive choice to avoid the birth affected children, or helped to prepare for the possible birth of a severely affected child.

Factors that need to be considered in planning a screening programme to detect at-risk couples:

- Knowledge of the frequency and heterogeneity of the haemoglobinopathies in the target population
- Knowledge of the frequency of the disease in endemic and non-endemic immigration countries
- Understanding of genotype/phenotype correlations
- The resources available (technical, infrastructure and financial) affect the choice of methods for carrier identification
- The social, legal, cultural and religious factors
- The purpose and timing of the screening programme: newborn, adolescent, premarital, preconceptional or antenatal stages.

SCREENING STRATEGIES

There are two types of screening: mass screening, provided to the general population before and at childbearing age, and target screening, which is restricted to a particular population group, such as couples preparing to marry, before conception or in early pregnancy.

- **Mass screening:** is more organisationally demanding than target screening, requiring careful planning and adequate technical and financial resources. This approach is most appropriate where there is a high frequency of thalassaemia, placing particular emphasis on pregnant women when they first present for antenatal care.
- **Target screening:** screening may target newborns or adolescents, or the premarital, preconceptional or antenatal stages in adults. For families wishing to avoid the birth of affected children, preconception or antenatal screening is the most effective approach and is widely applied in many high-risk populations. Details of the UK antenatal screening programme can be obtained from the NHS Sickle Cell and Thalassaemia Programme's laboratory handbook (1). Newborn screening is less effective for primary screening and prospectively informing carriers about their reproductive risks, but it is applied in certain non-endemic European countries such as the UK to support better infant healthcare through prompt identification of affected babies with sickle cell disease. Pre-operative screening of individuals from populations with increased incidence of sickle cell is also indicated.

RETROSPECTIVE SCREENING:

Screening may be "retrospective" - that is, when couples already have an affected child, or "prospective" - i.e. when carriers are identified before having an affected child. Retrospective screening is often performed in populations with a low frequency of thalassaemia, or at the initiation of a prevention program in a high frequency population. The method is relatively cheap and simple because it is restricted to a portion of the population. But the effect on the number of affected births is limited, since sick children may be born to undetected at-risk couples. For this reason, prospective carrier identification is more appropriate for populations with a high frequency of thalassaemia.

PROSPECTIVE SCREENING:

Numerous prospective carrier screening programmes are conducted around the world (for a review, see Cousens et. al., 2010, (2)). They can be divided into mandatory or voluntary programmes. Despite the WHO recommendation that no compulsory genetic testing should be carried out, some countries, including Iran, Saudi Arabia and Palestinian territories have laws in place making haemoglobinopathy screening mandatory for all couples before having the approval to get married. In Cyprus, couples waiting to get married are required by the church to be screened and counselled. In other countries, including Sardinia, Greece, Guandong province of China and in England, haemoglobinopathy screening programmes are offered on a voluntary basis.

CASCADE SCREENING:

Inductive screening (also known as cascade screening or extended family testing) involves the testing of relatives of identified carriers and/or patients, and is a powerful means of improving the efficiency of carrier identification. In Sardinia for instance, such a policy has led to the detection of 90% of expected at-risk couples through tests on only 15% of the adult population (2).

NEONATAL SCREENING:

Neonatal screening is relevant to areas where sickle cell disease is prevalent and where the at-risk couples are not detected by a population-based carrier screening programme. It is generally applied for the early recognition of babies affected by disorders that benefit from early treatment to avoid irreversible health problems, such as inherited metabolic disorders and sickle cell disease. The early detection of affected children with sickle cell anaemia enables timely interventions which reduce the likelihood of life threatening complications such as pneumococcal infections. In addition, neonatal screening provides invaluable epidemiological information regarding the frequency and geographical distribution of homozygous sickle cell disease, sickle cell thalassaemia, Hb S/D, Hb SC disease and Hb S/O-Arab. Despite its usefulness, practical application is limited to few countries which include the USA (3), Jamaica (4), Brazil (5) and some European countries (6).

In the UK, newborn screening is offered at 5-8 days of age as part of the newborn dried blood spot screening programme (7). The programme is designed to detect all the different forms of clinically significant sickle cell disorders: Hb SS, Hb SC, Hb S/D-Punjab, Hb S/O-Arab, Hb S/E, and Hb S/ β -thalassaemia. It also detects the rare non-sickling condition of Hb S/HPFH, which is followed up because it is not possible to distinguish it from Hb SS and Hb S/ β^0 -thalassaemia at birth on the initial screening test (these are distinguished by family studies). The Programme also reports conditions presenting as homozygous β -thalassaemia and Hb E/ β -thalassaemia from which the patient can benefit from early detection and follow up. These clinically significant conditions are not part of the screening programme but are detected by the screening methods.

In many high prevalence areas, including Africa and South America, the service is either targeted to high-risk groups, limited to parts of a country or not provided at all. Targeted screening has led to the possibility of discrimination, especially in countries where sickle cell disease is prevalent in ethnic minorities and universal screening may be preferable. The success of neonatal screening as a means to timely and effective patient care depends to a great extent on follow up of cases detected.

APPROACHES TO CARRIER IDENTIFICATION

There are two approaches to the delivery of a screening programme designed to detect carriers of β -thalassaemia, α -thalassaemia and the clinically significant haemoglobin variants:

1. A primary screen to determine red cell indices, followed by a secondary screen involving haemoglobin analysis in subjects with reduced MCV and/or MCH, and subjects identified to be at high risk of carry such a clinically significant variant by their ethnic origin.
2. Complete screening based on determining red cell indices, haemoglobin pattern analysis and Hb A₂ in all subjects from the outset.

A primary screening approach is recommended in countries with low frequency and limited heterogeneity of thalassaemia, while complete screening is recommended in populations where

both α - and β -thalassaemias are common, and where interaction of α - and β -thalassaemias could lead to missed diagnoses due to the normalisation of red cell indices. The UK antenatal screening programme is organised along similar lines, with a primary screening approach used for areas with a low prevalence of Hb S and a complete screening approach for high prevalence areas (defined as an area where the estimated fetal prevalence of sickle cell disease is 1.5 per 10,000 pregnancies or greater).

PROBLEMS ASSOCIATED WITH SCREENING PROGRAMMES

“Screening” is distinct from “definitive” diagnosis in that the purpose of screening is to test for a defined set of conditions using simple haematological/biochemical tests. Screening programmes are designed using a protocol of first and second line methods in order to obtain a reliable diagnosis, which is essentially a presumptive diagnosis. Antenatal screening programmes are also designed to minimise the follow up of partner testing in low risk cases in order to minimise the work load, costs and the stresses of being tested (see chapter 4). If an unequivocal, definitive diagnosis is required, characterisation methods based on either protein or DNA analysis must be utilized.

Some of the problems associated with haematological screening programmes are:

- **β -thalassaemia.** Some atypical and silent β -thalassaemia carriers may be missed when the Hb A₂ level is 3.5% or below. These are summarised in the next chapter with details of the best practice screening algorithm.
- **$\delta\beta$ -thalassaemia.** A raised Hb F level identifies carriers of $\delta\beta$ -thalassaemia and HPFH, but only DNA analysis can definitively distinguish the two conditions.
- **α -thalassaemia.** There is no specific screening test for the carrier of α -thalassaemia, which often remains a diagnosis made by exclusion. Many carriers of α^0 -thalassaemia have occasional red cells containing Hb H inclusions but these are not always detectable by routine screening. A definitive diagnosis of α -thalassaemia and the differentiation between α^+ and α^0 alleles can only be made by DNA analysis.
- **Hb variants.** When an abnormal haemoglobin is identified by a screening methodology, the results obtained constitute a presumptive identification of the haemoglobin. The only abnormal haemoglobins that can be identified definitively without resorting to molecular analysis are Hb S and the other rare sickling variants.

SCREENING PROGRAMME REQUIREMENTS.

The laboratory methods for carrier identification are relatively expensive (electronically determined RBC indices, HPLC analysis of haemoglobin) and the flowchart is laborious and includes complex methods such as DNA analysis when a definitive diagnosis is required. For countries with more limited resources, mass screening can be conducted using cheaper methods (such as single tube osmotic fragility tests, or chromatography for Hb A₂ determination) and a less complex flowchart.

The British Committee for Standards in Haematology, a subgroup of the British Society for Haematology, has published a series of guidelines over the years for UK haematologists and laboratory scientists

carrying out haemoglobinopathy screening (8,9), thalassaemia trait screening (10), foetal DNA analysis (11) and antenatal / newborn screening (1). The guidelines describe the requirements and approach to screening that are considered practical for the UK population. Although different requirements and strategies may be required for populations with a different prevalence of haemoglobinopathies, they are a useful source of advice to the advantages and disadvantages of the different methods and techniques available for the standard laboratory investigations.

Table 3.1 Standard laboratory investigations

Haematology diagnostics	Red blood cell count or complete blood count Reticulocyte count
Hb quantification	Hb A ₂ & F: HPLC, capillary electrophoresis. Hb F: Alkali denaturation, Hb F-cells: Kleihauer test
Hb pattern analysis	Electrophoretic methods: agarose gel, cellulose acetate, isoelectric focussing. HPLC, capillary electrophoresis (CE)
Molecular analysis	DNA analysis Protein analysis - mass spectrometry
Biochemical indices	Iron status: ZnPP, serum ferritin or serum iron + serum transferrin

HAEMATOLOGY DIAGNOSTICS

The basic diagnostics consist of the determination of a complete blood count (CBC) or a full blood count (FBC), usually carried out by an automated electronic cell counter. These counters produce many parameters of red blood cell indices, of which only a few, such as the red cell count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and haemoglobin (Hb) concentration, are strictly relevant and essential for haemoglobinopathies screening. However, some other parameters are useful, such as red cell distribution width (RDW), which is the standard deviation of the red cell size measurements expressed either as a percentage of the mean or a coefficient of variation).

MCV AND MCH:

These parameters are variably reduced in thalassaemia carriers. MCH is more reliable than MCV, since the MCV does not remain stable due to a tendency for the red cells to increase in size over time.

- Evaluation of CBC in samples >24hours old should be made with caution, as the red cells increase in size, leading to falsely raised MCV (although different analysers have variable sensitivity to this problem). The MCH, however, may be stable for up to 5 days, depending on storage conditions (4-20°C).

Important cut-off values indicating possible heterozygosity for thalassaemia include MCV <79fl and MCH <27pg. Values below these may indicate α - or β -thalassaemia or iron deficiency and an individual's iron status must be taken into account when evaluating MCH and MCV values for thalassaemia screening. Each laboratory should establish their own normal ranges for these parameters, based on the ethnicity of their patient population/s and patient age.

- Mild β -thalassaemia mutations cause less microcytosis and hypochromia than β^0 and severe β^+ mutations, although there is some overlap
- Carriers of silent β -thalassaemia mutations have normal or only slightly reduced MCV and MCH values
- Carriers for both β and α -thalassaemia may have normal MCV and MCH values because of less globin chain imbalance
- α^+ -Thalassaemia (- α / $\alpha\alpha$) carriers may have normal or reduced MCV and MCH values
- α^0 -Thalassaemia carriers (--/ $\alpha\alpha$) and α^+ -thalassaemia homozygotes (- α /- α) always have reduced MCV and MCH values.

RDW:

This parameter can potentially be used to discriminate between thalassaemia carriers and iron deficiency and sometimes between thalassaemia carriers and a thalassaemia disorder or other rare causes of microcytosis (decreased MCV). It is a measure of the degree of anisocytosis and is mostly increased in cases of iron deficiency, while normal values are seen in cases of thalassaemia trait.

RBC:

This CBC parameter can potentially distinguish between iron deficiency and thalassaemia. A high RBC (erythrocytosis) results from a mechanism that compensates for the chronic low MCH present in thalassaemia carriers. This compensating mechanism needs folic acid to be maintained and may restore the Hb level of a strongly microcytic thalassaemia carrier to near normal values because of a significant elevation of RBC, up to 6-7 ($10^{12}/l$) or more. On the other hand, RBC compensation might be less evident in case of folic acid deficiency. Also, RBC may not be a useful parameter in advanced pregnancy due to haemodilution, and iron deficient women who are responding to iron supplementation may have increased RBC.

HB QUANTIFICATION:

The most widely used methods for Hb A₂ measurement are high performance cation-exchange chromatography (HPLC), capillary electrophoresis (CE), and less frequently, microchromatography on DE-52 due to it being accurate but time consuming. Other methods that have been used are electrophoresis and elution (accurate but time-consuming) and Hb electrophoresis with automatic densitometry (not recommended by best practice guidelines).

Hb A₂:

Quantitative Hb A₂ determination is the most valuable test for β -thalassaemia carrier identification. The HPLC and CE methods are very fast, simple and accurate in the absence of variants in the Hb A₂ position, in contrast to microchromatography which is accurate but time-consuming. CE has an advantage over HPLC in that it separates Hb E from Hb A₂, thus providing a clean measurement of Hb A₂ in patients with Hb E.

- The important cut-off value indicating heterozygosity for β -thalassaemia is Hb A₂ >3.5%.
- Borderline levels of 3.2%-3.8% (depending upon laboratory) indicate further investigation is required as they may be due to an atypical type of β -thalassaemia mutation (see Table 4.1).

Hb F:

Usually measured by HPLC or CE. On some analysers, the Hb F values between 0 and 1% may be inaccurate, but this is the normal range from two years after birth and thus not significant. Measurement by the alkali denaturation method has excellent reproducibility in most ranges of Hb F. The intracellular distribution of Hb F in red cells with detectable amounts of Hb F (F-cells) can be detected on blood smears by two techniques: the acid elution test of Kleihauer and the immunofluorescence test using specific anti-Hb F monoclonal antibodies. Most HPFH disorders resulting from a DNA deletion have a uniform distribution of Hb F (pancellular) and most $\delta\beta$ -thalassaemias have a heterogeneous distribution, although some exceptions have been found.

Hb PATTERN ANALYSIS:

The five methods listed below can only give a presumptive identification of an Hb variant. A more accurate presumptive identification of abnormal haemoglobin may be made if at least two methods are used, as most Hb variants run in different positions when subjected to chromatography and electrophoresis. To aid identification, charts showing the electrophoretic positions of known variants are available from manufacturers or publications, and similarly for HPLC retention times of known variants. However, it must be emphasised that an accurate and definitive identification of a Hb variant can only be made by molecular analysis, apart from Hb S and Hb E:

- A sickle solubility test can be used to confirm the presence of Hb S if suspected.
- The DCIP (2,6 dichlorophenolindophenol) test can be used to confirm the presence of Hb E.
- For suspected high affinity Hb variants which co-elute with Hb A and are therefore electrophoretically silent, oxygen dissociation analysis may be useful, but definitive identification is only achieved by molecular analysis.

Cellulose acetate electrophoresis:

Haemoglobin electrophoresis at pH 8.6 using cellulose acetate membrane will reliably detect the common haemoglobin variants, ie Hb's S, C, D-Punjab, E, O-Arab and the Lepores (Hollandia, Baltimore and Boston-Wellington). Hb H and Hb Bart's may also be detected if suitable run times are used. Many other variants are also detectable, eg J's, N's, Q's, Hasharon.

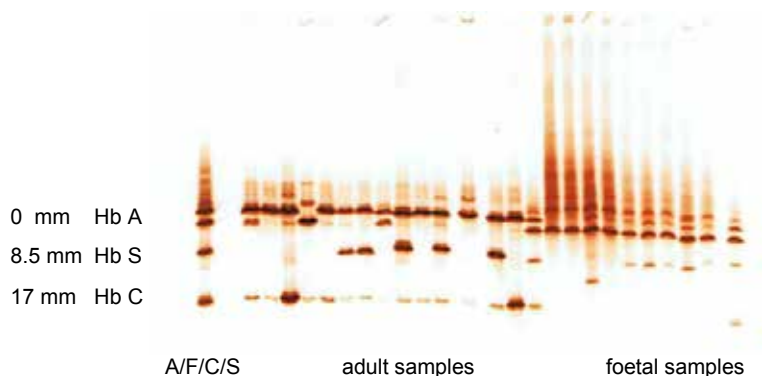
Citrate agar electrophoresis:

Haemoglobin electrophoresis at pH 6.0 using acid agarose or citrate agar gel is useful for distinguishing Hb's C, E, and O-Arab from each other, also Hb S and Hb D-Punjab from each other. Note that the migration patterns are different for acid agarose gels and citrate agar gel.

Isoelectric focusing (IEF):

IEF is a more sensitive method than electrophoresis methods, giving very good separation of haemoglobin variants which focus as sharp bands at unique positions for each variant (Figure 3.1), but requires considerably more expertise for interpretation than electrophoresis since adducted fractions also separate. This method is also being used for neonatal haemoglobinopathy screening programmes to screen for Hb S homozygotes. The position of an unknown variant can be accurately measured and calculated relative to the standard position of a control band such as Hb S or Hb C and used for presumptive identification by comparing to the positions of known variants (13) (see Table 4.4). Several isoelectric focusing maps of haemoglobin variants have been published that show the position of variants relative to those of Hb S and Hb C (14, 15).

Figure 3.1 Carrier screening for variants by isoelectric focusing gel electrophoresis (IEF)



High Performance Liquid Chromatography (HPLC):

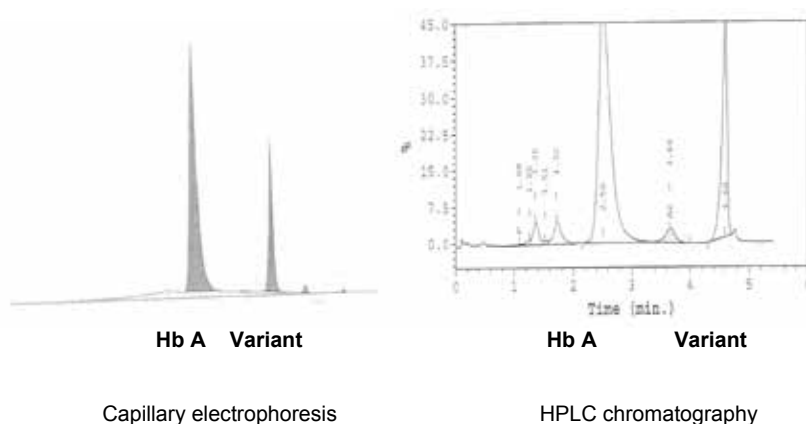
This method is recommended for simultaneous detection and quantitation of haemoglobin fractions. Since the systems are automated, operation of the analysers is simple, but interpretation of the chromatograms requires expertise. Also, attention must be paid to quality control, especially for measurement of Hb A₂. Although the cost per test is relatively high, the application is useful for large scale screening programmes. An advantage of this approach is that HPLC chromatograms with retention times for more than 300 rare alpha and beta chain variants have now been published to aid a presumed diagnosis (in a book by Barbara Bain et al (16) and in the Bio-Rad Hb Variant CD library, available from Bio-Rad). HPLC is also being used for neonatal haemoglobinopathy screening programmes to screen for Hb S homozygotes.

Capillary electrophoresis (CE):

Automated capillary zone electrophoresis is a relatively new and complementary screening technique to HPLC for the routine detection and measurement of haemoglobins and variants (17). CE patterns are simpler and easier to read than HPLC (as long as Hb A is present in the sample), as the method does not separate haemoglobin derivatives such as glycated fractions. CE also detects and measures Hb H and Hb Bart's more easily than by HPLC.

The HPLC chromatogram and CE pattern for Hb Ottawa are shown in **Figure 3.2** for comparison of the two techniques.

Figure 3.2 A comparison of the separation of Hb Ottawa by HPLC chromatography and Capillary Electrophoresis.



MOLECULAR ANALYSIS

Molecular analysis is carried out in specialist diagnostic centres to determine the genotypes of some carriers in screening programmes, the genotypes of patients with clinically significant haemoglobin disorders such as thalassaemia major and intermedia, the identification of the genotypes involved in complex cases involving combinations of different haemoglobinopathies, for clarification and identification of haemoglobin variants, prenatal diagnosis, and pre-implantation diagnosis. This is normally done by PCR-based DNA analysis techniques (18), although some labs are now adding next generation sequencing techniques and mass spectrometry to the haemoglobinopathy molecular diagnostic repertoire (19).

DNA analysis.

The application of DNA analysis is required at various stages of the carrier detection process to differentiate thalassaemia carriers, as specified in the carrier screening flowchart. In particular it is required to identify carriers of α -thalassaemia, to differentiate between carriers of $\delta\beta$ -thalassaemia and HPFH, to confirm the identity of the clinically significant haemoglobin variants and to confirm atypical cases of β -thalassaemia trait. The various methods used are summarised in chapter 5.

Mass spectrometry.

Identifies the amino substitution in Hb variants. It is a fast, cheap, specialized method based on analysing tryptic digests of whole blood in a mass spectrometer. Although there may be only a small shift in mass for some common variants, it is very effective for the characterisation of Hb variants especially if used in conjunction with other methods (28). It is now being used by some labs in the UK neonatal screening programme to screen for Hb S (20). However although the method usually identifies the specific amino-acid change, it does not necessarily confirm the DNA sequence variation or, in the case variants arising from the α - or γ - duplicated genes, it does not identify the gene involved.

BIOCHEMICAL TESTS FOR IRON STATUS

Analysis and determination of iron status is quite often necessary in screening for thalassaemia carriers. Iron status can be measured using many haematological and biochemical indices. Each parameter reflects changes in different body iron compartments (storage, transport, end product, receptors) and is affected at different levels of iron deficiency. However, the presence of iron deficiency should be evaluated with simple tests such as Zinc protoporphyrin (ZnPP), serum iron and transferrin and in some cases serum ferritin.

Zinc protoporphyrin (ZnPP):

The red blood cell ZnPP determination is the fastest and easiest method for screening of iron deficiency. Iron depleted red blood cells show increased levels of ZnPP. A diagnosis of iron deficiency has to be confirmed by serum iron and transferrin determination, to calculate transferrin saturation. Zinc protoporphyrin is elevated in iron deficiency, but may be falsely high in lead intoxication or if the bilirubin levels are raised.

Ferritin:

Some laboratories prefer to determine the iron status using serum ferritin, but it should be pointed out that with this parameter there are several limitations (i.e. false positive and negative results).

Serum iron:

Serum iron levels are reduced after the complete depletion of iron stores but before the haemoglobin level drops. Several manual and automated methods are available. Serum iron should be used in combination with serum transferrin to calculate the percentage of saturation. Serum iron has a low specificity as low levels may be found in pregnancy, during chronic infections and inflammations, pyrexia, and malignancy.

Serum transferrin.

Transferrin is the iron-transporting protein which can be determined using normal or automated techniques as total iron binding capacity (TIBC), ie. the amount of added iron specifically bound by plasma. Alternatively transferrin can be measured as protein using immunological methods. Serum transferrin increases in iron deficiency, and is falsely reduced in acute inflammation, chronic infections, renal diseases, and malignancy. Several manual and automated methods are available:

- Measurement of iron status in samples with hypochromic, microcytic indices but with a normal Hb A₂ and Hb F level is useful to distinguish between cases of iron deficiency and those with possible α -thalassaemia trait or some cases with silent β -thalassaemia trait in whom the iron status is normal. This is a useful approach not only to prevent unnecessary further investigation but in some cases inappropriate iron therapy.
- It is important to note that iron deficiency can co-exist with the thalassaemias, and such cases could be misinterpreted. It is sometimes necessary to recommend repeating the haematology screen once the individual is iron replete (assuming that there is no time limit with an on-going pregnancy and that the partner's result do not indicate a need for further investigation).

SUPPLEMENTARY HAEMATOLOGICAL METHODS

Many haematological methods assist the identification of haemoglobinopathy carriers which are supplementary to the essential basic techniques described above. These methods are often used as elements in the description of carrier status or as economical screening methods;

RED BLOOD CELL MORPHOLOGY

Morphological changes of red cells can be detected in most thalassaemia carriers. An examination of a stained peripheral blood smear may be helpful in the evaluation of cases.

- Microcytosis, hypochromia and anisopoikilocytosis (variation in the size and shape of the red cells) are the most typical changes in thalassaemia. Other less common findings are basophilic stippling and the presence of some target cells. A high percentage of target cells are found in Hb C syndromes.
- Nucleated red blood cells are indicative of bone marrow hyperactivity and can be found in homozygous β -thalassaemia. Polychromasia (blue-grey and slightly bigger red cells) is associated with the presence of reticulocytosis. Howell-Jolly bodies (fragments of nuclear DNA) can be found after splenectomy or in the functional asplenic condition in sickle-cell syndromes, where sickle shaped cells are sometimes seen on the stained film as well.

RETICULOCYTE AND HBH INCLUSION BODY DETECTION

In thalassaemia carrier screening reticulocyte count does not have a diagnostic value. However in the detection of α -thalassaemia, especially Hb H disease, the brilliant cresyl blue stain will detect the characteristic Hb H inclusion bodies.

- Reticulocyte number is useful in the evaluation of erythropoiesis and is significantly increased when haemolysis (or bone marrow regeneration) is present.

HEINZ INCLUSION BODIES

Inclusion bodies (Heinz bodies) are intracellular haemoglobin precipitates detected by supravital stains. These may be found in some forms of thalassaemia (mainly α -thalassaemia) and in unstable haemoglobin disorders.

- Heinz bodies are found in variable percentages (5-50%) in Hb H disease, and carriers of unstable haemoglobins.
- Also sometimes found in very low number (1:1000-10.000 RBC) in α^+ -thalassaemia homozygotes (genotype: $-\alpha/-\alpha$) and α^0 -thalassaemia carriers (genotype: $--/\alpha\alpha$). However in these carrier states they are rare and so in most cases it is time consuming to attempt confirmation of the presence of α^0 -thalassaemia through inclusion body detection. The absence of inclusions does not exclude α -thalassaemia.

OSMOTIC FRAGILITY TEST

Osmotic fragility test (OFT) was the first method used for screening of thalassaemia and was introduced as a simple approach to detect thalassaemia carriers by Silvestroni and Bianco in the 1940s. This fast and simple method has been applied as a screening test in large populations. The availability of electronic counters for the measurement of MCV and MCH has decreased the use of OFT. It is still used in low resource countries to screen large rural or tribal populations and in places where the electronic cell counters are not available (21). The most used test at present is NESTROFT, the acronym for Naked Eye Single Tube Red cell Osmotic Fragility Test.

- Although the method is easy to perform, fast, cheap and does not require sophisticated equipment, it needs careful standardization. It is particularly useful in places where the electronic cell counters are not available. In view of the false negative results seen in a small proportion of β -thalassaemia carriers, it is not recommended when an automated hematology analyzer is available (21).
- The test is positive both in β - and in α -thalassaemia carriers, in sickle cell trait and iron deficiency anaemia. False positive results are obtained in patients with iron deficiency and therefore subjects positive with NESTROFT need further investigation to define the diagnosis. False negative results have also been reported.

HB STABILITY TEST

Disruption of the normal structure of the haemoglobin molecule can result in reduced stability, which leads to precipitates in the erythrocyte causing its destruction. The clinical consequence is often a haemolytic anaemia of variable severity. Many Hb variants elute with Hb A by HPLC and CE, and thus if an unstable haemoglobin is suspected clinically, a specific test for unstable haemoglobin should be performed even if the electrophoretic pattern is normal. There are two stability tests: isopropanol test and heat test.

SICKLING TEST

When Hb electrophoresis, HPLC or CE shows a Hb fraction that runs in the position of Hb S, a functional test for Hb S should be undertaken. Some common haemoglobins migrate to the same position as Hb S but do not sickle. It should also be noted that there are other (rare) sickling haemoglobins that have reduced solubility, and have therefore a positive solubility test, but most of which do not migrate to the same position as Hb S. These very rare sickling variants are listed in Chapter 6.

- **Sickling test.** Many tests have been described and several commercial kits are available. In positive samples the typical sickle-shaped red blood cells will appear. Note a positive test does not distinguish sickle cell trait from sickle cell disease.
- **Solubility test.** HbS is quite insoluble when in the reduced state in high phosphate buffer solution. It forms tactoids (water crystals) which refract and deflect light rays and produce a turbid solution, permitting a solubility test.

HB E DETECTION BY DCIP TEST

Hb E is a mildly unstable haemoglobin with an exposed -SH group which can be oxidized by certain chemical agents including the dye DCIP (dichlorophenolindophenol) at the neutral pH (7.5). Hb E and other unstable haemoglobin molecules such as Hb H will be precipitated when exposed to this dye at 37°C.

- Useful for distinguishing between Hb EE and Hb E/β-thalassaemia. In homozygous Hb E, a heavy sediment will form at the bottom of the test tube. In Hb E trait, and Hb E/β-thalassaemia, the precipitation of Hb E produces a cloudy or an evenly distributed particulate appearance.
- The test is positive also in Hb H disease and other unstable haemoglobins. To overcome these false positive results a modified DCIP test has been described (22) and more recently the CMU-E (Chiang Mai University-E) has been found to have 100% sensitivity and 99.1% specificity (23).

OXYGEN DISSOCIATION CURVE.

Used to characterise Hb variants with an altered oxygen affinity. Requires specialist equipment to measure the oxyhaemoglobin dissociation curve and P50 value.

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CHAPTER 4

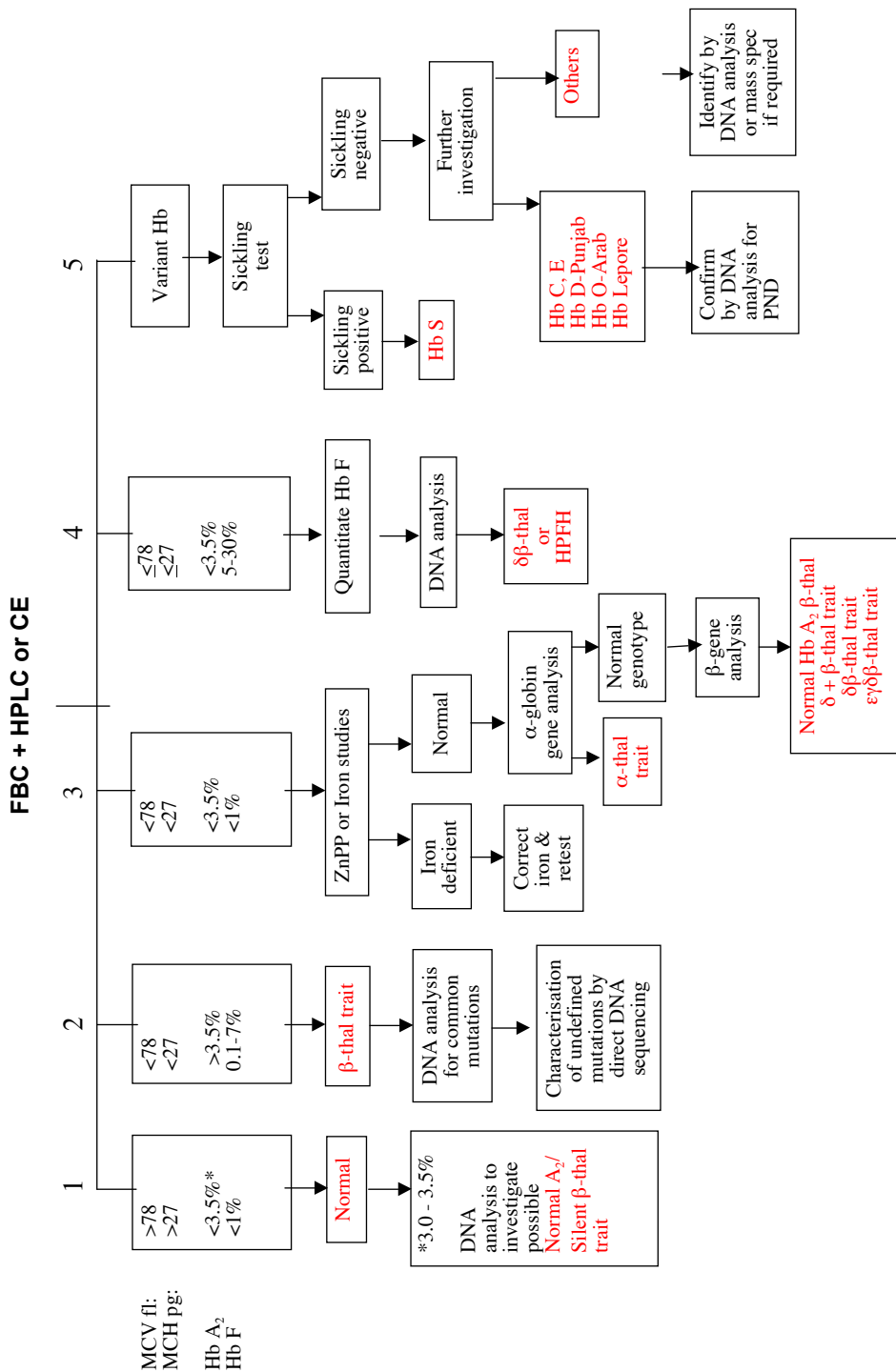
CARRIER SCREENING ALGORITHM

DIAGNOSTIC FLOW CHART

A flow-chart illustrating the best practice strategy used to diagnose carriers in high risk areas is shown in **Figure 4.1**. Five parameters are used in the algorithm, Hb A₂%, Hb F%, MCH, MCV, and Hb pattern which lead to five possible outcomes, as listed below. The cut-off indices in the algorithm are the ones most widely used, however appropriate reference values should be independently defined for each population as there may be slight differences according to the types of thalassaemia alleles present.

It must be noted that there is a difference between screening and diagnosis. National carrier screening programmes, such as the UK antenatal screening programme, are not designed to detect 100% of all possible haemoglobinopathies in a carrier, due to the costs and resources required of following up too many false negatives with partner testing. The antenatal screening programme's flow charts are based on genetic risks and ethnic origins, and so the rare cases of normal Hb A₂ β-thalassaemia are missed by the scheme, rare cases of α⁰-thalassaemia trait will be missed if the individual is not from a high risk ethnic group and cases of α⁺-thalassaemia trait are never followed up, so that the very rare couples potentially at risk for Hb H hydrops foetalis syndrome are not forwarded for DNA analysis by the antenatal screening programme's carrier algorithm.

Figure 4.1 Flowchart for thalassaemia carrier screening



FLOW CHART DIAGNOSTIC OUTCOMES 1 - 5

There are five outcomes of the carrier diagnosis flow chart using the well-established and generally accepted cut off values of 3.5% or below for a normal Hb A₂ level, 27pg and above for a normal MCH, 5% and above for a raised Hb F level, and with the presence of a Hb variant. Each of the five outcomes is designed to detect one of the main types of haemoglobinopathy, although there are diagnostic problems such as microcytosis due to iron deficiency which complicate the diagnostic algorithms of the flow chart.

- **Outcome 1:** Normal individual
- **Outcome 2:** β -thalassaemia trait
- **Outcome 3:** α -thalassaemia trait
- **Outcome 4:** $\delta\beta$ -thalassaemia trait or HPFH trait
- **Outcome 5:** Hb variant

OUTCOME 1: NORMAL INDIVIDUAL

Diagnostics: normal Hb A₂ level with normal MCH and MCV values

Reference intervals for Hb A₂ in normal subjects are usually between 2.0 and 3.3%, but slightly different values can be found depending on the method and differences in populations. The most widely used cut off for a normal Hb A₂ level is 3.5%, so values of 3.6% and over most likely indicate β -thalassaemia trait (see outcome 2).

Pitfall. However individuals with certain silent β -thalassaemia mutations can have an Hb A₂ level between 3.0%-3.5%, and for the +1480 (C→G) mutation, between 2.2%-3.5%. Such individuals will not be detected by antenatal screening programmes, but for diagnostic cases it is recommended that subjects with borderline Hb A₂ levels, particularly if their partner is a typical β -thalassaemia carrier, should be extensively investigated by α - and β -gene analysis.

Hb A₂ of 3.0% - 3.5% with normal red cell indices

In diagnostic cases, further investigation by DNA analysis is required to exclude silent β -thalassaemia in individuals with Hb A₂ values of 3.0-3.5% (depending upon the method, the laboratory reference range and coefficient of variation). A list of β -thalassaemia mutations that have been found to be associated with Hb A₂ of 3.5% or less are described in **Table 4.1**. The triplicated α -globin gene allele is also has silent β -thalassaemia phenotype:

- **Silent β -thalassaemia.** Very few normal Hb A₂ mutations are truly silent, most will be picked up in section 3 of the algorithm due to evidence of microcytosis and hypochromia (eg CAP+1 (A→C) and IVS1-6 (T→C) if the Hb A₂ is 3.5% or less). However there are two well-known silent mutations [-101 (C→T) and +1480 (C→G)] for which many carriers have normal red cell indices and a Hb A₂

level in the normal range of 3.5% and below. Graphs showing the ranges of the Hb A₂ and MCH values determined in carriers for the two silent mutations-101 (C→T) and +1480 (C→G) are shown in **Figure 4.2** (a and b).

- **The $\alpha\alpha\alpha$ -gene allele.** Triple and quadruple alpha globin gene alleles have silent β -thalassaemia phenotype. They result in a mild α/β -globin chain synthesis imbalance, but are associated with normal red cell indices and a normal Hb A₂. Co-inheritance with β -thalassaemia trait can sometimes result in a mild thalassaemia intermedia phenotype (see outcome 2).

Table 4.1 β -thalassaemia alleles with a Hb A₂ value below 3.6%*

Traditional nomenclature	HGVS nomenclature	MCV fl	MCH pg	Hb A ₂ %
Silent β-thalassaemia				
-101 (C→T)	c.-151C>T	88.5 ± 7.8	30.1 ± 1.0	3.1 ± 1.0
-92 (C→T)	c.-142C>T	83.0 ± 6.0	28.3 ± 2.0	3.5 ± 0.4
+33 (C→G)	c.-18C>G	82.0 ± 9.2	27.1 ± 3.4	2.5 ± 1.4
IVS2-844 (C→G)	c.316-7C>G	96.0 ± 4.0	30.3 ± 1.8	3.2 ± 0.2
+1480 (C→G)	c.*+6C>G	88.3 ± 9.5	27.9 ± 6.0	2.7 ± 0.8
$\alpha\alpha\alpha/\alpha\alpha$		85.5 ± 7.8	30.4 ± 5.0	2.8 ± 0.6
Normal Hb A₂ β-thalassaemia				
CAP+1 (A→C)	c.-50A>C	77.5 ± 2.5	25.4 ± 3.5	3.7 ± 0.7
IVS1-6 (T→C)	c.92+6T>C	71.0 ± 4.0	22.7 ± 3.5	3.9 ± 0.6

* Other β -thalassaemia mutations that have been reported to have a borderline-raised Hb A₂ level and thus potentially may occur in individuals with a value of 3.5% or below are: -102 (C→A), -101 (C→G), CAP+8 (C→T), CAP+10 (-T), CAP+45 (C→G), IVSII-844 (C→A), Poly A (AATAAA→CATAAA).

Figure 4.2 Graphs showing the range of Hb A₂ values (fig 4.2A) and MCH values (Fig 4.2B) in 41 carriers of the silent mutation -101 (C→T) and 18 carriers of the silent mutation +1480 (C→G) (data collected by J. Old for the EC framework 6 Itharet research project). Average Hb A₂ values were 3.8% and 2.9% respectively; average MCH values were 29.0pg and 28.2pg respectively.

Fig 4.2A Hb A₂ values

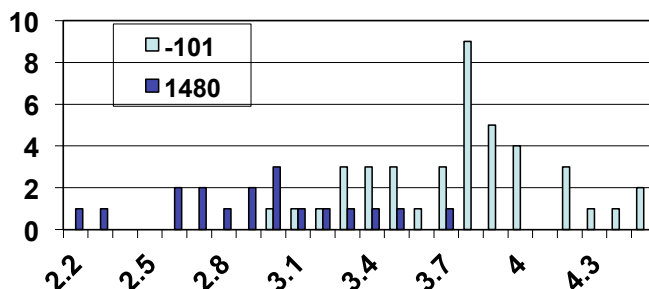
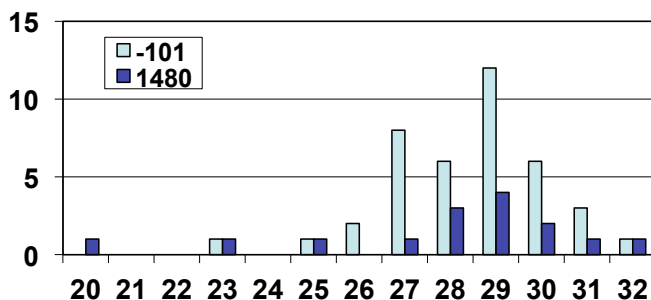


Figure 4.2 B. MCH values



Hb A₂ of 3.5% - 4.0% with normal red cell indices

Borderline-raised Hb A₂ levels in normal individuals can be explained as the extreme end of the distribution of the normal range. Borderline Hb A₂ values are not rare events in a population with a high prevalence of β -thalassaemia carriers (1), and it is necessary to investigate these cases at the molecular level, particularly if the partner is a carrier of β -thalassaemia. However there are some other possible reasons which need to be taken into consideration, such as:

- The co-inheritance of α -thalassaemia trait with a mild β^+ -thalassaemia mutation such as IVSI-6 (T→C) may restore the α/β chain imbalance to normal, leaving just a borderline-raised Hb A₂ level as a diagnostic clue to the β -thalassaemia trait.
- Individuals with a KLF1-gene mutation may have a borderline-raised Hb A₂ level of 3.4% - 3.8%.
- Individuals with some unstable Hb variants
- Acquired conditions, such as hyperthyroidism, megaloblastic anaemia, use of antiretroviral drugs (e.g. for HIV treatment).

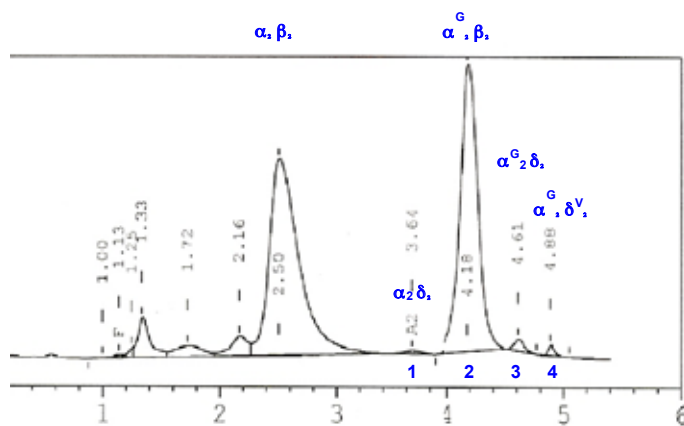
- Note that Hb S carriers often have borderline-raised Hb A₂ level in this range. This does not indicate co-inheritance of β -thalassaemia trait unless the Hb S level is <45%

Hb A₂ level between 0% - 2.0% with normal red cell indices

Individuals with an Hb A₂ level below 2.0% may have δ -thalassaemia trait, or may have a split Hb A₂ level due to a δ -chain variant or an α -chain variant which creates a variant Hb A₂ molecule which elutes in a different position to the normal Hb A₂ fraction. The presence of a δ -chain variant will split the Hb A₂ into two equal peaks on HPLC, whilst the presence of an α -chain variant will split the Hb A₂ into two unequal peaks, the abnormal one being 25% of the normal one. Note the ratios change if the individual also has co-inherited α -thalassaemia, ratio being altered according to the number of unexpressed α -genes). **Figure 4.3** shows an HPLC chromatogram of rare case of an individual heterozygous for both the δ -chain variant Hb A₂' and an α -chain variant, resulting in the Hb A₂ fraction split four ways (2).

Figure 4.3

HPLC chromatogram of an individual heterozygous for the δ -chain variant Hb A₂' and the α -chain variant Hb G-Philadelphia. The Hb A₂ ($\alpha_2\delta_2$) peak is split into four by the presence of the δ - and α -chain variants.



- 1: $\alpha_2\delta_2$ (3.64 min)
- 2: $\alpha_2\delta_2^V$ (4.18 min) (co-migrates with Hb G-Philadelphia)
- 3: $\alpha_2\delta_2$ (4.61 min)
- 4: $\alpha_2\delta_2^V$ (4.88 min)

OUTCOME 2: β-THALASSAEMIA TRAIT

Diagnostics: raised Hb A₂ level with reduced MCH and MCV values.

An Hb A₂ level of 3.6% or greater with a MCH value below 27pg is indicative of β-thalassaemia trait (3), although there are some rare pitfalls with this diagnosis as detailed below in which individuals with β-thalassaemia trait can exhibit normal or borderline reduced red cell indices, and some normal individuals with reduced red cell indices can have a slightly raised Hb A₂ level.

Hb A₂ of 3.6% - 4.5% with reduced red cell indices

The majority of β-thalassaemia carriers with an Hb A₂ value between 3.6-4.0% are carriers of mild β⁺-thalassaemia mutations, sometimes called normal Hb A₂ mutations as they are associated with clearly reduced red cell indices but with an atypical, borderline-normal Hb A₂ value. Some examples of which are listed below in [Table 4.2](#).

Table 4.2 Average values of some normal HbA₂ β-thalassaemia alleles

Allele	Hb A ₂ %	MCH pg
CAP+1 (A→C)	3.7	25.4
IVSI-6 (T→C)	4.2	22.7
Poly A (A→G)	3.9	24.7
Poly A (T→C)	4.0	22.4
Poly A (-AT)	3.8	22.7
Poly A (-AA)	4.0	23.6

- **Pitfall:** Normal individuals with reduced red cell indices arising from iron deficiency or α-thalassaemia may have a slightly raised Hb A₂ value in the region of 3.6-4.0% due to HIV drug therapy, hyperthyroidism, or KLF1 gene mutation (4).

Graphs showing the ranges of the Hb A₂ and MCH values determined in carriers for the two silent mutations CAP+1 (A→C) and IVSI-6 (T→C) are shown in [Figure 4.4](#) (a and b).

Figure 4.4 Graphs showing the range of Hb A₂ values (fig 4.4A) and MCH values (Fig 4.4B) in 34 carriers of the normal HbA₂ mutation IVSI-6 (T→C) and 76 carriers of the normal HbA₂ mutation CAP+1 (A→C) (data collected by J. Old for the EC framework 6 Itharet research project). Average HbA₂ values were 3.9% and 3.7% respectively; average MCH values were 22.7pg and 25.4pg respectively.

Fig 4.4A Hb A₂ values

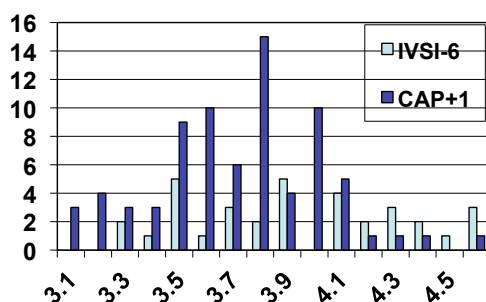
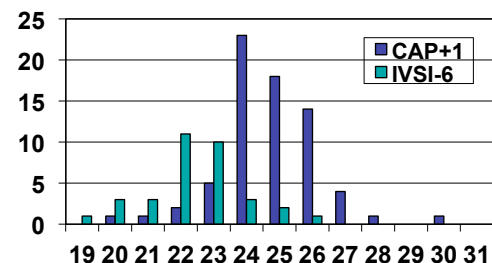


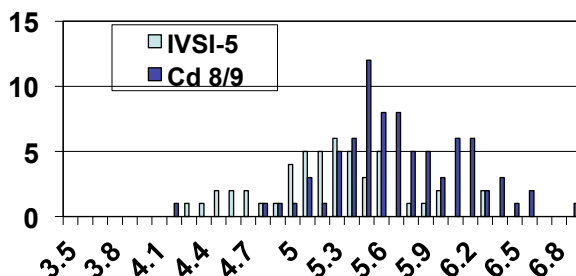
Fig 4.4B MCH values



Hb A₂ of 4.5% - 6.5% with reduced red cell indices

The majority of β -thalassaemia carriers fall into this category, either with a β^0 or severe β^+ -type mutation. They are characterised by a markedly low MCH (19-23pg), an elevated Hb A₂ level in the range of 4.5-6.5% and there is also a slightly raised Hb F level (1-3%) in about 30% of cases. The ranges of Hb A₂ values observed in carriers of a typical severe β^+ -thalassaemia allele, IVSI-5 (G→C), and a typical β^0 -thalassaemia allele, Cd 8/9 (+G) are shown in [Figure 4.5](#).

Figure 4.5. Graphs showing the range of Hb A₂ values of 49 carriers of the severe β^+ -thalassaemia allele IVSI-5 (G→C) and 81 carriers of the β^0 -thalassaemia allele Cd 8/9 (+G) (data collected by J. Old for the EC framework 6 Ithant research project).



Hb A₂ of 6.5% - 9.0% with reduced red cell indices

Some β -thalassaemia carriers have atypically high Hb A₂ levels in the range of 6.5-9.0% and a variable elevated Hb F level of 3-15%. The molecular lesions in such cases are large deletions that remove the 5' promoter region of the β -globin gene.

Hb A₂ level of 3.6% - 6.0% with a significant clinical phenotype

- **Dominant β -thalassaemia.** Some rare molecular lesions of the β -globin gene produce highly unstable β -chains that precipitate in bone marrow precursors. This results in ineffective erythropoiesis and a variable clinical phenotype of thalassaemia intermedia with a raised Hb A₂ and a low Hb level in heterozygotes. Because of the precipitation in early red cell precursors, the β -chain variant is usually undetectable in peripheral blood. These mutations, known as dominant inclusion-body β -thalassaemia alleles, are usually located in exon 3 of the β -globin gene (5).
- **Co-inherited triple α -gene** Co-inheritance of heterozygous β -thalassaemia with the triple or quadruple α -globin gene arrangement generally results in mild thalassaemia intermedia.
- **Co-inherited Hb H disease.** The presence of an Hb H disease genotype ($--/\alpha$ or $--/\alpha^T\alpha$) in interaction with heterozygous β -thalassaemia, results in a moderate to severe anaemia (Hb 8-10g/dl) with marked microcytosis (MCV < 60fl) and hypochromia (MCH < 19pg). It should be pointed out that Hb H inclusion bodies are absent.

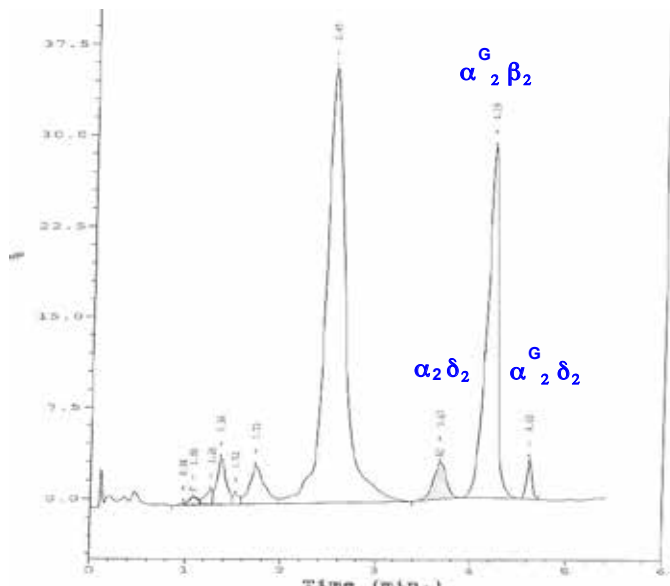
PROBLEMS IN β -THALASSAEMIA TRAIT DIAGNOSIS:

Variations of haematological findings not consistent with typical β -thalassaemia trait can occur. Some carriers with a clearly raised Hb A₂ value may have normal red cell indices, and some individuals appear to have clearly raised Hb A₂ value but do not have β -thalassaemia trait.

- Split Hb A₂ peak.** The presence of a δ -chain or α -chain variant may split the Hb A₂ peak, in which case the quantities of the two peaks must be added together (6). If the split peak is not visible, the individual would be diagnosed as having α -thalassaemia.

A β -thalassaemia carrier with a split Hb A₂ peak due to the co-inheritance of Hb G-Philadelphia trait is shown in **Figure 4.6**.

Figure 4.6 HPLC chromatogram of an individual with β -thalassaemia trait and heterozygosity for the α -chain variant Hb G-Philadelphia.



Hb peak	retention time	%
Hb A ₂	3.67 min	3.0*
Hb A ₂ hybrid	4.60 min	1.4*
Total Hb A ₂		4.4%

* Note: the ratio of normal Hb A₂ to hybrid Hb A₂ is 2:1 due to co-inherited α^+ -thassaemia trait linked to Hb G-Phildaelphia, which results in the expression of two normal α -genes and one Hb G α -gene.

- **Raised Hb A₂ level of 3.6% - 6.0% with normal red cell indices.** The co-inheritance of α -thalassaemia may result in a carrier having a nearly normal MCH value with a clearly raised Hb A₂ level. A high frequency of both α - and β -thalassaemia is found in some countries in Southeast Asia, the Mediterranean area, and possibly some Middle Eastern countries, and therefore the relevant α^0 -thalassaemia mutations should always be screened for by DNA analysis in β -thalassaemia carriers and Hb E carriers from these regions.
- **Raised Hb A₂ level of 3.6% - 6.0% with a normal β -globin genotype.** Normal individuals can appear to have β -thalassaemia trait due to a falsely raised Hb A₂ level arising from a Hb variant eluting with or close to Hb A₂ on HPLC (3), and may well have reduced red cell indices if they have iron deficiency or α -thalassaemia

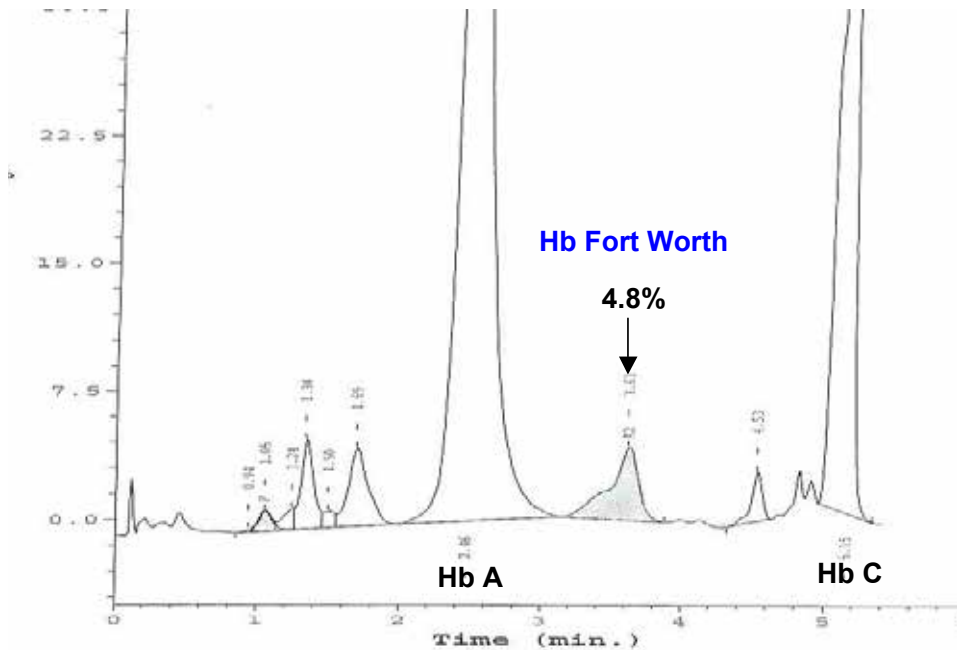
Pitfall: In particular, the α -chain variant Hb Fort Worth co-migrates with Hb A₂ on HPLC and is expressed at levels low enough to mistaken for a raised Hb A₂ value. Three normal individuals, initially misdiagnosed with β -thalassaemia trait and referred for confirmation by molecular analysis, were found by DNA sequencing to simply be carriers of Hb Fort Worth with peaks of 5.1, 5.2 and 6.4% in the Hb A₂ position. The HPLC chromatogram of an individual heterozygous for Hb C and Hb Fort Worth is shown **Figure 4.7**.

Table 4.3 lists several other Hb variants that ran close to the Hb A₂ window on HPLC in my lab at approx. 3.6 minutes on the Bio-Rad Variant 2. However these do not normally create a diagnostic problem, apart from the fact that a diagnosis of β -thalassaemia trait cannot be achieved by HPLC. These Hb variants are associated with a peak which is either too high to be mistaken for a raised Hb A₂ value or can migrate close enough to interfere with a true measurement of the Hb A₂ peak area.

Table 4.3. Some Hb Variants that can interfere with HbA₂ estimation by HPLC

Retention time (min)	Hb variant
3.4	Deer Lodge, Toulon, Loves Park
3.5	Lepore, G-Coushatta
3.6	Hb A ₂ , Fort Worth, E, D-Iran, Kenya
3.7	Ocho Rios, G-Honolulu
3.8	Osu Christiansborg, Ethiopia
3.9	G-Accra, Bethesda, Maputo

Figure 4.7 HPLC chromatogram of an individual with Hb C trait and heterozygosity for the α -chain variant Hb Fort Worth.



OUTCOME 3: α -THALASSAEMIA

Diagnostics: normal Hb A₂ level, reduced MCH and MCV values.

When the MCH is below the cut off value of 27pg, the Hb A₂ is 3.5% or below and the Hb F level is normal, the diagnosis indicates a form of α -thalassaemia, or iron deficiency, or possibly both together. A MCV cut of value of 78fl can also be used. although a recent study has shown that the use of MCH was better for thalassaemia carrier screening as the same cut off value was applicable using five different haematology analysers, in contrast to using the MCH cut of value (7).

However there are also a number of other thalassaemia genotypes that result in this outcome that need to be considered. After iron deficiency is excluded and DNA testing for α -thalassaemia has given a negative result, the different thalassaemia genotypes leading to this phenotype are identified by α -, δ -, and β globin gene mutation analysis: ($\epsilon\gamma\delta\beta$)⁰-thalassaemia, β - and δ -thalassaemia or mild β -thalassaemia trait with a normal Hb A₂ level.

Normal Hb A₂, MCH between 24 - 30 pg

This phenotype is consistent with α^+ -thalassaemia trait, when one α -globin gene is dysfunctional. α^+ -thalassaemia is an asymptomatic carrier state in which one α -globin gene is dysfunctional. Red cells are often not microcytic, and Hb A₂ and Hb F levels are always normal. In the neonatal period, small amounts (1-3%) of Hb Bart's (γ_4) may be detected. Thus the cut off values will only catch around 50% of individuals with α^+ -thalassaemia trait:

- A reliable diagnosis of α^+ -thalassaemia trait can only be achieved by DNA analysis.
- However α^+ -thalassaemia is not usually clinically significant condition and in antenatal screening programmes, partners are not followed up in this case to avoid unnecessary (and expensive) investigations and the engendering of anxiety.

Normal Hb A₂, MCH between 18 - 25 pg

This phenotype is consistent with either homozygous α^+ -thalassaemia or α^0 -thalassaemia trait, when two α -globin genes are dysfunctional. Carriers of α^0 -thalassaemia usually have a MCH below 25pg unless they also have β -thalassaemia trait. However individuals with homozygous α^+ -thalassaemia also have a MCH below 25pg and the different forms of α -thalassaemia can only be differentiated reliably by gene analysis.

α^0 -thalassaemia trait is usually associated with a slight reduction in haemoglobin concentration, and reduced red cell indices (MCV, MCH), hypochromia, microcytosis and anisopoikilocytosis, with decreased erythrocyte osmotic fragility and Hb A₂ levels in the low to low-normal range (1.5-2.5%). During the neonatal period, there are moderate amounts of Hb Bart's (3-8%) and cord blood erythrocytes are microcytic. Adult individuals with α^0 -thalassaemia trait may have a few red cells with Hb H inclusions but their absence does not exclude this genotype.

- A reliable diagnosis of homozygous α^+ -thalassaemia or α^0 -thalassaemia trait can only be achieved by DNA analysis.
- Although α^0 -thalassaemia is a clinically significant condition, in antenatal screening programmes, DNA analysis for a definitive identification of the α^0 -thalassaemia trait is only carried out when both have an MCH below 25pg, again in order to avoid unnecessary (and expensive) investigations and the engendering of anxiety. Therefore such screening programmes will not detect couples at risk of having a child with Hb H disease.

Normal Hb A₂, MCH between 16 - 20 pg

This phenotype most commonly results from the compound heterozygous genotype of α^+ -thalassaemia and α^0 -thalassaemia. This genotype results in Hb H disease, when three α -globin genes are dysfunctional, and Hb H is detected by electrophoresis, CE or HPLC at a level of 3-30% of total haemoglobin. The clinical presentation of Hb H disease varies widely, from a mild asymptomatic to a severe anaemia requiring intermittent red blood cell transfusions. In addition to anaemia, clinical features may include jaundice and hepatosplenomegaly.

- Patients with a non-deletion α^+ -thalassaemia allele have a more severe clinical expression than those with an α^+ -thalassaemia deletion.
- Patients with an α^+ -thalassaemic (hyperunstable) globin allele have the severest phenotype. Some very rare forms of unusually severe Hb H disease associated with hydrops foetalis, for which prenatal diagnosis can be indicated, are described in Chapter 6.

PROBLEMS IN α -THALASSAEMIA TRAIT DIAGNOSIS

Pitfall. There are a number of thalassaemia genotypes which are associated with similar haematological findings to individuals with α -thalassaemia trait and thus carriers could be misdiagnosed as having α -thalassaemia trait without any further investigation by DNA analysis. The main categories of problem genotypes are:

- **($\epsilon\gamma\delta\beta$) $^\circ$ -Thalassaemia trait.** This is a very rare condition described in just few families, arising from a small number of large DNA deletions that cause no expression of the ϵ , γ , δ , and β globin genes of one chromosome. Newborns heterozygous for this condition are severely anaemic, but the condition improves after 3 months and adults have a phenotype consisting of a mild anaemia with reduced MCH and MCV levels, a normal Hb F and a normal Hb A₂ value, similar to α^0 -thalassaemia trait.
- **δ -Thalassaemia + β -thalassaemia trait.** δ -Thalassaemia has mainly been described in individuals from Mediterranean countries and its co-inheritance with β -thalassaemia trait (on different chromosomes) reduces the Hb A₂ value to below 3.5%.
- **Corfu $\delta\beta$ -thalassaemia.** This condition results from the presence of a δ -thalassaemia and β -thalassaemia) in the same chromosome (in cis): a partial deletion of the δ -gene and an IVS1-5 (G \rightarrow A) β -thalassaemia mutation. Carriers of this form of ($\delta\beta$) $^+$ -thalassaemia have haematological findings comparable to the β -thalassaemia trait, but with normal or slightly reduced Hb A₂.
- **Normal Hb A₂ β -thalassaemia.** Some carriers of a mild β^+ -thalassaemia mutations may have reduced MCV and MCH values but HbA₂ level in the range of 3.1-3.5% and thus misdiagnosed as having α -thalassaemia trait. As listed in under outcome 1, the most common such mutations are the Asian Indian mutation CAP+1 (A \rightarrow C), the Mediterranean mutation IVS1-6 (T \rightarrow C) and the African mutation Poly A (T \rightarrow C).
- **Iron deficiency + β -thalassaemia trait.** Co-existing iron deficiency in a β -thalassaemia carrier has been shown to usually reduce the Hb A₂ level, but by only approximately 0.5%. Thus the values associated with typical β -thalassaemia mutations are not usually lowered to 3.5% or below, unless a very severe anaemia is present (5).

Pitfall. If the mutation is a mild β^+ -thalassaemia type or normal Hb A₂ type that would normally have given a Hb A₂ value around 4%, the presence of iron deficiency may produce a falsely low level below 3.5% or below, resulting in a misdiagnosis without further investigation by DNA analysis.

- **δ -globin chain variant + β -thalassaemia trait.** A co-inherited δ -globin chain variant, or possibly an α -chain variant can lead to a split Hb A₂ peak. The percentages of the two Hb A₂ fractions must be added together to measure the true Hb A₂ level.

Pitfall. If the variant hybrid Hb A₂ peak is mistakenly not taken into account, or it is masked by another Hb fraction so that it cannot be taken, the result will be a misdiagnosis of α -thalassaemia.

Pitfall. The phenotype of α -thalassaemia is masked by the co-inheritance of β -thalassaemia trait. In antenatal screening programmes, all individuals with β -thalassaemia trait from countries in which α^0 -thalassaemia is prevalent should be screened by DNA analysis for the possible co-inheritance of α^0 -thalassaemia.

OUTCOME 4: $\delta\beta$ -THALASSAEMIA OR HPFH

Diagnostics: normal Hb A₂ with a raised Hb F (5-30%).

Foetal haemoglobin is the prevalent haemoglobin type in the newborn (about 60-80%) then progressively decreases to very low levels, of less than 1%, by the second year of life. The decline is slower in β -thalassaemia carriers. Hb F levels may be increased in adults in a number of inherited conditions ($\delta\beta$ -thalassaemia, deletional and non-deletional hereditary persistence of fetal haemoglobin (HPFH), some β -thalassaemia mutations) and acquired conditions, including pregnancy, recovery after bone marrow transplantation and aplastic anaemia, myelodysplastic syndromes, juvenile chronic myeloid leukaemia (8). The Hb F is restricted to a sub-population of erythrocytes termed "F-cells". Individuals with $\delta\beta$ -thalassaemia or HPFH trait usually have a raised Hb level between 5% and 30%.

It is important to differentiate $\delta\beta$ -thalassaemia from HPFH for genetic counselling reasons, because compound heterozygotes for HPFH and β -thalassaemia or Hb S have a silent or very mild phenotype in contrast to the combination of $\delta\beta$ -thalassaemia and β -thalassaemia which results in clinically significant condition varying from β -thalassaemia intermedia to major.

- **$\delta\beta$ -Thalassaemia trait.** Typical carriers have reduced red cell indices and a Hb F level of 5-18%. Caused by more than 20 DNA deletions that result in no expression of the δ and β -globin genes, $(\delta\beta)^0$ -type, or δ , β and γ -genes, $(\gamma\delta\beta)^0$ -type.
- **HPFH trait.** Typical carriers have normal red cell indices and a Hb F level of 5-35%. The disorder is caused by at least 25 different mutations, either large deletions in the globin gene cluster or point mutations in the γ -gene or γ -gene promoter regions.

- **Pitfall:** Although HPFH is associated with normal red cell indices, individuals with HPFH trait often have co-inherited α -thalassaemia and thus the MCH is not a reliable parameter for the differentiation between HPFH and $\delta\beta$ -thalassaemia. The distinction may be made haematologically by analysing the red blood cell distribution of Hb F. Hb F is usually heterogeneously distributed in $\delta\beta$ -thalassaemia trait in contrast to HPFH trait in which it is homogeneously distributed. However, a definitive diagnosis can only be made by identification of the deletion mutation by DNA analysis.

PROBLEMS IN HB VARIANT IDENTIFICATION

OUTCOME 5: HB VARIANT

Diagnostics: abnormal haemoglobin detected by electrophoresis and/or HPLC.

Abnormal haemoglobins are usually detected by the first line screening methods of HPLC or CE. Both are accurate, fast and quantitate the variant, providing an elution time (HPLC) or an elution zone (CE) for a presumptive variant identification. Confirmation of the presumptive identification can be performed by electrophoresis, usually cellulose acetate electrophoresis at alkaline pH. However alternative methods may be used, such as isoelectric focussing (IEF). Although not the cheapest method, IEF offers the best resolution for identification of abnormal haemoglobins by haemoglobin electrophoresis.

Clinically important Hb variants.

There are six variants that produce a symptomatic disorder in combination with certain thalassaemias and themselves and therefore prenatal diagnosis may be required: Hbs S, C, E, D-Punjab, O-Arab and Lepore. They are characterized in antenatal screening programmes by two procedures before partners are tested, and then confirmed required by a sickling test for Hb S and DNA sequencing for the others if prenatal diagnosis is required.

Rare Hb variants.

Screening programmes are bound to reveal rare some variants that cannot be putatively identified from a combination of past experience of the chromatograms and electrophoresis patterns, IEF positions, HPLC retention times or CE time zones of known variants, ethnic origin, etc. Most of these will have no clinical significance, but just a few, particularly the unstable haemoglobins or those with altered oxygen affinity, can produce clinical manifestations, especially in combination with β -thalassaemia trait. It is recommended that rare variants are referred to a specialist laboratory for a precise identification by DNA analysis or protein analysis by mass spectrometry.

- The use of a single test to establish presumptive identification of a variant is inappropriate and second or even third line testing procedures should be in place. A recommended strategy is to use a combination of cation-exchange high performance chromatography, capillary electrophoresis and isoelectric focusing (9).

- Many variants have similar HPLC retention times and similar electrophoresis patterns, but a more accurate presumptive diagnosis can be made by combining two parameters, such as HPLC retention time and relative IEF position (10). These can be used to differentiate three different Hb Q variants as shown below:

Table 4.4 A more accurate presumptive diagnosis can be obtained by using both the HPLC retention time and IEF position.

Hb variant	HPLC rt	IEF position
Q-India	4.8 min	8 mm
Q-Iran	4.8 min	7 mm
Q-Thailand	4.6 min	7 mm

- Derivatives of Hb S may co-elute Hb A and Hb A₂, increasing it to 3.6-4.0%, and thus giving the appearance of co-existing β -thalassaemia trait. However for Hb S, and Hbs C and E, the presence of 50% or more Hb A (ie less than 50% Hb S, C or E) excludes a co-inherited β -thalassaemia mutation in almost every case.
- When the amount of Hb S is greater than 50%, the genotype may be Hb S/ β -thalassaemia, Hb S/ $\delta\beta$ -thalassaemia, Hb S/HPFH or Hb SS. DNA studies and/or haematological analysis of the patient's parents are required to identify the correct genotype.
- The quantity of Hb S and Hb E in heterozygotes is decreased by the presence of α -thalassaemia. The amounts measured by HPLC in my lab in individuals with a known α -genotype are shown below.

Table 4.5 Relationship of the %Hb S and %Hb E with α -thalassaemia

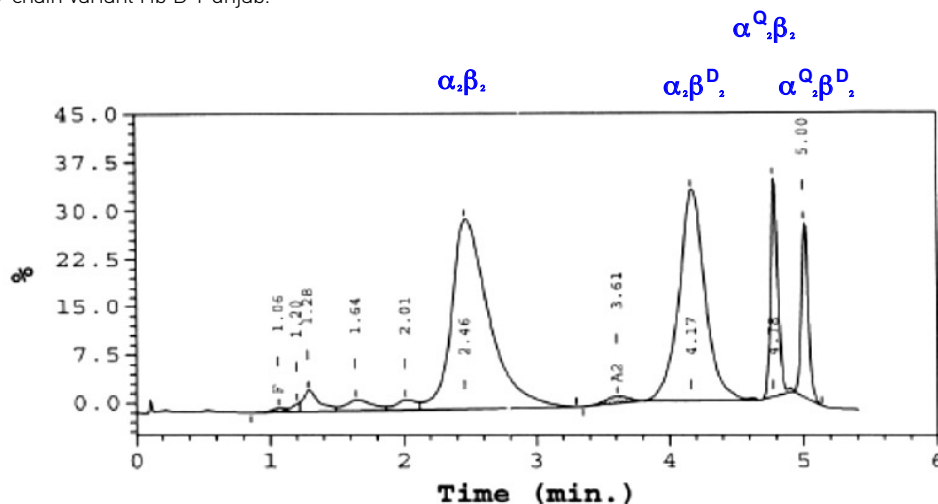
	$\alpha\alpha/\alpha\alpha$	$-\alpha/\alpha\alpha$	$-\alpha/-\alpha$ or $--/\alpha\alpha$
Hb S (%)	35-40	29-34	24-28
Hb E (%)	25-30	21-25	19-21

- The quantity of an α -chain variant is increased by the presence of α -thalassaemia. For example Hb G-Philadelphia is approximately 25% in normal individuals, 33% in those with the genotype ($-\alpha/\alpha\alpha$), 50% in heterozygotes with the genotype ($-\alpha/-\alpha$), and 100% in homozygotes with the genotype ($-\alpha/-\alpha$).

- Thirteen variants are known to sickle in addition to Hb S. These all have two amino acid substitutions, the Hb S substitution plus one other, and this often changes their separation characteristics from Hb S. These are Hbs S-South End, C-Harlem, S-Antilles, S-Oman, S-Providence, S-Travis and C-Zinquinchor, S-Cameroon, C-Ndjamena, S-Clichy, S-San Martin, S-Sao Paulo and Jamaica Plain.
- **Pitfall.** The co-inheritance of an α -chain variant and a β -chain variant creates a new hybrid variant with a different retention time and electrophoresis mobility to the parent abnormal haemoglobins, creating a complex or confusing pattern and making diagnostic interpretation difficult. An example of the complex pattern resulting from a patient heterozygous for Hb Q-India and Hb D-Punjab is shown in **Figure 4.8A**.

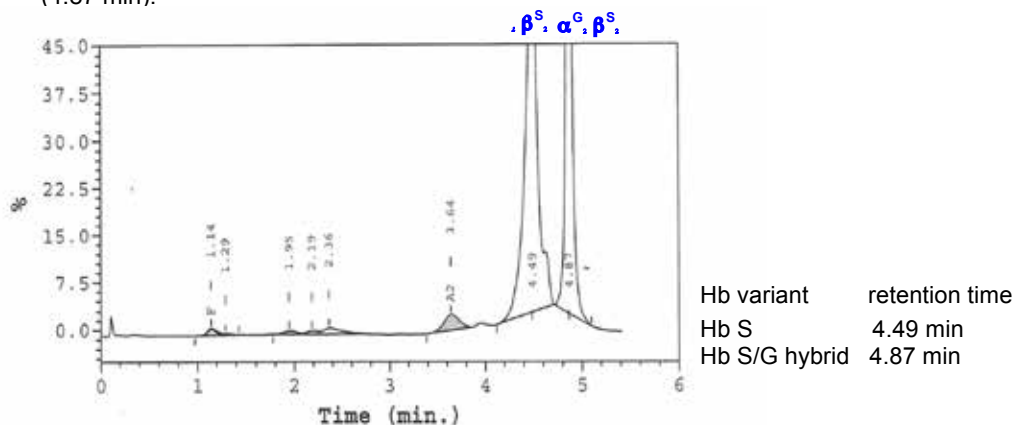
Interpretation of the pattern is especially difficult in homozygous cases, in which the expected Hb fractions may be missing (eg Hb AS individuals with homozygous G-Philadelphia have no Hb fractions in the Hb A or Hb S positions). An example of a homozygous case with a hybrid variant Hb is shown in **Figure 4.8B**. The sickle cell disease patient in **Figure 4.8B** was initially diagnosed incorrectly as having sickle cell disease due to Hb S and Hb O-Arab before referral for confirmation by molecular studies, which subsequently revealed that patient was Hb SS with Hb G-Philadelphia trait.

Figures 4.8 A and B. HPLC chromatogram of an individual heterozygous for the α -chain variant Hb Q-India and the β -chain variant Hb D-Punjab.



Hb variant	retention time
Hb D-Punjab	4.17 min
Hb Q-India	4.78 min
Hb D/Q hybrid	5.00 min

Figure 4.8 B. HPLC chromatogram of a patient homozygous for Hb S and heterozygous for the α -chain variant Hb G-Philadelphia. Note there is no peak in the Hb G-Philadelphia position (4.2 min) and the hybrid variant α^G, β^S , has a HPLC retention time the same as that observed for Hb O-Arab (4.87 min).



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CHAPTER 5

MOLECULAR DIAGNOSTIC TESTS

The haemoglobinopathies were the first genetic diseases to be investigated at the molecular level and consequently have been used as a prototype for the development of new techniques of mutation detection since the first prenatal diagnosis of the sickle cell gene in 1978. There are now many different PCR-based techniques that can be used to detect globin gene mutations, with each method having its advantages and disadvantages, and subject to a number of reviews (1-4). The particular ones chosen by a laboratory depends upon factors such as the technical expertise available in the diagnostic laboratory, the type and variety of the mutations likely to be encountered in the individuals being screened, as well as the budget available.

Molecular diagnostics is an excellent tool that any lab may consider when available. The first step in carrier identification is the accurate measurement of the hematological phenotype by standard hematology tests and the separation of the Hb fractions by HPLC or CE, and electrophoresis, and in many cases this is sufficient for carrier diagnosis to be made. It is only mandatory for:

1. genotype/phenotype correlation, prognosis and treatment.
2. confirmation of a putative hematological diagnosis when needed, in case of possible risk.
3. performing prenatal diagnosis.

DIAGNOSTIC METHODS

Almost all methods for DNA analysis of haemoglobinopathies currently in use are based on the polymerase chain reaction. There are many different PCR-based techniques that can be used to detect the globin gene mutations. Those in current use for detecting and/or characterizing nucleotide variations include the amplification refractory mutation system (ARMS), restriction-endonuclease PCR (RE-PCR), denaturing gradient gel electrophoresis (DGGE), high resolution melting analysis (HRMA), direct DNA sequencing by Sanger sequencing, pyrosequencing, real-time PCR, reverse dot blot analysis and microarrays (the latter two usually as commercially available systems rather than as in-house protocols). For detecting and/or characterizing DNA large deletions, the methods include gap-PCR, Multiplex Ligation-dependent Probe Amplification (MLPA) and array comparative genome hybridization (aCGH). All are recommended for use as best practice in the best practice guidelines (5)

Table 5.1 The principal methods currently used for the diagnosis of haemoglobinopathies by DNA analysis

Globin gene disorder	Diagnostic method
α^0 -thalassaemia	Gap-PCR, MLPA
α^+ -thalassaemia (deletional)	Gap-PCR, MLPA
α^+ -thalassaemia (non-deletional)	Sanger sequencing, ASO, RE-PCR, pyrosequencing
β -thalassaemia (deletional)	Gap-PCR, MLPA
β -thalassaemia (non-deletional)	Sanger sequencing, ASO, RDB, ARMS, RE-PCR
δ -thalassaemia	Sanger sequencing
$\delta\beta$ -thalassaemia	Gap-PCR, MLPA
$\epsilon\gamma\delta\beta$ -thalassaemia	MLPA
HPFH (deletional)	Gap-PCR, MLPA
HPFH (non-deletional)	Sanger sequencing, ASO, RE-PCR, pyrosequencing
All Hb variants	Sanger sequencing
Hb S	ASO, RDB, ARMS, RE-PCR, pyrosequencing
Hb C	ASO, RDB, ARMS, pyrosequencing
Hb E	ASO, RDB, ARMS, RE-PCR, pyrosequencing
Hb D-Punjab	ASO, RDB, ARMS, RE-PCR, Sanger sequencing
Hb O-Arab	ASO, ARMS, RE-PCR, Sanger sequencing
Hb Lepore	Gap-PCR, MLPA

DIAGNOSTIC STRATEGIES

The most common haemoglobinopathies of autosomal recessive inheritance are traditionally population-specific, with each population having a unique combination of abnormal haemoglobins and thalassaemia disorders. The spectrum of mutations and their frequencies have been published for most populations, and usually consist of a limited number of common mutations and a slightly larger number of rare mutations (6). Therefore knowledge of the ethnic origin and family history (including consanguinity) of a patient may support the diagnostic strategy, to expedite identification of the underlying defects in most cases. With the advent of global migration, however, this is becoming less practical and useful.

Thus there are two approaches for diagnosing haemoglobinopathy alleles. Some labs with a limited number of alleles in their population's repertoire use a simple and cheap screening method first to detect a small number of common mutations, and then resort to the more expensive approach of DNA sequencing for the small number of samples remaining unidentified. Other labs with a larger and more varied repertoire of alleles have switched to universal DNA sequencing of every suspected case of β -thalassaemia (such as the UK, in which more than 100 different β -thalassaemia mutations have been identified through routine DNA sequencing for the antenatal screening programme) (7).

α -THALASSAEMIA

α -Thalassaemia alleles result from mutations affecting either one α -globin gene (α^+ -thalassaemia) or both α -globin genes on the same chromosome (α^0 -thalassaemia). The majority of the commonest mutations are gene deletions but a number of point mutations within one of the two α -globin genes resulting in α^+ -thalassaemia have been described. More than 100 varieties of α -thalassaemia have now been identified (8).

SCREENING STRATEGY

The strategy for molecular screening for α -thalassaemia mutations is directed by the haematological findings and the ethnic origin and of the individual being screened. α^0 -Thalassaemia is found in mainly patients of Mediterranean or Southeast Asian in origin, although it may be found at lower frequencies in other ethnic groups such as Syrian and Iranian. Only one or two very rare α^0 -thalassaemia mutations have been described in patients of Asian Indian or African origin, and thus patients with a MCH value below 25pg will usually have the genotype of homozygous α^+ -thalassaemia rather than α^0 -thalassaemia trait. α^+ -Thalassaemia can reach high gene frequencies in parts of Africa and Asia, with the - α 3.7 deletion being the predominant mutation in African, Mediterranean and Asian individuals and the - α 4.2 being more common in Southeast Asian and the Pacific islands populations.

Thus a commonly used screening strategy is to:

1. Screen every sample for α^+ -thalassaemia deletions.
2. Screen for α^0 -thalassaemia if indicated by an MCH below 25pg, or by the presence of Hb H inclusions or suspected Hb H disease.
3. If negative results are still obtained, selective DNA sequencing of the two α -globin genes is used to investigate the possibility of non-deletional α^+ -thalassaemia.
4. At this point, if no α -thalassaemia defects are found and thalassaemia parameters are persistent, other options are considered, such as large β -gene deletions, β -thalassaemia trait + δ -thalassaemia trait, and normal Hb A₂ β -thalassaemia, as outlined in the flow chart.

DIAGNOSIS OF DELETION MUTATIONS BY GAP-PCR

This technique uses primers complementary to the sequences adjacent to the breakpoint ends of the deletion to create a characteristic amplified product which spans the deletion, but creates to large a product for any amplification with normal DNA. Gap-PCR provides a quick diagnostic test for α^+ -thalassaemia and α^0 -thalassaemia deletion mutations but requires careful application for prenatal diagnosis.

Seven of the most common deletional α -thalassaemia alleles can be diagnosed by multiplex gap-PCR: the two commonest α^+ -thalassaemia deletions ($-\alpha^{3.7}$ and $-\alpha^{4.2}$); two Mediterranean α^0 -thalassaemia alleles ($--_{MED}$ and $-(\alpha)^{20.5}$); and three Southeast Asian α^0 -thalassaemia alleles ($--_{SEA}$, $--_{FIL}$ and $--_{THAI}$) [9,10].

- Amplification of sequences in the α -globin gene cluster is technically more difficult than that of the β -globin gene cluster, requiring more stringent conditions for success due to the higher GC content of the breakpoint sequences and the considerable sequence homology within the α -globin gene cluster. Sub-optimal PCR conditions may result occasionally in unpredictable reaction failure and the problem of allele drop out. It is best practice to use a second method (MLPA) to confirm any prenatal diagnosis.
- The advantage of gap-PCR is that it provides a definitive diagnosis for the deletion.
- The disadvantage is that few deletions have had their breakpoint sequences determined to enable gap-PCR primers to be developed.

DIAGNOSIS OF DELETION MUTATIONS BY MPLA

Multiplex ligation-dependent probe amplification assay (MLPA) uses multiple probe pairs of different lengths which are hybridised to sequences across the α -globin gene cluster, ligated together, and then amplified in a semi-quantitative manner before quantitative fragment analysis on a genetic analyser. Probe pairs not hybridizing due to deleted sequences in an α -thalassaemia heterozygote result in a peak of half normal height. The usefulness of MLPA is that it detects any large α -thalassaemia deletion allele, and thus is more practical (but more expensive) than gap-PCR. (11).

- The technique will also identify gene rearrangements which lead to the duplication of the α -globin genes in the form of triple and quadruple α -gene alleles.
- MLPA cannot give a definitive identification of a deletion allele, as the probe sequences are not specific to any deletion breakpoint sequences. Therefore positive results should be reported as "consistent with" the particular allele that fits the pattern of half height peaks.

DIAGNOSIS OF NON-DELETION α -THALASSAEMIA

The term non-deletional α^+ -thalassaemia is used to describe the group of point mutations, small deletions or insertions that occur in one of the two α -globin genes resulting in little or no expression of the gene. These alleles can be detected by PCR using a technique of selective amplification of each α -globin gene followed by a general method of mutation analysis of the two amplified gene products.

- For screening and diagnosis of unknown non-deletional α^+ -thalassaemia mutations, DNA sequence analysis by Sanger sequencing is the gold standard method. An example is the molecular prenatal diagnosis of Hb H hydrops foetalis caused by the non-deletion mutation giving rise to Hb Adana (12).
- For the diagnosis of specific known mutations, other PCR-based methods have been applied, such as restriction enzyme digestion of amplified product (RE-PCR), reverse dot blotting and the amplification refractory mutation system (ARMS). Re-PCR has been used for the diagnosis of the mutation for Hb Constant Spring mutation (13), and also the $\alpha 2$ gene mutations: initiation codon (ATG→ACG) and the IVS1 donor site 5 base pair deletion (-GAGGT). Reverse dot blotting has been applied for diagnosing six Mediterranean α^+ -thalassaemia point mutations (14), and the amplification refractory mutation system (ARMS) for diagnosing six common Southeast Asian point mutations (15).

β -THALASSAEMIA

Although more than 170 different β -thalassaemia have been characterised and listed on the HbVAR database (16), only approximately 30 mutations are found at a frequency of 1% or greater in at risk groups. Most mutations are regionally specific and are divided into four regions: Mediterranean, Asian-Indian, Southeast Asian and sub-Saharan African countries. Countries without a large multi-ethnic immigrant population have just a few of the common mutations together with a larger and more variable number of rare ones (17). This makes it easy to screen for β -thalassaemia mutations in most cases if the ethnic origin of the patient is known.

SCREENING STRATEGY

The diagnostic strategy in many diagnostic laboratories screening for a limited mutation spectrum is to use a simple and cheap PCR based technique that allows the detection of the common mutations simultaneously. Although a bewildering variety of PCR technologies have been developed and applied for screening β -globin gene point mutations, most diagnostic laboratories are still using a simple and cheap technique based on allele specific oligonucleotide hybridisation or allele specific priming, eg reverse dot-blotting or ARMS. This approach will identify the mutation in more than 90% of cases and then a further screening for the known rare mutations will identify the defect in most of the remaining cases. Mutations remaining unidentified after this second screening are treated as unknown mutations and then characterised by DNA sequencing.

However, in many European laboratories, the impact of migration by different populations with high frequencies of haemoglobinopathies has led to a significantly enlarged the range of haemoglobinopathy mutations that need to be detected. This has increased the number of possible combinations and interactions of different mutations that require molecular analysis for prenatal diagnosis. Molecular diagnostic laboratories in such countries must have the technical expertise, equipment and diagnostic strategy to detect a large variety of mutations quickly for prenatal diagnosis, and these laboratories use DNA sequencing as the main screening method for the diagnosis of β -thalassaemia point mutations.

POINT MUTATIONS

Although a bewildering variety of PCR-based techniques have been developed and applied for screening β -globin gene point mutations, most diagnostic laboratories are either still using a simple and cheap technique based on allele specific oligonucleotide hybridisation or allele specific priming, eg reverse dot-blotting or ARMS-PCR, or routinely sequencing every suspected β -thalassaemia referral without any mutation screening approach. The main techniques used in diagnostic laboratories to characterize point mutations are summarized below.

REVERSE DOT-BLOTTING WITH ALLELE-SPECIFIC OLIGONUCLEOTIDES (ASO'S):

The first PCR based method to gain widespread use was the hybridisation of allele-specific oligonucleotide probes (ASOs) to amplified DNA bound to nylon membrane by dot-blotting. However, the method was limited by the need for separate hybridisation steps to test for multiple mutations. This was overcome by the development of the reverse dot-blotting technique, in which amplified DNA is hybridised to a panel of mutation specific probes fixed to a nylon strips. This technique is compatible with the optimum strategy for screening, using different panels for common and rare β -thalassaemia mutations. It has been applied to the diagnosis of β -thalassaemia mutations in Mediterranean individuals (18), African-Americans (19), and Thais (20).

COMMERCIALLY AVAILABLE ASO KITS:

The company Vienna Lab have marketed globin strip assays using allele-specific oligonucleotide probes which reverse-hybridise to biotinylated DNA and which are currently used in several diagnostic laboratories. The assays cover 21 α -thalassaemia mutations and 22 β -thalassaemia mutations, the latter optimised in separate strips for the detection of the common Mediterranean, Middle Eastern and Indian/Southeast Asian mutations.

OLIGONUCLEOTIDE MICROARRAYS:

The principle of reverse dot blotting has been brought up to date by the development of oligonucleotide microarrays for the simultaneous detection of multiple β -thalassaemia mutations, promising a one-step strategy for the identification of all possible β -globin gene mutations that result in both β -thalassaemia and β -chain variants (21). However, the commercial development of a single array that will identify any one of the thousand possible DNA sequence changes in the β -globin gene simultaneously is still awaited.

PRIMER-SPECIFIC AMPLIFICATION:

A number of PCR techniques for detecting β -thalassaemia mutations have been developed based on the principle of primer-specific amplification. The most widely used method is known as the amplification refractory mutation system (ARMS). The method provides a quick screening assay that is cheap and does not require high technology or dedicated instruments. ARMS primers have been developed to screen for the common β -thalassaemia mutations of all the main ethnic groups (22). The technique has been established for prenatal diagnosis in countries such as India and Pakistan, because of its rapid and inexpensive properties for screening and prenatal diagnosis, and ARMS primers can also be multiplexed to screen for multiple mutations in a single PCR assay (23).

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE):

Denaturing gradient gel electrophoresis (DGGE) is an indirect diagnostic method which has been widely used in the past to characterise β -thalassaemia mutations without any prior knowledge of the molecular defect. This approach detects at least 90% of β -thalassaemia mutations through shifted band patterns of amplified homoduplex and heteroduplex products (24). DGGE has been used for prenatal diagnosis of β -thalassaemia in Greece and India (25).

RESTRICTION ENZYME PCR (RE-PCR):

Restriction enzyme PCR (RE-PCR) has had a limited diagnostic role because very few β -thalassaemia mutations create or abolish a restriction endonuclease site, although its use can be widened by the artificial creation of a restriction enzyme site which includes the target mutation. The main use of RE-PCR has been for the analysis of β -globin gene haplotypes to determine the origin of globin gene mutations in different ethnic groups (26).

OTHER METHODS:

Many other methods have been applied for the diagnosis of known β -globin gene point mutations, including DHPLC, multiplex minisequencing, multiplex primer extension analysis, pyrosequencing, real time PCR quantification and melting curve analysis. All provide rapid and accurate genotyping of the common mutations and are worth considering as alternative diagnostic approaches for point mutations.

DELETION MUTATIONS

The strategy to identify large β^0 -thalassaemia deletion alleles is the same as that for the α^0 -thalassaemia deletion alleles - a combination of gap-PCR and MLPA. A considerable number can be identified definitively by gap-PCR, because the deletion breakpoint primer sequences have been published. These are the 290bp, 532bp, 619bp, 1393bp, 1605bp, 3.5kb, 10.3kb and 45kb β^0 -thalassaemia deletion alleles, plus Hb Lepore. As with α -thalassaemia, all but the smallest deletion alleles may be detected by MLPA with a "consistent with" diagnosis (11).

$\delta\beta$ -THALASSAEMIAS AND HPFH

GAP-PCR

The strategy to identify the $\delta\beta$ -thalassaemias, HPFH, Hb Lepore and Hb Kenya deletion mutations is the same as that for the β^0 - and α^0 -thalassaemia deletion alleles - a combination of gap-PCR and MLPA. Gap-PCR can be used for the diagnosis of nine of the common $\delta\beta$ -thalassaemia and HPFH alleles, plus Hb Kenya. These are: the Spanish, Sicilian, Vietnamese and the Macedonian/Turkish $\delta\beta$ -thalassaemia alleles; the Indian and Chinese $\gamma\delta\beta$ -thalassaemia alleles; and the HPFH1 (African), HPFH2 (Ghanaian) and HPFH3 (Indian) alleles (27).

- Gap-PCR provides a quick and simple genotype screening method for distinguishing the phenotype of HPFH trait from $\delta\beta$ -thalassaemia trait in Asian Indian, African and Mediterranean individuals with raised Hb F levels.

MLPA

MLPA may be used as the front line screening method, or in combination with gap-PCR for diagnosing those individuals carrying a novel or one of the rarer $\delta\beta$ -thalassaemia, $\epsilon\gamma\delta\beta$ -thalassaemia and HPFH deletion mutations (11).

- MLPA is a fast and effective way of screening for the spectrum and frequency of β -globin gene cluster deletion mutations in a particular population, as has been demonstrated for the UK populations (28).

ABNORMAL HAEMOGLOBINS

More than 700 haemoglobin variants have been described to date and listed on HbVAR, many of which have been identified by protein analysis and have never been characterised by their DNA sequence change. Positive identification at the DNA level for all is achieved by selective globin gene amplification and DNA analysis by Sanger sequencing, or by determination of the amino acid change by mass spectrometry, or by protein sequencing as molecular studies alone cannot detect post-translational changes in the Hb molecule.

The identification of the clinically important variants, namely Hb S, Hb C, Hb E, Hb D-Punjab and Hb O-Arab, is required routinely for antenatal screening and prenatal diagnosis. These can be diagnosed by simple and cheap DNA analysis techniques, such as ASO & dot-blot hybridization, ARMS, or RE-PCR (except Hb C) (29). Some of these techniques have also been developed for the definitive diagnosis of other reasonably common variants, such as Hb Q-India by ARMS-PCR, in order to replace diagnosis by DNA sequencing with a simpler and cheaper method (30).

CHAPTER 6

PND, PGD, & GENETIC COUNSELLING

The thalassaemia mutations and various abnormal haemoglobins interact to produce a wide range of thalassaemia and sickle cell disorders of varying degrees of clinical severity. There are four categories of disorders for which prenatal diagnosis is indicated: beta thalassaemia major & thalassaemia intermedia, Hb Bart's & Hb H hydrops foetalis syndrome, sickle cell syndromes and Hb E thalassaemia. The interactions of Hb E with Hb S and α -thalassaemia result in milder symptomatic disorders for which prenatal diagnosis is not required.

HOMOZYGOUS β -THALASSAEMIA

β -THALASSAEMIA MAJOR

The majority of individuals homozygous for β -thalassaemia have the transfusion-dependent condition called β -thalassaemia major. At birth, β -thalassaemia homozygotes are asymptomatic because of the high production of Hb F but as this declines, affected infants present with severe anaemia during the first or second year of life.

Clinical phenotype:

Treatment of the anaemia is by regular blood transfusion with iron chelation therapy to control iron overload, as recommended in the TIF guidelines, otherwise death from heart failure results in the second or third decade. This treatment does not cure β -thalassaemia major, although many patients now reach the fourth and fifth decade of life in good health and are often married with children. Although there has been some good progress at last with research into gene therapy, bone marrow transplantation remains the only cure for β -thalassaemia for the foreseeable future. Although this form of treatment has proved successful when carried out in young children, it is limited usually by the requirement of an HLA-matched sibling or relative.

Genotypes:

β -Thalassaemia major may result from the homozygous state for one or two different β^0 -types of β -thalassaemia mutation (genotype: β^0/β^0), homozygosity for one or two different types of severe β^+ -thalassaemia mutations (genotype: β^+/β^+), or from the compound heterozygous state for a β^0 and severe β^+ -thalassaemia mutation (genotype: β^0/β^+).

β -THALASSAEMIA INTERMEDIA

Thalassaemia intermedia is a milder clinical condition compared to thalassaemia major. Such Patients with this condition present later in life and are capable of maintaining a haemoglobin level above 6g/dl without transfusion. Thalassaemia intermedia is caused by a wide variety of genotypes, as described below, and the disease covers a broad clinical spectrum ranging from a very mild to a severe phenotype.

Clinical phenotype:

Patients with a severe condition present between 2 and 6 years of age and although they are capable of surviving with an Hb level of 5-7g/dl, they will not develop normally and are treated with minimal blood transfusion. At the other end of the spectrum are patients who do not become symptomatic until they reach adult life and remain transfusion independent. However, even these clinically milder patients tend to accumulate iron with age and many thalassaemia intermedia patients eventually develop clinical problems relating to iron overload in later life. Thalassaemia intermedia is caused by a wide variety of genotypes, as described below, and the disease covers a broad clinical spectrum of severity. Prenatal diagnosis is often requested by couples at risk of having a child with thalassaemia intermedia due to the unpredictability of the phenotype, particularly in cases in which one partner carries a severe β -thalassaemia mutation, but also in some cases where both partners carry mild mutations.

Genotypes

The genetic interactions leading to the phenotype of β -thalassaemia intermedia are very heterogeneous. The simplest genotypes are homozygosity or compound heterozygosity for one of the mild β -thalassaemia mutations. A genotype that should result in thalassaemia major can be ameliorated to thalassaemia intermedia by the co-inheritance of α^0 -thalassaemia trait or homozygous α^+ -thalassaemia. Compound heterozygous genotypes causing thalassaemia intermedia include the combination of a severe β -thalassaemia mutation with either $\delta\beta$ -thalassaemia trait, Hb Lepore trait or a triple / quadruple α -gene allele. It also results from the heterozygous state for one of the rare β -thalassaemia mutations in exon 3 that cause inclusion-body haemolytic anaemia, sometimes called dominantly inherited β -thalassaemia.

Finally, amelioration of a severe condition to a milder condition may result from the co-inheritance of a raised Hb F determinant, such as the Xmn1 polymorphism in the G γ -globin gene [$^6\gamma$ -158 (C \rightarrow T)], [HbG2:c.-211C \rightarrow T], which is linked to several β -globin gene haplotypes and causes the elevation of Hb F under circumstances of erythropoietic stress. For example Hb SS patients with the Indian β^S haplotype have a milder form of sickle cell disease, homozygotes for in particular to two β^0 -thalassaemia mutations linked to the Xmn1 polymorphism in Iranian and some Turkish patients (IVSII-1 (G \rightarrow A) and Cd 8 (-AA) have the milder condition of thalassaemia intermedia compared to patients with a different haplotype, and similarly homozygosity for the -29 (A \rightarrow G) mutation has a mild phenotype in African patients, but a severe one in Chinese patients due to linkage of the Xmn1 polymorphism.

- A few β^+ -thalassaemia mutations that are associated with an unusually mild phenotype are sometimes designated β^{++} -type in the literature (eg the silent β -thalassaemia mutation -101 (C \rightarrow T)). Thalassaemia intermedia patients that are homozygous for a β^{++} -type mutation usually have a very mild disorder and prenatal diagnosis is not normally indicated for this genotype.

- However, the combination of a mild β^{++} and a severe β^{+} or β^0 mutation results in a severe disorder in some cases and predicting the phenotype of thalassaemia intermedia on the basis of a known genotype can be difficult. Because the mutations are very uncommon, homozygotes do not exist and there is a general lack of data on cases with the co-inheritance of other β -thalassaemia alleles. The unpredictability of the phenotype in compound heterozygotes for these mutations remains a diagnostic and counseling problem.

Table 6.1 β -Thalassaemia disorders and Hb variant interactions: Indications for prenatal diagnosis and preimplantation genetic diagnosis

homozygous genotypes	Clinical Phenotype	PND indicated
β^0 or severe β^{+} -thal	Thal major	Yes
Mild β^{+} -thal	Thal intermedia	Occasionally*
Mild β^{++} -thal (silent)	Very mild thal intermedia	No
$\delta\beta^0$ -thalassaemia	Thal intermedia	Occasionally*
Hb Lepore	Thal intermedia to major (variable)	Occasionally*
HPFH	Not clinically relevant	No
Hb C	Not clinically relevant	No
Hb D-Punjab	Not clinically relevant	No
Hb E	Not clinically relevant	No
Hb O-Arab	Not clinically relevant	No
Compound heterozygous genotypes	Clinical Phenotype	PND indicated
β^0 /severe β^{+} -thal	Thal major	Yes
Mild β^{+}/β^0 or severe β^{+} -thal	Thal intermedia to major (variable)	Occasionally*
Mild β^{++}/β^0 or severe β^{+} -thal	Mild thal intermedia (variable)	Occasionally*
$\delta\beta^0/\beta^0$ or severe β^{+} -thal	Thal intermedia to major (variable)	Occasionally*
$\delta\beta^0$ /mild β^{+} -thal	Mild thal intermedia	Occasionally*
$\delta\beta^0$ /Hb Lepore	Thal intermedia	Occasionally*
Hb Lepore/ β^0 or severe β^{+} -thal	Thal major	Yes
Hb C/mild β^{+} -thal	Not clinically relevant	No
Hb C/ β^0 or severe β^{+} -thal	β -thal trait to intermedia (variable)	Occasionally*
Hb D-Punjab/ β^0 or severe β^{+} -thal	Not clinically relevant	No
Hb E/ β^0 or severe β^{+} -thal	Thal intermedia to major (variable)	Yes
Hb O-Arab/ β^0 -thal	Severe thal intermedia	Yes
aaa/ β^0 or severe β^{+} -thal	Mild thal intermedia	No
aaaa/ β^0 & aaaaaa/ β^0 -thal	Mild to severe thal intermedia (variable)	Occasionally*

* Occasionally, depending upon patient choice following genetic counselling
Abbreviations: thal, thalassaemia

HOMOZYGOUS α-THALASSAEMIA

HB BART’S HYDROPS FOETALIS SYNDROME.

The phenotype of the homozygous state for α⁰-thalassaemia is known as Hb Bart’s hydrops foetalis syndrome. This condition results from a deletion of all four globin genes and an affected fetus cannot synthesise any α-globin chains to make Hb F or Hb A. Fetal blood contains only the abnormal haemoglobin Bart’s (γ₄) and a small amount of Hb Portland (which can facilitate the application of prenatal diagnosis based on HPLC analysis of foetal blood). Without interuterine transfusion or perinatal treatment, the resulting severe foetal anaemia leads to asphyxia, hydrops fetalis, and stillbirth or neonatal death. Even with perinatal treatment, hydrops fetalis is a very severe condition, and thus prenatal diagnosis is indicated. In addition prenatal diagnosis helps to avoid the severe toxaeemic complications that occur frequently in pregnancy with hydropic fetuses, which endanger the pregnant mother (31).

HB H HYDROPS FOETALIS SYNDROME

In rare cases, the interaction of α⁰-thalassemia with a severe type of non-deletion α⁺-thalassemia allele can lead to Hb H hydrops foetalis syndrome, eg the interaction of the --SEA allele with the hyperunstable α-globin variants Hb Adana (6), Hb Agrinio and Taybe. Homozygosity for the α2 gene Poly A signal mutation (AATAAA>ATA--) has also been reported to result in Hb H hydrops foetalis syndrome (32). In these families prenatal diagnosis is indicated.

Table 6.2 α-Thalassaemia disorders: indications for prenatal diagnosis and preimplantation genetic diagnosis

Homozygous genotypes	Clinical Phenotype	PND indicated
α ⁰ -thalassemia (--/--)	Hb Bart’s hydrops foetalis	Yes
α ⁺ -thalassemia (-α/-α)	Not clinically relevant	No
α ⁺ -thalassemia (α ^I α/α ^I α)	Severe α-thal trait to severe Hb H disease	Occasionally*

Compound heterozygous genotypes	Clinical Phenotype	PND indicated
α ⁰ -thal/α ⁺ -thal (--/-α)	Hb H disease	No
α ⁰ -thal/α ⁺ -thal (--/α ^I α)	Severe Hb H disease to Hb H hydrops fetalis	Occasionally*

* Occasionally, depending upon patient choice following genetic counselling. Couples with genotypes that may lead to offspring with unpredictable but potentially severe phenotypes occasionally select to have prenatal diagnosis or PGD.

Abbreviations: thal, thalassaemia

SICKLE CELL DISORDERS

Sickle cell disease is characterised by a lifelong haemolytic anaemia, the occurrence of acute exacerbations called crises, and a variety of complications resulting from an increased propensity to infection and the deleterious effects of repeated vaso-occlusive episodes. With active management, the proportion of patients expected to survive to 20 years of age is approximately 90%. The course of the illness is very variable, even within individual sibships let alone different racial groups.

GENOTYPES

Sickle cell disease results from not only from homozygosity for the sickle cell gene, (β^S/β^S), but also from a number of different compound genotypes, most commonly Hb S/ β^0 thalassemia, Hb S/ β^+ thalassemia, Hb S/ $\delta\beta$ thalassemia, Hb S/Hb C, Hb S/Hb D-Punjab and Hb S/Hb O-Arab. A few rare β -chain variants have also been found to cause sickle cell disease in the compound heterozygous state with Hb S in one or two reported cases, namely Hb Quebec-Chori, Hb C-Njamena and Hb O-Tibesti. There are also 12 other rare sickling Hb variants with two amino acid substitutions which potentially will result in sickle cell disease in the homozygous state although no such cases have been reported. These are Hbs S-South End, C-Harlem, S-Antilles, S-Oman, S-Providence, S-Travis and C-Ziquinchor, S-Cameroon, C-Ndjamenia, S-Clichy, S-San Martin, S-Sao Paulo and S-Jamaica Plain. Two of these, Hb S-Antilles and Hb S-Oman, have such a severe sickling phenotype that they result in a form of sickle cell disease in the heterozygous state. Several of these rare variants have been reported to result in severe sickle cell disease in the compound heterozygous state with either Hb S or Hb C: namely Hb C-Harlem, Hb S-Southend and Hb S-Antilles.

Table 6.3 Sickle cell disorders: indications for prenatal diagnosis and preimplantation genetic diagnosis.

Homozygous genotype	Clinical Phenotype	PND indicated
Hb S/Hb S	Sickle cell disease	Yes
Compound heterozygous genotype		
Hb S/ β^0 or severe β^+ -thalassaemia	Sickle cell disease	Yes
Hb S/mild β^+ -thalassaemia	Mild sickle cell disease	Occasionally*
Hb S/ $\delta\beta^0$ -thalassaemia	Mild sickle cell disease	Occasionally*
Hb S/Hb Lepore	Mild sickle cell disease	Occasionally*
Hb S/HbC	Sickle cell disease (variable severity)	Yes
Hb S/Hb D-Punjab	Sickle cell disease	Yes
Hb S/Hb O-Arab	Sickle cell disease	Yes

continues...

Hb S/Hbs C-Harlem, S-Southend S-Antilles, S-Oman	Sickle cell disease	Yes
Hb C/Hb S-Antilles	Sickle cell disease	Yes
Hb S/Hbs Quebec-Chori, C-Ndjamena, O-Tibesi	Sickle cell disease	Yes
Hb S/Hbs I-Toulouse, Shelby, Hope, North Shore	Haemolytic anaemia	No
Hb S/Hb E	Mild to severe sickle cell disease	Occasionally*
Hb S/HPFH	Very mild sickle cell disease	No

* Couples with genotypes that may lead to offspring with unpredictable phenotypes occasionally select to have prenatal diagnosis or PGD.

Hb E syndromes

Many types of Hb E syndromes are observed, due to various and complex interactions with α -thalassaemia, β -thalassaemia, or other haemoglobin variants. The only symptomatic form for which prenatal diagnosis may be considered result from the compound heterozygosity of Hb E trait with β -thalassaemia trait.

HB E / β THALASSAEMIA.

Hb E thalassaemia, the compound heterozygous state of Hb E and β thalassaemia, is a common disease in Thailand and parts of Southeast Asia. It results in a variable clinical picture similar to that of homozygous β thalassaemia, ranging from a condition indistinguishable from thalassaemia major to a mild form of thalassaemia intermedia. The severest conditions are found in individuals with β^0 thalassaemia who usually have about 40-60% Hb F, the remainder being Hb E. Compound heterozygotes for Hb E and β^+ thalassaemia usually have a milder disorder and produce variable amounts of Hb A. As with homozygous β -thalassaemia, the genetic factors that account for a mild phenotype in some, but not all patients, are mild β^+ - type mutations, the co-inheritance of α -thalassaemia, and the homozygosity for the XmnI restriction site due to the C→T polymorphism at position -158 5' to the $\epsilon\gamma$ -globin gene.

OTHER HB E INTERACTIONS

Many types of Hb E syndromes are observed due to the interaction of Hb E with α -thalassaemia or α -thalassaemic haemoglobin variants such as Hb Constant Spring. These are summarised in the table below. The two most commonly observed symptomatic disorders are Hb AE Bart's disease and Hb EF Bart's disease, for which prenatal diagnosis is not normally indicated. These can result from many different genotype combinations, which can only be differentiated by family studies and further investigation by DNA analysis.

- **Hb AE Bart's disease.** Results from the interaction of Hb E trait with co-inherited Hb H disease. Characterised by the presence of Hb A, Hb E (15%) and Hb Bart's. Two common subtypes of Hb AE Bart's disease have been observed: without or with Hb Constant Spring. The latter disorder has the more severe clinical syndrome.
- **Hb EF Bart's disease.** Results from the interaction of homozygous Hb E with co-inherited Hb H disease, or Hb E β -thalassaemia with Hb H disease, again both types can be with or without Hb Constant Spring. Characterised by the presence Hb E (80%), Hb F (10%) and Hb Bart's.

Table 6.4 Hb E syndromes: indications for prenatal diagnosis and preimplantation genetic diagnosis.

Asymptomatic forms	Genotype	Anaemia	PND indicated
Hb E trait	β^A/β^E	No	No
Hb E trait & α^+ -thal trait	β^A/β^E & $\alpha\alpha/\alpha^+$ -thal	No	No
Hb E trait & α^0 -thal trait	β^A/β^E & $\alpha\alpha/\alpha^0$ -thal	No	No
Hb E homozygote	β^E/β^E	No	No
Hb E homozygote & α -thal trait	β^E/β^E & $\alpha\alpha/\alpha$ -thal	No	No
Hb E/Hb C	β^E/β^C	No	No
Symptomatic forms	Genotype	Anaemia	PND indicated
Hb E homozygote / & Hb CS homozygote	β^E/β^E & Hb CS/Hb CS	Mild	No
Hb E/ β^0 -thalassaemia	β^0/β^E	Moderate to severe	Yes
Hb E/ β^+ -thalassaemia	β^+/β^E	Mild	Yes
Hb EA Bart's:			
Hb H disease with Hb E trait	α^0 -Thal / α^+ -thal & β^A/β^E	Moderate	No
Hb H-CS disease with Hb E trait	α^0 -Thal / Hb CS & β^A/β^E	Moderate	No
Hb EF Bart's:			
Hb H disease with Hb E homozygote	α^0 -Thal / α^+ -thal & β^E/β^E	Moderate to severe	No
Hb H-CS disease with Hb E homozygote	α^0 -Thal / Hb CS & β^E/β^E	Moderate to severe	No
Hb H disease with Hb E/ β -thalassaemia	α^0 -Thal / α^+ -thal & β^0/β^E	Moderate to severe	No
Hb H-CS disease with Hb E/ β -thalassaemia	α^0 -Thal / Hb CS & β^0/β^E	Moderate to severe	No

Abbreviations: A, normal; CS, Constant Spring; thal, thalassaemia

REPRODUCTIVE CHOICES

Depending on acceptable practices in each society, after carrier screening and genetic counselling, at-risk couples can then be offered the reproductive choice to avoid the birth affected children, or helped to prepare for the possible birth of a severely affected child. Amongst the available options to avoid having affected children are, before marriage, a change in partner choice; or, after marriage, by remaining childless, opting for gamete donation or adoption. However couples rarely take these options. Instead it is more common for at-risk couples to avoid the birth of an affected child by opting for a conventional prenatal diagnosis in early pregnancy, or before pregnancy by choosing pre-implantation genetic diagnosis (PGD). Both conventional prenatal diagnosis and PGD involve prior mutation characterization in the parents and subsequent foetal (or embryo) DNA analysis.

PRENATAL DIAGNOSIS

Prenatal diagnosis is normally carried out by analysis of foetal DNA prepared from amniotic fluid cells or preferably, from chorionic villi in the first trimester of pregnancy from 11 weeks of pregnancy. The DNA analysis techniques used are those best suited to the laboratory's infrastructure, expertise and target population.

An exception to DNA analysis is possible for women at risk of α -thalassaemia hydrops foetalis when, ultrasound examination shows hydropic features in the foetus. In these cases a quick diagnosis may be obtained in a few minutes by analysing foetal blood using haematological techniques such as HPLC, as Hb F will be absent if the foetus is affected.

DIAGNOSTIC CENTRES.

Prenatal diagnosis is usually carried out in specialist diagnostic laboratories due to the problems related to PCR-based prenatal diagnosis, which include the high sensitivity of PCR techniques to maternal DNA contamination and the requirement that laboratories need quick access to a complex battery of probes and primers necessary to detect a wide range of thalassaemia mutations. It is important to consider that all CVS and AF samples (with or without culture) may be contaminated with maternal tissue or cells. Chorionic villus samples should be carefully dissected to remove maternal tissue. This is usually performed by cytogeneticists.

The diagnostic laboratory may or may not be associated with a cytogenetics laboratory which will also offer foetal sample backup cultures as well as karyotype analysis for detection of aneuploidies. However it is highly recommended that women also be evaluated for risk of transmitting other conditions, for example they should be offered karyotype analysis if appropriate.

DNA SOURCES

There are three possible procedures, chorionic villus sampling, amniocentesis and foetal blood sampling (no longer used in most centres). Prenatal diagnosis is preferably carried out by chorionic villus DNA analysis in the first trimester of pregnancy from 11 weeks of pregnancy.

- **Chorionic villi.** Chorionic villus sampling (CVS) usually provides sufficient villi to give an excellent yield of DNA, and the risk of maternal contamination is low with careful microscopic dissection to remove contaminating maternal decidua. If the sample is small, and has to be cultured it should be noted that there is an increased risk of maternal contamination.
- **Amniotic fluid.** Amniotic fluid (AF) samples can be used for molecular analysis directly with amniocytes spun down from the amniocentesis sample. The DNA yield is generally lower than from CVS samples but usually the yields are sufficient DNA for analysis with PCR-based methods. However there is a risk of maternal cell contamination in the spun down cells.
- **Cultured Amniocytes.** Amniotic fluid (AF) samples cultured for 10-14 days provide a greater yield of DNA than uncultured samples. Culturing the sample normally decreases the risk of maternal contamination, but the reverse has been observed, although it is very rare.

DIAGNOSTIC PROBLEMS.

It is recommended that the laboratory evaluates all samples undergoing prenatal diagnosis for maternal contamination. Apart from maternal DNA contamination, diagnostic errors may be introduced by technical pitfalls (for example partial digestion by restriction enzymes), by inherent properties of the DNA sample (eg rare nucleotide variations that may prevent annealing of the PCR primers or probes), or from sub-optimal reaction conditions eg allele dropout or nonspecific priming (33). All logical steps should be taken to monitor, and thus preclude, such events. If identical methods are used to identify the mutations in the parents and subsequently to analyse the foetal DNA, then any pitfalls caused by rare nucleotide variations will be previously identified and can be addressed by adapting the diagnostic strategy accordingly.

BEST PRACTICE PROCEDURES.

The following best practice procedures and precautions (5) are intended to minimise the diagnostic error rate.

- Blood samples and copies of the haematology results should be obtained from both parents with every prenatal diagnosis to confirm the phenotype of parents by haematological techniques and to provide a source of control DNAs for the molecular analysis.
- Before performing a prenatal diagnosis, the genotypes in the prospective parents should be accurately characterized and confirmed.

- If the women's partner is not available for testing, it is important to evaluate the risk of a major haemoglobinopathy in the foetus. When the foetal diagnosis identifies a heterozygote foetus, it is recommended that the entire β -globin gene of the foetal DNA is sequenced to exclude other β globin gene mutations.
- Always analyse the fresh parental DNA sample(s) alongside the foetal DNA, and always include appropriate control DNA's within the test batch, preferably all as duplicates. Always include PCR blanks and, optionally, sample blanks (e.g. some labs include a DNA extraction control sample using reagents from the extraction procedure).
- Perform a duplicate mutation test(s) on the foetal DNA, along with relevant controls, possibly with different DNA concentrations.
- Use a limited number of amplification cycles to minimise co-amplification of any maternal DNA. This is especially important when using highly sensitive techniques such as ARMS PCR for prenatal diagnosis, as there may be a risk of preferential amplification of maternal alleles.
- For the optimal accuracy of a prenatal result, one approach is to base all prenatal diagnosis results on two independent diagnostic methods to identify/investigate each parental risk allele (mutation). For example to test for α 0-thalassaemia mutations by both MLPA and gap-PCR. In laboratories or for some rare β -thalassaemia mutations for which Sanger sequencing is the only diagnostic method, then all diagnoses (on foetal and parental DNA) should involve sequencing in both the forward and reverse directions. If a rare nucleotide variation is detected within the timeframe of a PND, and confirmation by both forward and reverse sequencing or another method is not possible, then confirmation can be addressed by performing a second forward sequencing analysis using alternative primer sets to produce the target PCR template.
- It is recommended to check for maternal DNA contamination in every case. This is best performed using a panel of Short Tandem Repeat polymorphisms (STRs), available in commercial kits for the analysis of up to 16 markers. When the foetal genotype is the same as the mother's, and no informative marker to indicate the presence or absence of maternal contamination is found, the foetal diagnosis report should state these findings and indicate a greater risk of error in the foetal result.
- If the paternal DNA sample is analysed in addition to the foetal and maternal DNA sample, then these tests may also identify non-paternity. Such incidental findings should be handled according to local practice. In laboratories which do not routinely analyse paternal samples when performing prenatal diagnosis, paternity is assumed to be true and the prenatal report should state that the accuracy of the diagnosis is based on declared relationships.

- External Quality Assessment (EQA) is also an intrinsic part of best practice. EQA provides a long-term, retrospective assessment of laboratory performance, allowing laboratories to demonstrate consensus with their peers and providing information on inter-method comparability. Participation in EQA is encouraged and is essential, where available for an investigation, for any laboratory already accredited or seeking accreditation to international standards eg ISO 17025, ISO 15189 or equivalent.

CELL-FREE FETAL DNA IN MATERNAL PLASMA.

Maternal plasma contains a relatively low amount of cell-free DNA, of which approximately 3 - 6% is foetal DNA derived from dying placental cells. Non-invasive prenatal diagnosis (NIPD) by the analysis of cell-free fetal DNA in maternal plasma is potentially a more attractive approach than PND using chorionic villi DNA, but much more technically difficult due to the presence of cell-free maternal DNA and the fact the cell free DNA is degraded into small fragments of approximately 300bp in size. This approach has been used for determining foetal sex and Rhesus D blood group typing for several years, and currently, screening programmes are being introduced in the UK to detect Down's syndrome by determining the copy number of chromosome 21 in cell free foetal DNA using new approaches such as digital PCR and next generation sequencing (34). However the use of this approach for NIPD of thalassaemia and sickle cell disease is still at the research stage and some way off implementation as a routine alternative approach for PND.

PREIMPLANTATION DIAGNOSIS

Preimplantation genetic diagnosis represents a "state-of-the-art" procedure that allows at-risk couples to have disease free children without the need to terminate affected pregnancies. PCR-based diagnostic methods can be used on three types of cells: a polar body from the oocyte/zygote stage, 1-2 blastomeres from cleavage stage embryos, or on 5-10 trophoctoderm cells from blastocysts (35). Although the technique requires a combined expertise in both reproductive medicine and single-cell PCR techniques, a small number of centres around the world are now routinely performing this procedure for both α -thalassaemia (36) and β -thalassaemia (37).

The approach is a useful alternative to PND for couples who have fertility problems and require assisted reproduction therapy anyway and/or who have already had one or more therapeutic abortions and/or for whom religious or ethical beliefs will not permit the termination of pregnancy. The latter reason is usually the least common reason for requesting PGD and, furthermore, a study of the attitude of Muslim women to PGD demonstrated that parents concerns were complex and PGD was only acceptable to 27% of couples questioned (38).

Preimplantation genetic diagnosis is a technically challenging, multi-step and expensive procedure. The PCR protocol must be able to diagnose the required genotype in single cells reliably and accurately and it also has to be optimised to minimise the problems of total PCR failure, allele drop out (the failure of one allele to amplify to detectable levels), and the problems of DNA contamination. PGD for haemoglobinopathies has been reported using nested-PCR protocols coupled with various mutation detection techniques: denaturing gradient gel electrophoresis (DGGE), single strand conformation analysis (SSCA), minisequencing and real time PCR (39). The birth of a healthy unaffected baby depends not only on an accurate molecular diagnosis, but also on the success of each of the multiple stages of the assisted reproduction procedure. Overall, the success rate of the procedure has been only 20-30% and thus this approach has not been used routinely as an alternative to prenatal diagnosis of the haemoglobin disorders. One specific use of this approach is to allow the birth of a normal child that is HLA identical to an affected sibling, thus permitting a possible cure by stem cell transplantation (40).

However the recent development of a technique using SNP arrays with customized bioinformatics, commercially available as a method known as "karyomapping" offers a new way of detecting genetic conditions in IVF embryos (41). It can be used to simultaneously detect both a chromosomal count (euploidy/aneuploidy) and a monogenic disorder in PGD, eliminating the time consuming and expensive process of developing a specific molecular test for monogenic PGD in each couple. Using trophoctoderm biopsy, karyomapping and cryopreservation rates as high as 60-70% for achieving a pregnancy after a PGD cycle have now been achieved (M. Petrou, personnel communication). However, karyomapping has several disadvantages, including the need to establish phase of SNPs to determine haplotypes requiring co-analysis other first degree relatives of the prospective parents (either other children, or, if unavailable, prospective grandparents), the much higher cost of genetic analysis and finally the need to cryopreserve embryos, which adds an additional unknown factor with respect to long-term safety of outcomes..

GENETIC COUNSELLING

It is best practice for all couples undergoing prenatal diagnosis to be counselled by a qualified health professional who is well versed in the molecular diversity of the haemoglobinopathies. No woman should undergo prenatal diagnosis unless she has been counselled by a qualified health professional, and preferably been provided with appropriate information materials. A good selection of these are available on the web-pages www.chime.ucl.ac.uk/APoG1, <http://www.enerca.org>; and the NHS web-page <http://sct.screening.nhs.uk/>. Irrespective of the couples' previous experience, counselling should be offered for each pregnancy found to be at risk. Genetic counselling should be "non-directive": that is, the genetic counsellor's main role is to provide at-risk individuals with full information, give them time to consider that information, and support them in making the decision they feel to be most appropriate for them.

The essential requirements for genetic counselling are:

- A correct diagnosis in the presenting family member.
- An explanation of the nature and prognosis of the disorder, the treatment options available and where to find them.
- The estimation of genetic risk for parents and family members. This requires drawing a family tree. It may also call for investigations on other family members.
- Communication of genetic risks and the options for avoiding them, including the chances of parents and other family members passing the disorder on to their children, and an explanation of that risk. The options for avoiding further affected children must also be addressed, including techniques of prenatal diagnosis and associated risks including risk of error and pregnancy complications.
- Support for the individual or couple in making the decision that is right for them.
- Accessibility for long-term contact: people at risk often need counselling and support at several points in their life.

B-THALASSAEMIA.

The problem of mild phenotypic heterogeneity requires a genetic counsellor to be knowledgeable in the molecular genetics of the thalassaemias, so that he/she can understand the molecular mechanisms involved and communicate this information to families. More than 200 β -thalassaemia mutations have been described wide range of phenotypic severity (from silent to very severe), all of which can potentially interact to produce a β -thalassaemia disorder ranging from a very mild form of intermedia to the severe form, thalassaemia major. The severity of homozygous β -thalassaemia is also affected by ameliorating factors such as the co-inheritance of α -thalassaemia or gene modifiers that increase the Hb F production

For instance, the mild β^+ -type mutation -88 (C \rightarrow T), [HBB:c.-138 C>T], generally results in a disorder with a very mild clinical phenotype in patients homozygous for this mutation. Thus in the case when both partners carry the mild β^+ -type mutation -88 (C \rightarrow T), the question arises whether the couple should consider prenatal diagnosis (PND). This is also the case with a number of other mild β -thalassaemia mutations in the homozygous state, such as IVS1-6 (T \rightarrow C), [HBB:c92+6T>C], which may present as thalassemia intermedia or major. However the mild β^+ -thalassaemia mutation Cap+1 (A \rightarrow C), [HBB:c50A>C] has been reported to result in thalassaemia major in some homozygous patients.

The phenotype of mild thalassaemia intermedia poses an ethical dilemma as far as termination of pregnancy is concerned. There are silent β -thalassaemia mutations, such as -101 (C \rightarrow T), [HBB:c-151C>T], that produce a mild or very mild clinical phenotype in the homozygous state or even interacting in the compound heterozygous state with a severe β -thalassaemia mutation, such as IVS1-110 (G \rightarrow A). PND is not considered in such cases, or where one partner has β -thalassaemia trait and the other carries a triplicated α -gene allele or a hereditary persistence of foetal haemoglobin (HPFH) gene.

SICKLE CELL DISORDERS.

Counselling couples at risk for sickle cell disorders is often perceived as relatively simple but it is also in fact quite complex because of the wide range in severity of sickle cell disorders, ranging from the very mild to the very severe. As a result, parents face considerable difficulty in deciding whether or not to request PND. Similarly to β -thalassaemia, the above modifying factors may also influence the severity of sickle cell.

α^0 -THALASSAEMIA.

Counselling couples at risk for homozygous α^0 -thalassaemia (hydrops foetalis) is more straightforward because of the usually hopeless prognosis for an affected foetus and the possibility of life-threatening obstetric risks for the mother (42). In such cases it is rare for a couple to decline PND, although the fact that regular intra-uterine transfusion therapy can be instigated when an affected foetus is identified early enough brings added complications to the decision-making process (43). But more information is needed about the outcomes of such before intra-uterine transfusion can be offered widely. To date, the main problems found in babies surviving intra-uterine transfusion therapy have been severe neurological function, although some appear to be doing well and the long term neurological function appears good (44). The question, however, is whether it is justified to provide treatment that saves the life of an α -thalassaemia major foetus but then creates all the challenges associated with the treatment of β -thalassaemia major, such as regular blood transfusions and iron chelation therapy.

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APPENDICES

Appendix I. Traditional and HGVS nomenclature of globin gene mutations referred to in these guidelines

Traditional nomenclature	HGVS nomenclature
Hb variant mutations involving <i>HBB</i> gene	
Hb C	c.19G>A
Hb C-Harlem	c.[20A>T;220G>A]
Hb C-Ndjamena	c.[20A>T;112T>G]
Hb C-Ziguinchor	c.[20A>T;176C>G]
Hb D-Punjab	c.364G>C
Hb E	c.79G>A
Hb Hope	c.410G>A
Hb I-Toulouse	c.199A>G
Hb Jamaica Plain	c.[20A>T;205C>T]
Hb Kansas	c.308A>C
Hb Koln	c.295G>A
Hb Lepore Hollandia	NG_000007.3:g.63290_70702del
Hb Lepore-Baltimore	NG_000007.3:g.63564_70978del
Hb Lepore-Boston-Washington	NG_000007.3:g.63632_71046del
Hb North Shore.	c.404T>A
Hb O-Arab	c.364G>A
Hb O-Tibesi	c.[34G>A;364G>A]
Hb Quebec-Chori	c.263C>T
Hb S	c.20A>T
Hb S-Antilles	c.[20A>T;70G>A]
Hb S-Clichy	c.[20A>T;26A>C]
Hb S-Cameroon	c.[20A>T;271G>A]
Hb S-Oman	c.[20A>T;364G>A]
Hb Jamaica Plain	c.[20A>T;205C>T]
Hb S-Providence	c.[20A>T;249G>T or 249G>C]

Hb S-Sao Paulo	c.[20A>T;196A>G]
Hb S-San Martin	c.[20A>T;317T>C]
Hb S-South End	c.[20A>T;399A>C]
Hb S-Travis	c.[20A>T;428C>T]
Hb Shelby	c.394C>A
Thalassaemia mutations involving <i>HBB</i> gene	
-102 (C→A)	c.-152C>A
-101 (C→G)	c.-151C>G
-101 (C→T)	c.-151C>T
-92 (C→T)	c.-142C>T
-88 (C→T)	c.-138C>T)
-29 (A→G)	c.-79A>G
Cap+1 (A→C)	c.-50A>C
Cap+8 (C→T)	c.-43C>T
Cap+10 (-T)	c.-41delT
Cap+33 (C→G)	c.-18C>G
Cap+45 (C→G)	c.-3C>G
Cd 8 (-AA)	c.25_26delAA
Cd 8/9 (+G)	c.27_28insG
IVSI-5 (G→A)	c.92+5G>A
IVSI-5 (G→C)	c.92+5G>C
IVSI-6 (T→C)	c.92+6T>C
IVSI-110 (G→A)	c.93-21G>A
IVSII-1 (G→A)	c.315+1G>A
IVSII-844 (C→G)	c.316-7C>G
IVSII-844 (C→A)	c.316-7C>A
+1480 (C→G)	c.*+6C>G
Poly A (A→C) (AATAAA→CATAAA)	c.*+108A>C
Poly A (T→C) (AATAAA→AACAAA)	c.*+110T>C
Poly A (-AT) (AATAAA→A--AAA)	c.[*+109_.*+110delAT or *+110_.*+111delTA]
Poly A (-AA) (AATAAA→AAT--A)	c.*+111_.*+112delAA

Hb variant mutations involving <i>HBA1</i> or <i>HBA2</i> genes	
Hb Adana	HBA2:c.179G>A or HBA1:c.179G>A
Hb Agrinio	HBA2:c.89T>C
Hb Constant Spring	HBA2:c.427T>C
Hb Chesapeake	HBA1:c.278G>T
Hb Fort Worth	HBA2:c.83A>G
Hb G-Philadelphia	HBA2:c.[207C>G (or HBA1) or 207C>A]
Hb Hasharon	HBA2:c.142G>C
Hb M-Boston	HBA2:c.175C>T (or HBA1)
Hb Ottawa	HBA2:c.46G>C
Hb Taybe	HBA1:c.118_120delACC
Hb Q-India	HBA1:c.193G>C
Hb Q-Thailand	HBA1:c.223G>C
Hb Q-Iran	HbA2:c.226G>C
Thalassaemia deletion mutations involving <i>HBA1</i> or <i>HBA2</i> genes	
--FIL	NG_000006.1:g.11684_43534del31851
--MEDI	NG_000006.1:g.24664_41064del16401
--SEA	NG_000006.1:g.26264_45564del19301
--THAI	NG_000006.1:g.10664_44164del33501
-(α) ^{20.5}	NG_000006.1:g.15164_37864del22701
- α ^{3.7} (Type I)	NG_000006.1:g.34164_37967del3804
- α ^{4.2}	Not defined
Thalassaemia non-deletion mutations involving <i>HBA1</i> or <i>HBA2</i> genes	
α 2 initiation codon (ATG→ACG)	HbA2:c.2T>C
α 2 IVS1 donor site 5 base pair deletion (-GAGGT-)	HbA2:c.95+2_95+6delTGAGG
α 2 Poly A signal AATAAA->AATA- -	HbA2:c.*+93_.*+94delAA
α 2 Cd 142 (TAA→CAA) [→172aa]	HBA2:c.427T>C

Appendix 2. About TIF

The Thalassaemia International Federation (TIF) is a non-profit, non-governmental organisation founded in 1987 by a small group of patients and parents representing mainly National Thalassaemia Associations in Cyprus, Greece, UK, USA and Italy - countries where thalassaemia was first recognised as an important public health issue and where the first programmes for its control, including prevention and clinical management have started to be promoted and implemented.

TIF works in official relations with the World Health Organisation (WHO) since 1996 and has been awarded by WHO, the Dr Lee Jong-wook Memorial Prize for Public Health in 2015 (www.thalassaemia.org.cy)

Its mission is the development of National Control Programmes, including both components of prevention and patient management and the promotion of their establishment across the world.

The objective is to gain equal access to quality health care for every patient with thalassaemia wherever he or she may live. The means to achieve these aims include:

- The establishment of new and promotion of existing National Thalassaemia Patient/Parents Associations, and improving their capacity to advocate for necessary improvements in services
- The encouragement and support for studies and research for further improving prevention strategies, clinical care and for achieving the long-awaited final cure and
- The extension of knowledge and experiences gained from countries with successful control programmes to those in need. This is achieved through an extensive educational programme which includes publications, workshops, conferences and electronic courses
- The promotion of policies to regional and national health authorities.

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*Joanne Traeger-Synodinos, Cornelis L Hartevelde, John M Old, Mary Petrou, Renzo Galanello, Piero Giordano, Michael Angastiniotis, Barbara De la Salle, Shirley Henderson and Allison May. "EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies". Eur J Hum Genet 2014;1-12;1018-4813/14

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