PREVENTION OF THALASSAEMIAS AND OTHER HAEMOGLOBIN DISORDERS

VOLUME 1: PRINCIPLES

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DEDICATED TO PROFESSOR RENZO GALANELLO

The devastating news regarding the premature death of Professor Renzo Galanello reached the medical, research, scientific, patients' and parents' communities around the world on the morning of the 13th of May, 2013. The global patients thalassaemia community has not only lost a great scientist and doctor, but also a great friend and supporter of the patients' rights. His death will be a loss not only to his family and to the local thalassaemia services in Cagliari but also to the National Thalassaemia Services in Italy, to the European region and to the global medical/research communities.

Professor Renzo Galanello was a pioneer in the field of thalassaemia research, diagnosis prevention and management. For many decades, Professor Galanello's Centre in Cagliari has been a leading international research laboratory in this field and was one of the first to be assigned as a collaborating centre of expertise by the World Health Organisation.

Professor Renzo Galanello has not only been an author and co-author of numerous peer reviewed publications on the prevention and management of haemoglobin disorders, but also of a number of educational books published by Thalassaemia International Federation (TIF).

The news about his premature death found 'Prevention of Thalassaemia and other Haemoglobin Disorders volume 1, 2nd updated edition', in the printing process. Upon unanimous consensus of the authors of this book and the publishers, TIF, the printing process was suspended to include this dedication.

''We consider this dedication a very small expression of our immense and deep respect towards the invaluable contribution of a true scientist, a great researcher and a companionable doctor. ''

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PREFACE

Volume 1 of the Prevention Book presents the principles of a programme for the prevention of the thalassaemia and other haemoglobin disorders, including a description of the various types of disorders requiring prenatal diagnosis, the strategies used for carrier screening, and a number of annexes listing upto date epidemiological and mutation data on thalassaemia. This book was written for use in combination with Volume 2, which describes many of the laboratory protocols in great detail.

The haemoglobin disorders are the most common clinically serious single gene disorders in the world with an estimated 300,000 affected births each year, of which approximately 60,000-70,000 are ß-thalassaemia major. However, these figures are just an estimate - and as there have been few in-depth, reliable epidemiological studies into the incidence of Thalassaemia in many parts of the world, they are likely to be a gross underestimate. Although a detailed analysis of molecular variance in the thalassaemias exists in almost every affected country of the world, true gene frequencies for many populations are unfortunately still not available. Indeed, this book indicates how, in addition to preventing new births of affected children, a greater emphasis on prevention is likely to improve knowledge of the epidemiology of thalassaemia through pilot studies assessing the prevalence of the disease in different areas.

The benefits of prevention programmes have been clearly demonstrated by a number of Mediterranean countries over the past 30 years. The purpose of this book is to draw on this experience, and to lay out the issues and approaches involved in preventing new births of children affected with haemoglobin disorders.

The authors present the advantages for establishing prevention programmes, as well outlining the tools required for such programmes to be truly effective - understanding the epidemiology of the diseases concerned and developing appropriate health education programmes that are sensitive to prevailing cultural and religious norms.

As research into genetic disorders continues apace, the screening for and diagnosis of haemoglobin disorders, including prenatal diagnosis, are becoming increasingly significant aspects of prevention - along with genetic counselling and the need for medical specialists to be ever more sensitive to the power and complexity of the information with which parents are being presented. These subjects are addressed in detail, including problems associated with screening for the haemoglobinopathies.

Many innovations have occurred since the publication of the First Edition of this book in 2005, and therefore every chapter in this Second Edition has been revised and updated. In addition, we have included a chapter on newborn screening of the haemoglobinopathies, as several European countries have established national screening programmes for sickle cell disease and other haemoglobinopathies since the publication of the first edition. We are confident that this book, together with the revised Volume 2, will continue to provide a valuable tool and resource for all diagnostic laboratories engaged in the prevention of the thalassaemias and other haemoglobin disorders.

Of course the prevention of thalassaemia remains a delicate issue and the authors remain sensitive to this fact and have no wish to simplify or minimise the significance of the issues involved. Rather, we hope this book will contribute to constructive debate on the issue while encouraging ongoing pioneering work in the field of prevention.

The Editors

TABLE OF CONTENTS

FOREWARD

Thalassaemia and the haemoglobinopathies are a major health problem, placing an immeasurable emotional, psychological and economic burden on millions of people around the world. Substantial progress has been made towards understanding the pathology of thalassaemia and its treatment. But the fact remains that the treatment of thalassaemia is a costly and painful process. More important still, for many, many patients, particularly in poor countries, knowledge of the disease remains sparse and treatment is an unaffordable luxury.

It is in this context that the role of prevention is best understood.

Without any kind of prevention, it expected that there will be around 60000 births of children suffering from beta thalassaemia syndromes each year and around 300000 suffering from sickle cell syndromes, although these figures may be underestimates and do not include the serious alpha thalassaemia syndromes. Most of these children are born in Asia and Africa, in low resource countries. Over the past 25 years, a number of countries around the world, mainly those of the Mediterranean basin, have seen a dramatic fall in the number of affected births as a result of prevention programmes based on health education, widespread screening and genetic counselling. For many involved in the field of thalassaemia, the experiences of these countries have provided a wealth of expertise from which other countries can and indeed, must draw. This book provides a thorough overview of the issues involved in prevention. As such, it represents a major step towards a fundamental goal of the Thalassaemia International Federation (TIF) - that is, to establish prevention programmes based on the same high standard of best practice in every country of the world. Advances in genetic research have made prevention a feasible option everywhere. TIF is determined not only to encourage prevention programmes but also to ensure that lessons already learned are not lost - determined that those countries that can least afford a period of trial and error are provided with the essential knowledge needed to establish an effective prevention programme.

Of course, the issue of prevention is a difficult one, fraught with moral and emotional complexity. But no one is insisting that a blanket solution be applied everywhere - only that each country take a hard-headed look at the costs of inaction, before assessing what is feasible in a local context, based on well-established international norms. The fight against disease always requires dedication - both in terms of time and resources. For TIF, the overriding concern is that families should be supported in finding ways to avoid the burden and pain of dealing with a serious genetic disease, the haemoglobin disorders that can so easily be avoided.

As Chairman of the Thalassaemia International Federation, I welcome this thorough and wide-ranging book as a vital contribution to the work of TIF, and an invaluable resource for all those working in the field. On behalf of the Board of TIF, I would also like to express our most sincere appreciation for the hard work and devotion the authors have shown in compiling this publication.

Panos Englezos
TIF Chairman

THE PUBLISHERS-THALASSAEMIA INTERNATIONAL FEDERATION (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 with a number of other official health bodies and patient oriented organizations.
(www.thalassaemia.org.cy)

MISSION: The development of National Control Programmes, including both components of prevention and management and the promotion of their establishment across 'affected' countries.

VISION: Establishment of equal access to quality health care for every patient with thalassaemia wherever he or she may live.

OBJECTIVES: The objectives of the Federation in addressing effectively the needs of the world thalassaemia family have since its establishment remained the same and include:

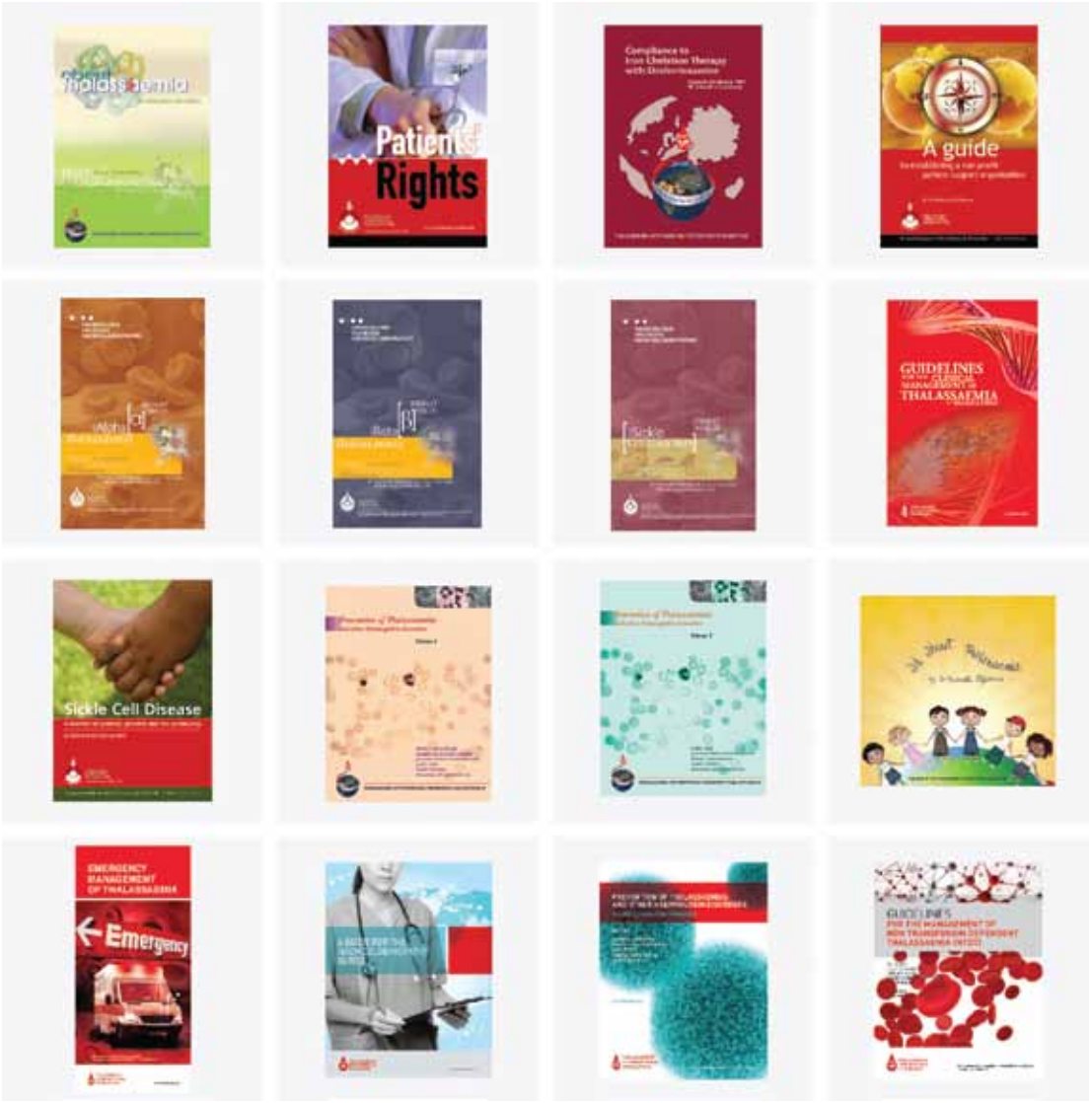
- The establishment of new and promotion of existing National Thalassaemia Patient/Parents Associations
- Encouraging, motivating and supporting studies and research for further improving prevention strategies, clinical care and for achieving the long-awaited final cure and
- Extending the knowledge and experiences gained from countries with successful control programmes to those in need.

TODATE: TIF has developed into an umbrella federation with 102 member associations, from 60 countries of the world, safeguarding the rights of patients for quality health care.

Its educational programme, focused on the needs of patients/parents, medical health professionals and the community at large, has been, and still is, amongst its strongest tools towards achieving its objectives.

TIF since 1990 has organised 60 national/local, 6 regional workshops and 14 international conferences and has prepared, published, translated and distributed more than 15 books todate in more than 50 countries worldwide.

OTHER TIF PUBLICATIONS



Please note that some of the publications maybe out of stock.
You can find more information regarding our educational program on our website
http://www.thalassaemia.org.cy/

CHAPTER 01

Michael Angastiniotis

WHY PREVENTION

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, 2]. 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 inefficient or inequitable health services, then survival remains poor and the quality of life seriously compromised [3, 4, 5].

1.1 THE THALASSAEMIA SYNDROMES

These are genetically determined conditions in which globin chains, mainly the alpha and the beta globin chains, which make up the haemoglobin molecule, are not produced or are produced in significantly reduced amounts. This leads to an anaemia of variable severity, largely determined by the responsible mutation and by the degree of globin chain imbalance which results. The clinical spectrum varies from those that are dependent on regular blood transfusions for survival and those that can survive without transfusion support, at least in the first few years of life. The pathophysiology of the thalassaemias is well known and will not be described here (demonstrated schematically in Figure 1.1). Clinically the transfusion dependent anaemias become multi-organ disorders as a result of ineffective eryhtropoiesis and iron overload resulting from the apoptosis of the donor red cells. Treatment is lifelong and involves a multidisciplinary team of health professionals, able to monitor and deal with each complication as it arises.

The most serious of the thalassaemia syndromes is thalassaemia major: This syndrome is characterised by a severe anaemia which is transfusion dependent, and complicated by iron overload, which is the result of repeated blood transfusions. Iron overload is the cause of cellular and tissue damage, affecting major organs such as the heart, the liver and the endocrine glands.

Table 1.1: Thalassaemia as a multi-organ disorder - the effect of complications

- Chronic anemia: poor vitality and growth
- Expansion of haemopoietic tissue - deformities and pressure effects
- Hypersplenism
- Infections
- Complications of blood transfusions: iron overload, immune reactions, infections

- Cardiac complications: siderosis, heart failure, arrhythmias, myocarditis
- Pulmonary hypertension
- Liver complications: siderosis, viral hepatitis, fibrosis, cirrhosis and liver failure, hepatocellular carcinoma
- Endocrine complications: growth failure, hypogonadic hypogonadism, diabetes, hypothyroidism, hypoparathyroidism
- Bone disease: osteopenia, fractures
- Thrombo-embolism
- Psychosocial stress

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. For this reason the basic treatment, apart from blood transfusion is iron chelation. While patients are still young this regime will guarantee survival, provided it is strictly adhered to and of course available. In the past there was great difficulty in providing this combined treatment due both to economic considerations and to poor patient adherence, since iron chelation was available only as a daily subcutaneous infusion which had to be given over 10-12 hours, even Mediterranean countries where the disease is prevalent and there is a relatively robust economy. In the present this very basic treatment is still not provided as it should to the majority of patients across the world [6].

Table 2.1: 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

As mentioned above if no treatment is provided, patients with thalassaemia major will not survive beyond 5-10 years. It has also been shown that if patients receive only blood transfusions survival may be prolonged but death from iron overload will occur later. Optimum basic therapy will ensure survival to adult life see (see Figure 1.2) [7]. Management must include the involvement of several medical disciplines and specialised care by a multidisciplinary team of experienced physicians and nurses working in a collaborative fashion in specialised centres. Patients being treated in peripheral centres due to distance, should benefit from a networking process with a centre of expertise where their condition should periodically be reassessed.

Comprehensive management results in survival which has now become open ended and the quality of life has improved allowing patients to fulfil their expectations in education, employment and marriage.

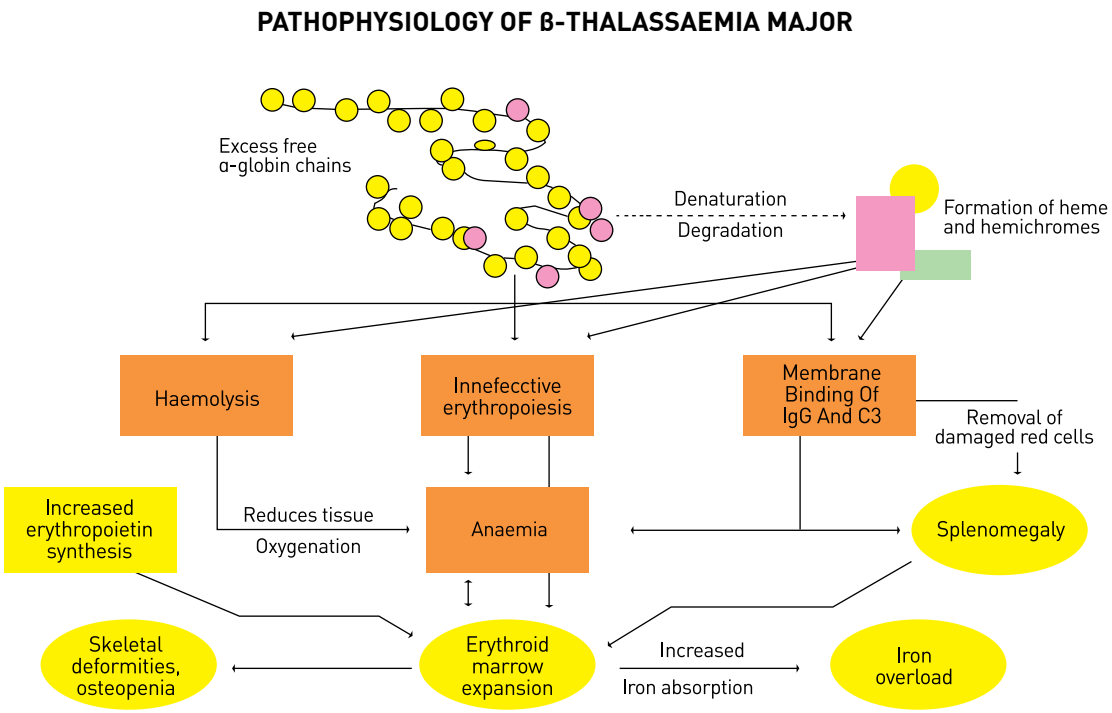


Figure 1.1: The pathophysiology of beta thalassaemia [courtesy of Olivieri NF, The beta thalassaemias N Engl J Med. 1999]

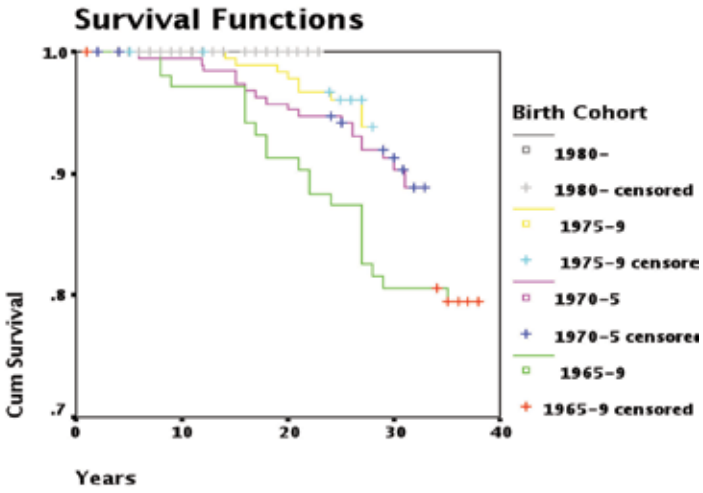


Figure 1.2: Survival of thalassaemia patients in Cyprus by birth cohort. [Courtesy of Telfer P et al; 2006]

1.2 THE SICKLE CELL SYNDROMES

These result from a mutation on the beta globin gene which produces a variant protein, known as haemoglobin S (HbS) which polymerises when oxygen concentration falls. The clinical outcome is significant when HbS is inherited in the homozygous form or co-inherited with beta thalassaemia or other variants, such as HbC, HbD and others (see chapter 7). The pathophysiology of the sickle cell syndromes is well known and will not be described here. Clinically these syndromes result mainly in vaso-occlusive episodes due the polymerised haemoglobin molecules in red cells which become inflexible, and in varying degrees of anaemia. Patients suffer from recurrent acute painful episodes, often necessitating strong analgesia and in-patient care, but also possible serious complications in vital organs, such as stroke, acute chest syndrome, priapism, renal failure and others. The result is a multi-organ disease with life threatening events occurring in acute episodes. The picture is summarised in Table 1.3.

Table 1.3: Sickle cell disease as a multi-organ condition

- Painful vaso-occlusive crises
- Anaemia: chronic and /or severe acute, haemolysis, splenic sequestration, gallstones
- Thrombo-embolism
- Acute chest syndrome
- Stroke: hemiparesis, aphasia, seizures, silent infarct
- Bone disease: osteopenia, osteonecrosis
- Leg ulcers
- Cardiac complications: heart failure
- Pulmonary hypertension
- Infections
- Priapism
- Renal disease

Long term management of this condition includes careful follow up to detect complications, neonatal screening to identify patients early and initiate infection prophylaxis and expertise in the management of vaso-occlusive crises, identification of patients at risk of serious complications with the aim of prevention or early intervention [8, 9]. Once tissue and organ damage have occurred then decisions must be taken on further protection for example by initiating regular transfusions or even haemopoietic stem cell transplantation (HSCT).

Table 1.4. Management of Sickle cell disease

- Neonatal screening for early monitoring and prevention of complications
- Infection prevention: vaccinations, penicillin prophylaxis
- Transcranial Doppler: stroke control
- Education of patients/families: early recognition and response to symptoms and signs, hydration, pain management at home
- Pain management in hospital
- Psychosocial support

- Blood transfusions and exchange transfusions according to criteria: regular or periodic
- Monitoring and management of iron overload

HSCT is at present the only possible cure for both the thalassaemia syndromes and sickle cell disease. This procedure is both expensive and involves risk if conditions are not right, which means that a compatible sibling donor is best available as opposed to a matched unrelated donor and the patient is fit to accept the procedure, especially the conditioning regimen.
Are the requirements for long survival and good quality of care, available for all patients suffering from these haemoglobin disorders? Is there equity of care and universal access to expert and optimum care? The answer to these questions is of course no. Only a very small fraction of the global patient population benefits from this level of care, although there is evidence of improvement in some countries.

Table1.5: Factors limiting the availability of optimum care

- Low health development index (HDI): 80% of affected children are born in middle and low resource countries, with low per capita health expenditure
- Other health priorities pre-occupy the health services, especially the high prevalence of communicable diseases leading to high infant and childhood mortality
- The rarity of the haemoglobin disorders in many countries and the high prevalence in ethnic minorities
- Health systems which do not adequately support chronic and rare conditions allowing out of pocket expenses, inadequate supply of drugs and non-provision of specialised care
- Lack of patient support organisations

Note: these last three limiting factors are equally important in high resource countries where not all patients have access to optimum care

As described in the next chapter on the epidemiology of these disorders, most affected children are born in low resource areas of the world [10]. In addition these areas face many health problems which are regarded as priorities and which include malnutrition and infectious diseases and which affect infant and under-five mortality. Children suffering from the haemoglobin disorders are even more susceptible to these conditions and their death is mostly assigned to an infection without the thalassaemia or indeed an anaemia being recognised. It is for this reason that the diagnosis is missed and the problem goes unrecognised. Haemoglobin and other genetic disorders become 'visible' to health services once the infant mortality from other disease is reduced. Experience has shown that this generally happens once infant mortality falls below 50/1000 [11]. In more recent years health authorities have been sensitised by patient associations and by treating physicians, and by the realisation of the true prevalence and public health significance . Awareness of the issue has led to demands for improved treatment of patients, which results in increased survival and so more patients in need of treatment. This leads to a demand on resources and requires organisation of services to deliver care.

1.3 THE POSSIBILITY OF PREVENTION IN HAEMOGLOBIN DISORDERS

Prevention of various genetic diseases, such as Tay-Sachs disease and haemophilia, had been initiated long before the haemoglobinopathy programmes [12]. Programmes aiming at the prevention of new affected births of genetic diseases have been the cause much controversy and debate since issues of bioethics, culture and religion as well as legal issues are involved. Despite the possible objections to population based prevention, programmes for the prevention of haemoglobin disorders, especially thalassaemia, have been applied and been accepted in high prevalence areas for several decades. In fact these programmes are being still being adopted in many parts of the world, developed and adapted to the local culture.

The World Health Organisation has advocated and promoted the adoption of these programmes from the early 1970s [13-17]. In the WHO resolution on thalassaemia and other haemoglobinopathies [EB118.R1, 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."

The directive to reduce incidence, new affected births, is clear. The reasons why this position has been accepted and promoted lies in the nature of these hereditary conditions and their natural history. This natural history has been significantly modified by a difficult, demanding and expensive treatment which has been adopted by few countries with a fraction of the affected patients benefitting across the globe. The remaining majority has a poor quality of life and the possibility of premature death.

However the decision to adopt a centrally planned and controlled prevention programme, also requires that the motives and objectives are clear. Reducing the health budget because it is cost effective to prevent rather than to treat is not defensible. A prevention policy, as any health policy, has at its centre the interests and rights of the individual, and especially the patient who suffers from such a condition.

In a policy which aims to limit new affected births the following beneficiaries should be considered:

1. **The patient**, who has the right to long survival and good quality of life, with the opportunity to fulfil normal life expectations including education, work and marriage. This means full integration into the community as a productive member and without stigmatisation. How does the patient benefit from the limitation of new births? Providing care for existing patients is limited for the majority, even those living in high resource countries. The addition of new cases each year further reduces the ability or willingness of health services to provide adequate supplies of necessities such as

blood and drugs, without considering the other complex requirements of optimum care. Health systems make compromises according to economic considerations and the need to divide limited resources in as equitable a manner as possible. This is a reality which however in an ideal world is a violation of human rights. Nevertheless it is a reality which is true for not only for those limited in funds. The one example is Cyprus, a very high prevalence area where one in a thousand (1:1000) of the population is a homozygote of beta thalassaemia. Without limitation of new births it was calculated that in twenty years the prevalence could reach 1:138 if all patients survived [18]. Such an increase in transfusion dependent patients may lead to 600% increase in blood requirements which would then be impossible to satisfy. The successful prevention programme has succeeded in allowing as good a patient survival as anywhere with a now adult population which has fulfilled most of their life expectations (see Fig 1.2). This may be an extreme example since most patients in the world, live where the prevalence is much less and in theory optimum services should be provided. In such areas however there is less sensitisation to real patient needs and health insurance systems simply provide basic treatment (transfusion and chelation) and very little else. Other examples of the successful balance between prevention and patient care are seen in Italy and Greece and services are improving in other parts of the world.

2. **The prospective parents**, ie the couples at risk of having a child with a serious hereditary disease. These couples need to be identified and offered informed choice and the chance to avoid such a birth if they so wish. All couples have fundamental rights:
   1. The right to healthy children
   2. The right to information
   3. The right not to know if this is their choice
   4. The right to choose for themselves what to do in their reproductive life, after reliable and non-directive counselling (see chapter 4).

Haemoglobin disorders are autosomal recessive disorders with a simple Mendelian inheritance. This means that in each pregnancy there is a one in four (1:4) or 25% chance of having an affected child.

This means that without knowledge of their risk they have a possibility of having one or more children who will need to be supported in the manner described and to accept the possibility of early death. If it is their choice it should be acceptable to all. In reality many parents face the existence of such a child without prior knowledge, as a result of no information and of poor services and so suffer a psychological burden and often a financial one as well as a struggle in many areas with poor quality services for their child. The fear of having a second child with the disease led to the termination of up to 75% of at-risk pregnancies in the past [19], with the loss of healthy as well as affected pregnancies. The provision of informed choice is therefore an ethical duty of health planners irrespective of whether couples live in high or low prevalence areas.

1.4 THE ELEMENTS OF A PREVENTION POLICY

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
- 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 (see chapter 5). Respect however of the wishes and choices of the couple should be a priority of these authorities.

All these elements are described in the chapters that follow. It must be stressed here that such a policy is difficult to maintain if it is limited to a few academic or hospital services or provided purely for profit. Standards of prevention practices are necessary, ranging from counselling standards, to laboratory standards and ethical control of practices. For these reasons national policies and central control and coordination are recommended. In addition there is need for budgetary support, monitoring and evaluation of the programme. Other elements of the health service may need to be involved such as the primary care system for the purposes of education and screening. The patient support organisations also have a role in raising awareness and promoting services. Prevention of hereditary disease becomes a community affair as it affects marriage practices and reproduction.

The final policy will be adapted to each countries needs and population characteristics. The population size may affect the screening policy, such as choosing target groups to screen and the tests that will be most cost effective in screening. The marriage practices especially if consanguineous marriage is prevalent, or arranged marriages are customary will influence both screening and counselling. The birth rate if high is a significant factor in prevention. The main influences on policy, however, are the carrier rates and whether carriers are across a homogeneous population or if prevalence differs in ethnic, religious or new migrant groups. In a heterogeneous population targeted screening may be necessary which may raise sensitive issues like requesting information on the origins of an individual. In a low prevalence area ante-natal clinic screening is tends to be the preferred strategy whereas in a high prevalence area pre-marital or school screening may be preferable, giving couples more choices since they will know their carrier status before any pregnancy. The level of education, especially on health matters, is also a consideration and this is particularly important in countries where the haemoglobinopathy genes are prevalent among new migrants, where the language barrier may also be a consideration in counselling.

A community will commit to a prevention programme if there is clear understanding of the disease and its consequences and that effective prevention will benefit the existing patients and the affected families. For political commitment there is need for evidence based information on the size of the problem and its contribution to the community's health burden. This requires epidemiological surveys and patient registries.

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CHAPTER 02

Michael Angastiniotis

EPIDEMIOLOGY OF HAEMOGLOBINOPATHIES

Hereditary haemoglobin disorders cause a variety of syndromes, all with anaemia as the common characteristic, and with a wide spectrum of clinical severity. The most important clinically are those in which the anaemia is so severe that life cannot be supported without regular blood transfusions. These include the beta thalassaemias, the compound beta and HbE thalassaemias and some forms of non-deletional alpha thalassaemias. They are caused by mutations affecting the production α-globin chains and ß-globin chains of the haemoglobin molecule. The severity of the anaemia and its consequences, depend on the molecular defects which are involved in each affected individual. In addition to the thalassaemia syndromes there are phenotypically different syndromes which are caused by variants of the haemoglobin molecule, mainly HbS and HbC, which cause sickle cell disease. In this chapter we discuss the importance of epidemiological information in the management of these disorders.

The thalassaemia syndromes, particularly those requiring multiple blood transfusions, are a serious burden on health services and a problem which may be increasing on a global scale (1, 2). Even milder syndromes, known as thalassaemia intermedia or non-transfusion dependent thalassaemia, require careful follow up since complications are expected over time, in the natural course of the disease. This is also true of the sickle cell syndromes. The need for lifelong follow up and care and the occurrence of complications affecting major organs such as liver, heart and endocrine glands, creates the need for organised expert services and also the need for major resources in terms of essential drugs and donated blood for transfusions. In terms of clinical outcomes, we expect that patients will survive with the best possible quality of life, if treated holistically in an expert centre.

The need for prevention and its inclusion in national health planning has already been discussed in the previous chapter. In addition to this the importance of neonatal screening for sickle cell disease in many populations must be emphasised. The need for both preventative and clinical services and the complexity of such services, together with the size of the problem in many parts of the world, makes the need for epidemiological information a necessary prerequisite to proper service planning. There is need for governments to develop national policies and strategies to manage the disease, both by control programs and by improved patient care.

2.1 EPIDEMIOLOGY AND SERVICE DEVELOPMENT

Epidemiology, which is the study of the distribution of a disease in human populations as well as the factors influencing this distribution (3) has, beyond any academic interests, very practical applications in the planning of services. The questions posed by an epidemiologic investigation should lead to concrete information which will result in rational planning aimed at both clinical care and prevention of disease. Developing appropriate health policies depends to a great extent on the availability of accurate epidemiological information concerning the size and distribution of the problem.

There is great variability in the prevalence of haemoglobin disorders across the globe but also in their distribution within a country. The recognition of the effect of the problem depends partly on prevalence, the number of affected individuals, but also on the overall health picture of each country and its socio-economic development. Low resource countries for example with several other serious health agendas, such malnutrition and infectious diseases, often with a high infant mortality from these conditions, may not see the hereditary disorders as a priority even though the affected children will be more susceptible and be the first to die from an infection (4). Visibility of hereditary disorders is often more clear when the infant mortality falls, and by experience this is when the level is around 50 per thousand live births.

In order to understand the impact of the hereditary anaemias on a population, especially with service development in mind, simple numbers are not enough. More complex data must be collected and used to both assess the country situation and promote the planning of services. Such data include:

- **Data which indicate the the impact of haemoglobin disorders in terms of numbers:** prevalence data such as patient numbers and their location; incidence, which is the number of new affected births which in turn depends on carrier frequency; data on the frequency of consanguinity which may have an impact on birth frequency . These figures are usually given as the overall data for a country but are more useful, if available, for various locations within a country or among the various population groups – this is known micromapping, which is particularly relevant to large populations with divergent ethnic groups. Information on incidence depends also on knowing the crude birth rate, the total number of births per annum, the overall life expectancy etc.

- **Data which will indicate the overall health status of a country:** basic data includes the infant mortality rate, the under-five mortality rate, the health expenditure per capita and knowledge of the way health provision is financed and the need for out-of-pocket expenditure, which is particularly relevant to chronically sick patients.

- **Service indicators:** these are the figures derived from the basic data, which will more directly lead to planning services either for patient care or for prevention. The term 'service indicators' was coined by Prof Bernadette Modell (1) and these will be further discussed below.

- **Changing epidemiology:** none of the information mentioned above is constant and changes may

occur in the short term but also in the long term. The most important short term change in recent years has been migrations from high prevalence regions of the world, such as Africa, the Mediterranean basin and Asia to low prevalence regions such as northern Europe and the Americas. Long term effects may come about by the reduction of habitual consanguineous marriage (5), by the falling crude birth rate of many populations and by the removal of environmental factors said to favour the carrier rate such as the eradication of malaria (6).

- **Outcome measures:** these include complication rates, survival rates, causes of death, and quality of life studies, the number of annual births of affected individuals and the effects of prevention measures. These are part of necessary motoring and evaluation processes which will audit the whole service provision and its dynamic evolution.

- **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 which will determine the viability and sustainability of these national programmes. These will not be considered in detail in this chapter since they are part of a separate science of health economics. However some comparisons in the past have been made between the cost of patient care and the cost of prevention which are useful indicators for health planners (7- 9).

It is with this service orientated perspective that we approach the study of the epidemiology of the haemoglobin disorders.

2.2 EPIDEMIOLOGICAL INFORMATION: METHODOLOGY

Epidemiology provides the foundation of public policy and the means for monitoring and evaluating these policies, and studying the changes over time and place.

2.2.1 THE NUMBER OF PATIENTS

This is usually the first question that is asked and is certainly very important for health planning. The number of homozygotes is known only in a few countries with advanced services. In many poorly developed economies, the early death of patients makes it impossible to give figures and most countries, even with developed services, do not maintain a national registry. In fact such a registry is the most important tool for planning patient services (10, 11). The registry will not only provide numbers but is a database which can collect other useful data: the location of patients, so that services can be accessed in areas of high concentration; the ethnicity or other characteristic of patients; their age, from which an age distribution can be derived, providing information on both prevention and case management outcomes; records of deaths; causes of death which is a basic source of information directing in many cases the treatment choices.

2.2.2 PATIENT REGISTRIES

A patient registry has been defined as a 'systematic collection of a clearly defined set of health and demographic data for patients with specific health characteristics, held in a central database for a

prescribed purpose'(12). Such a database is a key instrument for epidemiological research, which, as mentioned above, can support health planning but can also support clinical record keeping, auditing, clinical research including drug use surveillance and health outcomes. The possible use of a database and the type of database, is defined by the data specified from the onset of each registry design. There are various types of registries which include hospital databases, ad hoc surveys, observational studies, repositories of cases, each with different characteristics and applications. A registry may be locally based, national or international.

A national patient register for haemoglobin disorders is essentially a list of names of diagnosed patients to which demographic and health data are added:

- The demographic data will provide information on age, sex and location.
- Health data includes the basic diagnosis to which may be added additional clinical information according to the needs of the health service. Such clinical information may be limited to the recording of health outcomes, such as survival and complication rates which are retrospective data.
- The register may also be expanded to contain all clinical monitoring, including prospective information, making it a complete patient health record.

Registers may be paper based but for national registries and especially if prospective clinical data are to be included, then registries should be electronic, preferably centrally controlled by health authorities. The quality of data must be assured (13) and errors minimised. Data analysis will provide information both for planning services, for research also for medical auditing and program evaluation. Governance will allow for maintenance of internationally accepted standards on issues such as confidentiality. The registry should also be adequately funded to ensure sustainability and standards.

2.2.3 DO PATIENT REGISTRIES EXIST? ARE PATIENT NUMBERS KNOWN?

Maintaining national registries which can be regarded as accurate and according to accepted standards are limited to very few countries. In the European WHO Region, for example, only 6 out of the 34 member countries have such a registry for thalassaemia and sickle cell disease. In addition to these 6 countries, TIF has gathered estimates from another 7 countries derived from local medical contacts and the patient associations. From these approximations, which include most of the high prevalence counties, the total number of known thalassaemia major and intermedia is estimated to be over 17000 and 22000 sickle cell patients. The remaining 21 countries are mostly of low prevalence. With similar sources of information and without controlled registries, in the East Mediterranean WHO region around 108000 thalassaemia patients are estimated and 74000 sickle cell patients – the estimated new annual births for this region are 9100.

2.2.4 SCREENING AND SURVEYS TO IDENTIFY THE NUMBER OF CARRIERS

Identification of healthy carriers can be achieved through simple haematological tests is possible and this makes screening on a population scale possible. The same tests may be used for epidemiological surveys designed to estimate the proportion of carriers in a given population. The carrier rate can be measured from both surveys and screening programmes and it will give an overall indication on the size of the problem in a given population and identify at-risk groups within a population.

In a prevention programme the number of individuals to be screened will depend on whether it is necessary for the whole population of reproductive age to be identified, as is the case in high prevalence areas, or whether targeted screening of at-risk groups is required, as is the case where the genes are present in ethnic minorities. The service indicator (1) for screening varies therefore according to the policy which suits the population structure.

The methodology of screening is discussed in detail in chapter 4. The haematological characteristics of thalassaemia carrier states should be established for each population since in many populations there is interaction of various genes, which may alter the usual haematological manifestations of the heterozygote. This is necessary in order to avoid errors in carrier identification.

In many populations surveys have already been carried out. In these surveys, samples of 1000 or less have been used in most cases. The smaller the sample the more likely it is that a selection error has been introduced. A 'biased', selected sample will not represent the whole population. Such bias often occurs because target groups are chosen for convenience and accessibility and may not reflect the characteristics of all the people. For example, if university students are chosen because the investigating team has easy access to this group, care must be taken that they do not come from a racial or social minority.

The target population should therefore be defined according to how much it reflects the total population or whether it represents a particular sub-group. It is acceptable to use inclusion criteria based on accessibility e.g. 17-18 year old school leavers, provided schools from all areas are included. Army recruits or blood donors may be used if they are drawn from all sections of society. In a small country with a small homogeneous population (e.g. Cyprus or Malta) a random sample of 1000 is acceptable. In large, compound populations, it will be necessary to divide the population into well-defined subgroups (stratification). A sample from each group separately should be taken, ensuring random sampling from within each group. The final total sample must include the right proportion of each subgroup. In this way information will be obtained both about the total population and how the subgroups differ from each other. Knowledge of the distribution, both geographically and in specific groupings of the population (micromapping), is important for the accuracy but also for practical application of the epidemiological information such as directing services, both preventive and curative. In this respect knowledge of population size and characteristics derived from basic demographic data, including size, composition (ethnic groups, migrants), birth rates, and whether consanguineous marriage is common are all important data to be recorded. Migration has introduced haemoglobinopathy genes to areas of low prevalence, especially in Europe and the Americas.

The host countries are often unprepared for these new health problems while the migrants themselves, especially in the first generation, are often of a low socioeconomic and educational level and make poor use of local services. The language and cultural differences are barriers to effective educational and counselling activities in such sensitive areas as genetic prevention (14). Customary consanguineous marriage may be common in many migrant populations as it is in the countries of origin. This practice will tend to increase the affected births of rare recessively inherited disorders and must be considered when assessing the expected births. Where this is the case there will be a

clustering of cases within extended family groups, making family screening a productive exercise. It also means that patients with thalassaemia have a greater chance of having an HLA compatible sibling donor for stem cell transplantation, which is a consideration in planning services for patient care.

This kaleidoscope of epidemiological situations emphasises the need for a clear understanding of the individual country situation.

2.3 CALCULATING THE EXPECTED NUMBER OF HOMOZYGOTE BIRTHS

From the carrier rates a calculation of the number of affected conceptions per 1000 live births can be made based on the Hardy-Weinberg equation for a recessively inherited single gene disorder. This depends on certain assumptions:

a. That the population is mating randomly i.e. there are no consanguineous marriages.
b. There is no selection process (e.g. an ongoing prevention program) or genetic drift or frequent spontaneous mutations.

With these assumptions satisfied, the equation is as follows: $p^2 + 2pq + q^2 = 1$
p = thalassaemia gene frequency (½ 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 normals

Example:
In a country with a carrier frequency of 3% the gene frequency is approximately 1.5% or 0.015

- p = 0.015 q = 1-0.015=0.985
- The birth rate of homozygotes = p^2
  p^2 = 0.000225 = 0.0225% or 0.225/1000
- 2pq = proportion of carriers born 0.02955 ~ 3%
- The proportion of normals at birth = q^2 = 97%
- $p^2+2pq+q^2$ = 0.000225 + 0.02955 + 0.970225 = 1

If the country has 500,000 births/yr, then 112.5 births/yr will be homozygotes.

A more rough calculation is this:
Carriers - 3% of the population or 1/33
Marriages between carriers 1/33 x 1/33 = 1/1089
Affected births = 1/1089 x $^1/_4$ = 1/4356
500,000 x $\frac{1}{4356}$ = 114.8 births / year

These calculations must be modified in two situations:

1. If two haemoglobin disorders co-exist in a population, which may interact: e.g. ß-thalassaemia with HbS, or with HbE .
   In the case of co-existence of interacting haemoglobin disorders the Hardy-Weinberg equation is still applied. The sum of the carrier frequencies is used. For example where ß-thalassaemia and HbE coexist then p=the combined ß-thalassaemia and HbE frequency (e.g. if ß-thal=3% and HbE=3.5% then the total is 6.5% and so p= 0.035). A nomogram, derived from this calculation is here reproduced from the WHO publication "Guidelines for the control of Haemoglobin disorders", B. Modell, 1994).

2. Where consanguinity is frequent.
   Where consanguineous marriage is frequent the frequency of homozygote births increases for a given carrier frequency. The importance of consanguinity is inversely related to the gene frequency. If a mutation is rare, a carrier who marries another member of his/her own family group is more likely to have married another carrier since both may have inherited the rare trait from a common ancestor. A partner from the general population is unlikely to carry the same rare gene (15, 16). The contribution of consanguinity is expressed as the coefficient of consanguinity (or inbreeding), which is half of the probability that a couple share an allele (F). For a population the coefficient of consanguinity is equal to the mean of individual coefficients (a). The population coefficient is included in the Hardy-Weinberg equation when calculating the annual affected births:

$(p^2 + apq) + 2(pq - apq) + (q^2 + apq) = 1$

Values for population coefficients of various populations and sub-groups can be viewed on line (in www.consang.net).

Finally in order to calculate the annual affected births the demographics of each location must be known, which includes the total population, the general birth rate (per 1000 live births), the infant mortality rate, and the total number of births.

The number of expected births of children with clinically significant syndromes is derived from the calculations discussed above. This will also provide an indicator [3] for planning services for patient care if there is no prevention, since it is the number of patients expected to be added to the existing patients who will need blood and other resources.

2.4 THE REQUIREMENTS FOR HEALTH PLANNING – THE SERVICE INDICATORS

The needs for effective planning of prevention and control programmes can only be based on epidemiological information and this are summarised in the "service indicators", already mentioned above. These were first described by Professor Bernadette Model as practical measures to be considered for service developed (1). These indicators are the following:

- Indicator for patient care: the annual affected new births in the absence of prevention – the potential increase in patients and their needs in blood, medications, laboratory and other services, including manpower planning.
- Indicator for carrier screening: this requires prior epidemiological information on carrier rates and carrier distribution. A screening policy is derived from such information such as premarital screening of the whole population or ante-natal screening according to the annual number of pregnancies in at-risk groups. This indicator will help in planning appropriate laboratory services.
- Indicator for the number of carriers expected for counselling and information. This information is derived from annual number of carriers detected in screening.
- Indicator for the need of expert services such as risk assessment and genetic counselling. This is the annual number of carrier couples detected and the pregnancies to carrier couples.
- Indicator for prenatal diagnosis: the annual number of at-risk pregnancies.

2.5 NEONATAL SCREENING

Neonatal screening, discussed in detail in Chapter 10, will provide information on the size of the problem in a given population and also is an indicator for patient care. In haemoglobin disorders there are three situations in which it may be usefully applied:

- The detection of sickle cell disease
- The detection of alpha thalassaemia
- The detection of other haemoglobin variants

2.5.1 SICKLE CELL DISEASE

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. The rationale for its adoption historically was that early detection of affected children will lead to timely interventions which will reduce the likelihood of life threatening complications such as pneumococcal infections. These interventions include penicillin prophylaxis, vaccinations, education of parents in early detection and response to complications such as infections or splenic sequestration, Transcranial Doppler to detect intracranial vascular stenosis; these methods have been shown to reduce both morbidity and mortality (17, 18). In addition, neonatal screening provides invaluable epidemiological information regarding the frequency and geographical distribution of homozygous sickle cell disease, sickle cell thalassaemia, HbS/D, HbSC disease and HbS/O-Arab (19 - 22). Despite its usefulness, practical application is limited to few countries which include the USA, Jamaica and some European countries (see Table 2.1, provided by M de Montalembert and B. Gulbis). 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. This has been reported to be a problem in several African settings (23).

Table 2.1 Neonatal screening for sickle cell disease (SCD) in Europe.

Country	Programme	Incidence of SCD	Reference
Brussels (Belgium)	Universal but regional	1/1203	20
France	Targeted	1/2352	21
Netherlands	Universal	1/4394	22
Spain	Universal but regional	?	
UK, generalised since 2006	Universal	1/1851	19

2.5.2 NON-SICKLING HAEMOGLOBIN DISORDERS

Detection of other haemoglobin variants and of alpha thalassaemia through neonatal screening is usually limited to epidemiological surveys (24) and not used for early detection of disease. This is particularly important for the epidemiology of alpha thalassaemia which in adult screening programmes will only be suspected through microcytosis, low haematological indices, difficult to differentiate from iron deficiency. In contrast in neonatal samples (capillary or cord blood), the presence of Hb Bart's (γ4) has been used to identify carriers in epidemiological studies (25, 26). Despite a correlation of the quantity of Hb Bart's with the number of the alpha genes that are deleted, molecular studies are needed for more positive identification of the genetic structure of alpha thalassaemia genes in any given population (27). It has been suggested, especially by US authors that newborn screening for alpha thalassaemia may usefully be included in the national screening programme (28, 29). The reasons given for this are the increasing influx of migrants from high prevalence areas, especially from Southeast Asia, and the frequency of severe non-deletional forms alpha thalassaemia that can cause severe HbH disease which frequently requires regular blood transfusions. The most common of these non-deletional mutations is Hb Constant Spring. Some Asian countries are also considering this option (30).

The Thalassaemia International Federation maintains a global database on the magnitude of the problem in most populations. The data is gathered from published surveys but also from unpublished reports, questionnaires and information obtained from local visits by TIF professional advisors. The quality of this data is often questionable and represents the best available estimates (as indicated in the tables). Epidemiological information is found in Annex 1.

2.6 CHANGING EPIDEMIOLOGY - THE EFFECT OF MIGRATIONS

Changes in the epidemiology of inherited disease can be brought about by several possible factors which include natural selection, new mutational events, founder effects, genetic drift and migrations, and reproductive behaviour which includes customary consanguineous marriage and the reduction of crude birth rate in a population. It is not the purpose of this chapter to discuss the field of population genetics but to emphasise the practical consequences of these events, especially the migrations of the recent past. Migrations have been going on for some centuries and have led to

population mixes in many situations, as are the migrations from south to north Italy. The same can be said of Georgia where over the centuries Azeris, Armenians, Greeks and Jews have mixed with indigenous Georgians so that it is difficult today to identify genetically distinct groups as far thalassaemia is concerned (31). In other situations racial differences and minimal intermarriage have kept haemoglobinopathy genes within ethnic or racial groups, as is the case of sickle cell genes in north America. However the more recent migrations of the last two centuries have been to destinations in Europe and the Americas. These are mostly economic migrations and their effects in terms of genetic disease, are difficult to estimate in terms of numbers with accuracy, since many factors need to be considered. Such factors include the permanency of the migration, whether it is a migration of single people or of families, whether the migrant will marry locally or from the country of origin, whether consanguineous marriage will still be practiced in the host country, whether there will be free choice partner or an arranged marriage, whether birth rate of immigrant families will be that of the home or the host country and whether there is a second generation of migrants and the customs that they have adopted. The effects of these many variables are difficult to estimate and so in the TIF estimates of the effect of migration we consider country reports on the total number of migrants and their countries of origin, the carrier rate in the home country, and at the same time assume intermarriage between members of the same community. All calculated estimates are therefore based on assumptions which can lead only to approximations. However the size of the problem in each country can be assessed more accurately through surveys (32 - 35) and in the case of variants through neonatal screening programmes and through national registries.

2.7 OUTCOME STUDIES

Beyond the studies aimed at health planning, epidemiological techniques must be employed in outcome studies to monitor and evaluate the effectiveness and quality of services. Such studies include patient survival rates and age distribution of patients (36, 37), quality of life studies (38), complication rates (39) and health economic studies. Knowledge of the causes of death provides an important indicator to direct clinical research and therapeutic interventions aimed at improving survival of thalassaemia patients. The identification of cardiac complications of iron overload as the cause of 60%-70% of deaths (40), led eventually to the early detection of heart iron deposition by cardiac MRI and early intensification of iron chelation to increase survival. Surveillance of prevention programs is also important and is discussed fully in chapter 3. Continual surveillance of outcomes is, therefore, an important epidemiological exercise.

2.8 THE CURRENT SITUATION

Accurate epidemiological data are difficult to collect and a major challenge is collecting data from non-indigenous population groups in Europe (35). From these groups much information is derived from epidemiological information in the country of origin and not always from direct surveying of local ethnic minority. It is assumed that the migrant group is a representative sample of the population of the country of origin, with the same birth rate, etc. and calculations are made on these

assumptions. Much of the regional and global data may not be accurate but are the best estimates on available information.

Based on such data, the most recently published global estimates are the March of Dimes Global Report on Birth Defects published in 2006 (41) with a more detailed focus on haemoglobin disorders, presented in the WHO bulletin in 2008 (1). In their comments Modell and Darlison suggest that policies for treatment and prevention are required in 71% of 229 countries globally: this means 162 countries worldwide.

Of the 42,409 annual ß-thalassaemia conceptions that the authors estimate is the global incidence, how many survive to receive treatment? Of the estimated 1.33 million at-risk pregnancies, how many are aware of their risk, how many are counselled and given choices? This is difficult to know. In Southeast Asia - which includes some of the poorer countries – of 9983 expected transfusion dependent patients, 962 (10%) are actually transfused. It is clear that socio-economic development is not the only factor - or even the most important one – as to why patients do not receive appropriate treatment. It seems that the attention paid by health services to such rare (in some populations) and chronic disorders, which require lifelong and expensive medical care, is not adequate and the concept of using epidemiological data to plan and locate services has not yet sufficiently penetrated bureaucratic health systems. Another factor, as stated earlier, is the difficulty in catering for "immigrant" diseases in many parts of Europe. Some countries in Europe have tackled the problem in an organised way, partly because they host several generations of immigrants. One example is the United Kingdom where 7% of all residents are from minorities at-risk and 366 pregnancies per year are affected (42, 43). There are 900 registered patients, centres of expertise in London and a developing network with physicians who see less than 10 patients. Neonatal screening, ante-natal clinic screening and counselling services carried out by trained personnel who speak the language and share the culture of the immigrant groups. Such services need to be established in many countries across Europe.

In the meantime, more detailed epidemiological information is required at national level, which should include national registers of patients, carrier rates among immigrant populations, micro-mapping and the calculation of service indicators. It is on such an epidemiological basis that rational planning can lead to effective and equitable services both for the treatment of patients and for disease prevention.

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CHAPTER 03

Androulla Eleftheriou

HEALTH EDUCATION

Health education has been described by the WHO as the: "Consciously constructed opportunities for learning involving some form of communication designed to improve health literacy, including improving knowledge, and developing life skills, which are conducive to individual and community health."

The WHO health promotion glossary describes health education as not limited to the dissemination of health-related information but also "fostering the motivation, skills and confidence (self-efficacy) necessary to take action to improve health", as well as "the communication of information concerning the underlying social, economic and environmental conditions impacting on health, as well as individual risk factors and risk behaviours, and use of the health care system". A broad purpose of health education therefore is not only to increase knowledge about personal health behaviour but also to develop skills that "demonstrate the political feasibility and organizational possibilities of various forms of action to address social, economic and environmental determinants of health"[1].

Health education forms an important part of each country's health promotion activities, which aim to contribute significantly to the reduction of excess mortality, morbidity, to addressing leading risk factors and underlying determinants of health, help strengthen sustainable health system and place health at the centre of the broad development agenda.

Much has been written over the years about the relationship and overlap between health education, health promotion and other concepts such as health literacy and attempting to describe these and their relationship is not easy.

As defined by the Ottawa Charter and the Bangkok Charter for health promotion in a Globalized World (2005)[2], health promotion is the process of enabling people to increase control over and to improve their health and in the majority of cases, this is viewed as a combination of health education activities and the adoption of health public policies.

Today many authorities hold the view that health promotion comprises three overlapping components: (i) health education, (ii) health Protection and (iii) prevention, the combined efforts of which stimulate a social environment that is conductive to the success of preventive health protection measures.

Tones[3] suggested the formula that health promotion derives from the product of health education and healthy public policy. Health education according to this, focuses on building individual's ca-

pacities through educational, motivational skills building and conscious-raising techniques which healthy public policies provide and on other important elements such as those shown in the figure below:

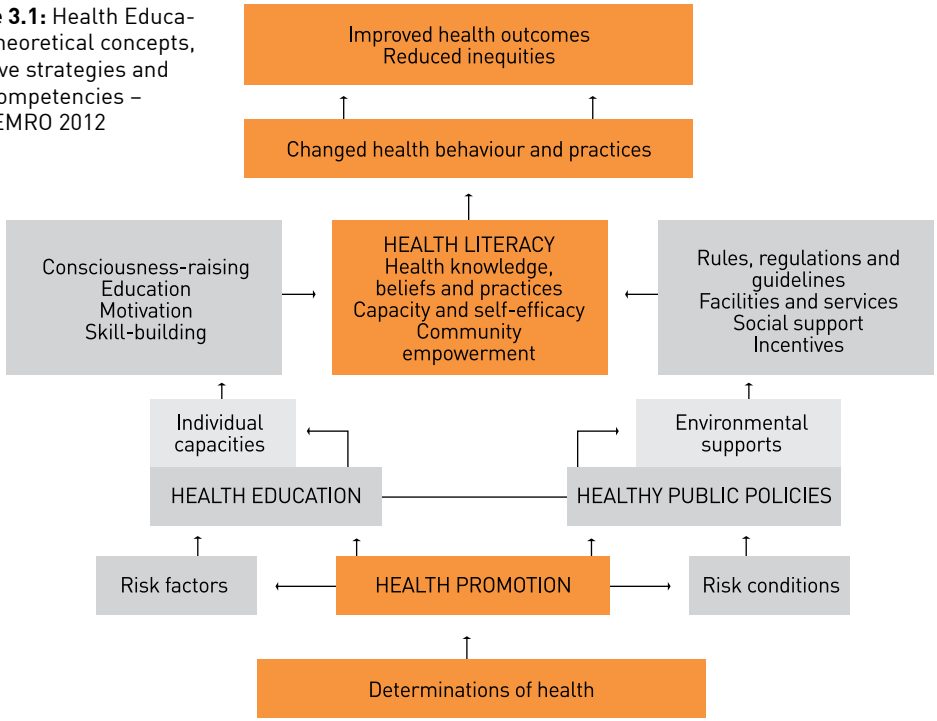

Figure 3.1: Health Education: theoretical concepts, effective strategies and core competencies – WHO EMRO 2012

Health literacy on the other hand, is the major outcome of effective health education, increasing individuals' capacities to access and use health information to make appropriate health decisions and maintain basic health or as WHO defines it: "The degree to which people are able to access, understand, appraise and communicate information to engage with the demands of different health contexts in order to promote and maintain good health across the life-course."[4]

Given the numerous health education initiative that have been undertaking over the past 30-40 years, by numerous involved stakeholders and official bodies in the context of addressing a wide range of disease oriented programmes, the multiple target groups and issues that have been addressed and the different evaluation methods that have been used, the methods described in brief below, appear to have stood the test of time and comprise essential components of health education programmes and services aimed at enhancing an individuals' and a community's health.

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2. Glossary of terms used in Health for All series. Geneva, WHO, 1984
3. Tones K. Health Education, beahaviour change and the public health: Oxford textbook of public health, 3rd edition, 1997
4. Health Education: theoretical concepts, effective strategies and core competencies – WHO EMRO 2012

- **Participant involvement:** Community members should be involved in all phases of a programme's development: identifying community needs, enlisting the aid of community organizations, planning and implementing programme activities, and evaluating results.
- **Planning:** This involves identifying the health problems in the community that are preventable through community intervention, formulating goals, identifying target behaviour and Health education: theoretical concepts, effective strategies and core competencies environmental characteristics that will be the focus of the intervention efforts, deciding how stakeholders will be involved, and building a cohesive planning group.
- **Needs and resources assessment**: Prior to implementing a health education initiative, attention needs to be given to identifying the health needs and capacities of the community and the resources that are available.
- **A comprehensive programme:** The programmes with the greatest promise are comprehensive, in that they deal with multiple risk factors, use several different channels of programme delivery, target several different levels (individuals, families, social networks, organizations, the community as a whole) and are designed to change not only risk behaviour but also the factors and conditions that sustain this behaviour (e.g. motivation, social environment).
- **An integrated programme:** A programme should be integrated: each component of the programme should reinforce the other components. Programmes should also be physically integrated into the settings where people live their lives (e.g. worksites).
- **Long-term change:** Health education programmes should be designed to produce stable and lasting changes in health behaviour. This requires longer-term funding of programmes and the development of a permanent health education infrastructure within the community.
- **Altering community norms:** In order to have a significant impact on an entire organization or community, a health education programme must be able to alter community or organizational norms and standards of behaviour. This requires that a substantial proportion of the community's or organization's members be exposed to programme messages or, preferably, be involved in programme activities in some way.
- **Research and evaluation:** A comprehensive evaluation and research process is necessary, not only to document programme outcomes and effects, but to describe its formation and process and its cost-effectiveness and benefits.

Today health education as a social science draws from the biological, environmental, psychological, physical and medical sciences to promote health and prevent disease, disability and prenatal death through education. Education can occur in many settings and the health educator plays a pivotal role in the success of each programme:

- **In schools** health is target as a subject and health educators promote and implement coordinated school health programmes, including health services and student, staff and parent health education; and promote healthy school environments and school–community partnerships. At the school district level they develop education methods and materials; coordinate, promote and evaluate programmes; and write funding proposals. A health education component should be incorporated into both basic and continuing teacher training, regardless of subject.
- **In college/university campus**, health education is part of a team effort to create an environment

in which students feel empowered to make healthy choices and create a caring community. Health educators identify needs; advocate and do community organizing; teach whole courses or individual classes; develop mass media campaigns; and train peer educators, counsellors and/or advocates. They address issues related to disease prevention; consumer, environmental, emotional and sexual health; first aid, safety and disaster preparedness; substance abuse prevention; human growth and development; and nutrition and eating issues. They may manage grants and conduct research.

- **In the work place** health education focus on performing or coordinating employee counselling as well as education services, employee health risk appraisals, and health screenings. Health educators, design, promote, lead and/or evaluate programmes and develop educational materials and write grants for money to support these projects. They help companies meet occupational health and safety regulations, work with the media and identify community health resources for employees.
- **In health care settings** health educators educate patients about medical procedures, operations, services and therapeutic regimens, and create activities and incentives to encourage use of services by high-risk patients. They conduct staff training and consult with other health care providers about behavioural, cultural or social barriers to health, and promote self-care. They
develop activities to improve patient participation on clinical processes, educate individuals to Health education: theoretical concepts, effective strategies and core competencies protect, promote or maintain their health and reduce risky behaviour, and make appropriate community-based referrals and write grants.
- **Health education in the community** is undertaken by health educators from NGOs and government sector and help a community identify its needs, draw upon its problem-solving abilities and mobilize its resources to develop, promote, implement and evaluate strategies to improve its own health status.

The professionals involved in health education come from wide range of disciplines including medical, nursing, genetics, biological and social backgrounds. Their work is very important and is mainly directly concerned with communities and individuals. It is crucial that the rights and privacy of individuals and communities are respected, and that programmes are developed on an equitable basis, addressing the needs of the most vulnerable population groups and embracing the following principles:

- respect for human dignity and rights
- respect for individual and family independence
- full consent
- confidentiality
- nondiscrimination or stigmatization
- equity in access, coverage and service delivery
- respect for cultural values and cultural diversity
- refraining from conflict of interest, particularly commercial interest
- integrity and good personal conduct.

In the field of genetic diseases, it is important for countries to develop strategies to ensure that the advances which have taken place in the area of genetics and genomics are harnessed to benefit the health of their citizens, and to undertake an appraisal of the potential of genomics for their citizens.

This means taking careful account in the creation of policy of not only the scientific and technical aspects of this area, but also of the not inconsiderable ethical and social implications.

For policies to be sustainable and effective particularly in the area of genetic diseases with considerable family/social repercussion, they need to be socially acceptable. This does not mean that they ought to be constructed to appease the masses; rather, it means that they should be informed by the views of a public that is itself well informed. There is therefore a need to educate ordinary citizens in a balanced manner about advances in the science, including the ethical, legal and social implications they pose. Many encouraging examples of successful medical interventions that were preceded by public educational efforts exist today, as demonstrated by the example of genetic screening and counselling programmes in the USA, Canada, Italy, Greece and Cyprus and in more recent years in the UK, the Netherlands, Belgium and outside Europe: Iran, Bahrain, UAE just to name a few success stories in the filed of Hb disorders. Mediums of communication like the television, workshops and print campaigns have proved to be effective for raising public awareness. An ongoing analysis of public perception of genetics must be incorporated into policy making so that it truly addresses the areas of public concern, and constructively addresses the apprehensions of society towards genetics.

Countries interested in empowering their citizens to make better choices and to participate in shaping the direction of technological development and its application, will need to do more than educate a passive constituency. Raising awareness is important for providing a general knowledge base, but this heightened awareness has only limited value if citizens are not engaged in public dialogue that encourages their active debate and participation in policy-making. While public engagement, particularly in large and diverse communities, is not easy to achieve, various methods-such as surveys, collaboration with local leaders and opinion shapers, and even innovative approaches such as interactive theatre and creating modules for classroom discussion in local schools-can be employed in different contexts to inform, generate discussion, and provide important input to policy-makers about how to approach often difficult and controversial issues.

Health education programmes in every country particularly in the field of genetics may be confronted with significant difficulties, as effective communication involves a range of sensitive issues including marriage, divorce, child breading and termination/intervention of pregnancy.

In addition, in large, heterogeneous populations with Hb disorders prevalent in their indigenous populations for example, in India and Pakistan, and with fragmented health services, promotion of health education may be confronted with many additional and significant difficulties. In these cases, the inclusion of many and different components related to language, dialects and culture need to be seriously considered and taken onboard in order to achieve effectiveness and avoid creation of stigmatization and/or other ethical problems and dilemmas.

In the same way but from a different angle, Europe, where in the majority of countries Hb disorders are diseases literally introduced into the indigenous populations (the first countries being UK and France) through immigration, has faced, faces and will continue to face huge difficulties and chal-

lenges in developing appropriate services including health education programmes to reach across many and different language, social, culture and religious barriers. The UK constitutes the best such example, where, despite existence and availability of high quality genetic services for long, the poor update of prenatal diagnosis, by ethnic minorities most of which are now in their third or fourth generation of settlement, as shown in a published analysis in 2000, resulted in poor prevention results. The promotion of nationally controlled programmes since then in the UK however, and the particular focus paid on health education programmes targeting to reach ethnic minorities and make services both available and accessible, has already started to show significant improvements.

Inaccurate, misinterpreted, unrealistic, overoptimistic or overly pessimistic information can have a very serious impact on public opinion leading to misguided conclusions and decisions. Hence the language barrier in transmitting the correct desired messages amongst ethnic minorities or even amongst populations of different dialects within a country should never be underestimated as well as the style and ways in which those messages reach the public and the individual.

Last but not least, health education should never be an isolated component of a strategy, particularly with regards to the control of a genetic disease but one, which, although considered as a priority and a first step, is part of a country's national programme. Political commitment is essential to build up the next steps of a strategy and ensure the sustainability of these. Otherwise, expectations raised to the public will not be met and essential services for correct diagnosis, appropriate counselling, care and other services including prenatal diagnosis, necessary to support the individuals' decision, will not be adequately available.

In conclusion, and with regards to Hb disorders, every country should have, in addition to the support from its Government, the Ministry of Health, WHO and other official bodies, a designated reference centre for health education in Hb disorders, equipped with resources to produce and regularly update reference materials.
Such centres should also be able to produce a range of high quality material on the treatment and prevention of Hb disorders, providing information that:

a. raises public awareness of the existence and magnitude of the problem, and the value of screening;
b. provides details of laboratories and screening tests available for the detection of carriers, including clear advice on interpreting test results;
c. provides specific and correct information to be given to individuals identified as carriers

The centre needs to meet the changing needs of the population by continually updating information (including video material) on every aspect of thalassaemia, in line with increasing familiarity with the disease and rising public awareness. Part of this process involves the continuing education of staff members through their participation in international workshops and worldwide conferences.

In addition, relevant NGOs should be implicated actively in these efforts and the contribution of Thalassaemia International Federation (TIF) for example, on the subject of health education and awareness, has been of pivotal value to many countries (please refer to http://www.thalassaemia.org.cy).

At a time when many of the world's religions are still struggling to define their position on genetic diagnosis and the termination of pregnancy, educational material must be very carefully designed. Even where religious leaders do succeed in finding common ground in their thinking on such controversial issues, in many societies it is likely to take some time for the majority to reach the same conclusions -- particularly in countries with low rates of literacy and powerful additional cultural and religious constraints. Clearly, it would be better if methods of controlling the disease that do not involve terminating pregnancy, such as pre-implantation diagnosis (PGD), were to become more widely available and accessible.

Given that it is likely to be some time before better forms of treatment or cure are developed, countries in which thalassaemia and other Hb disorders pose a major challenge in the new millennium, will have to seriously consider whether prevention programmes of this type are most appropriate to their circumstances. Each country will need to develop an individual strategy, backed by strong health educational programmes, appropriate to the local epidemiology of the disease, religious and cultural characteristics, current service structure and economic resources. In addition every effort should be made by Governments to integrate this strategy into national programmes on Rare Diseases, Non – Communicable Diseases (NCD) or birth defects programmes to ensure its sustainability.

CHAPTER 04

Renzo Galanello

SCREENING AND DIAGNOSIS FOR HAEMOGLOBIN DISORDERS

The haemoglobinopathies include quantitative and/or qualitative genetic disorders caused by mutations affecting the genes responsible for haemoglobin synthesis. Based on the gene(s) involved and the type of defect, the haemoglobinopathies can be broadly classified into thalassaemias (α, ß, δß) and abnormal structural variants. However, there are also structural variants such as Hb Lepore and HbE that result in a thalassaemic phenotype. The term Hereditary Persistence of Foetal Haemoglobin (HPFH) is used to define a group of conditions characterised by increased levels of HbF in adults, due to a persistent synthesis of γ-globin chains after birth without any significant clinical or haematological manifestations.

In general terms, the haemoglobinopathies 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, HbE syndromes). Heterozygotes are symptom-free but present haematological characteristics, often useful for their identification. The heterozygous states for the most common haemoglobinopathies are summarised in Table 4.1.

Table 4.1 Classification of carriers for most common haemoglobinopathies

ß-thalassaemia	- typical
	- atypical
α-thalassaemia	- typical: homozygous and heterozygous α+-thal,
	heterozygous α°-thal, non-deletion α^{+}-thal defects
	- silent: heterozygous α^{+}-thal
δß-thalassaemia	- $\delta\beta^{+}$-thal
	- δß°-thal
Hereditary persistence of fetal haemoglobin	- pancellular
	- heterocellular
Haemoglobin variants	- common variants (HbS, HbE, HbC)
	- unstable
	- altered oxygen affinity
	- M haemoglobins
	- asymptomatic

4.1 SCREENING STRATEGIES

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. Ideally, screening is performed before pregnancy (1). There are several possible strategies for screening, depending on factors such as the frequency of the disease, heterogeneity of the genetic defects, resources available, and social, cultural and religious factors.

Knowing 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 (see below). 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 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. The laboratory methods for carrier identification are relatively expensive (electronically determined RBC indices, HPLC analysis of haemoglobin) and the flowchart is laborious and may include complex methods such as globin chain synthesis and DNA analysis. For countries with more limited resources, mass screening can be conducted using cheaper methods (such as single tube osmotic fragility tests, or chromatography for HbA2 determination) and a less complex flowchart. The laboratory methods for carrier identification are described in detail in the second volume of this book (Volume 2: Laboratory Protocols).

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.

4.2 WHEN TO SCREEN

Screening can be targeted at different age groups (Table 4.2), with genetic counselling adjusted according to the age of the individual or target group being screened. Currently, newborn screening

(discussed in detail chapter 10) is only recommended for sickle cell disease, since early recognition of the disease can prevent mortality and morbidity caused by bacterial sepsis or sequestration crisis in the first months of life. Newborn screening for ß-thalassaemia is less frequent, as it requires expensive DNA analysis. Advances in molecular biology and biotechnology may in the future provide faster and cheaper methods (e.g. microchips) for newborn screening of the haemoglobinopathies and other genetic diseases and traits (i.e. cystic fibrosis, G6PD, Wilson disease, etc). However, even if neonatal carrier identification were to become feasible, the problem of providing genetic counselling and relevant information throughout adult life remains.

Table 4.2: Timing for haemoglobinopathy screening
Newborn
Adolescence
Premarital
Preconceptional
Antenatal

Experience with adolescent screening suggests that acceptance rates are high (usually above 80%) and the information is well understood and maintained (2-4). The advantages of screening in schools include the ability to reach a majority of the population and a sense of providing increased options to those identified as carriers (i.e. not to marry another carrier). However, the approach requires an intense and well-organised educational programme, relying on the support of motivated teachers and professional genetic counsellors.

Premarital testing is carried out in several Mediterranean countries (Greece, Italy and Cyprus) but it is not suitable in countries where the identification of a genetic risk prior to marriage can result in stigmatisation, particularly for the woman.

Preconception screening is directed at couples planning a pregnancy, while antenatal screening focuses on pregnant women. Screening during pregnancy may be disadvantageous in at-risk cases, since the only option is prenatal diagnosis. Furthermore, carrier identification may be too late to allow prenatal diagnosis, resulting in marked emotional stress.

Numerous carrier screening programmes are conducted around the world (for a review, see Cousens et. al., 2010, (5). 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.

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 (5).

4.3 CHARACTERISTICS OF THE β AND δβ-THALASSAEMIAS

4.3.1 THE β-THALASSAEMIAS

The β-thalassaemias are very heterogeneous at the molecular level, with more than 200 point mutations and deletions of different severity described (Annex 2) (6, http://globin.cse.psu.edu/). The degree of severity generally corresponds to the magnitude of the residual output of the defective β-globin gene, and accordingly, the β-thalassaemia mutations are classified into severe, mild and silent types. The different types of β-thalassaemia mutation produce clinical and haematological phenotypes of variable severity, both in homozygotes (or genetic compounds) and in carriers. The clinically significant forms of β-thalassaemia (major and intermedia) are described in Chapter 7.

β-Thalassaemia carriers of either the β^0 or severe β^+ type are characterised by modified red blood cell indices, haemoglobin pattern and globin chain synthesis ratio. The red blood cell count (RBC) is relatively high, while mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) are markedly reduced (MCV 60-70fl, MCH 19-23pg). Mean haemoglobin levels may be up to 2g/dl lower than normal, but vary widely. Red cell morphology is modified and typically includes microcytosis, hypochromia and variation in the size and shape of red cells (anisopoikilocytosis), target cells and basophilic stippling. The most characteristic haematological finding related to the β-thalassaemia trait is an elevated level of HbA_2, typically between 4–6%. Occasionally, β-thalassaemia carriers may have unusually elevated levels of HbA_2 (>6.5%). Such carriers generally have point mutations, or more rarely, deletions involving the 5' promoter region of the β-gene. HbF may be slightly elevated (1–3%) in about 30% of carriers, and globin chain synthesis analysis shows an imbalanced α/β-globin ratio, with values ranging from 1.5-2.5. Finally, the decrease in osmotic fragility is another characteristic haematological finding in β-thalassaemia carriers (see Table 4.3).

Heterozygotes for mild β-thalassaemia mutations generally have higher MCV and MCH values compared to those in β-thalassaemia heterozygotes with severe β^0 and β^+ mutations. Their HbA_2 levels usually range from borderline-normal to slightly increased values (3.4-4%), as in carriers of the IVSI-6 (T→C) mutation, up to manifestly increased levels (4.5-6%), as in carriers of mild β-gene promoter mutations

In carriers of very mild or silent β-thalassaemia alleles, the minimal deficit of β-globin production is not associated with any consistent or significant phenotype. In most cases, MCV, MCH, HbA_2, total haemoglobin and even α/β-globin synthesis ratios are within the normal range, although sometimes borderline-raised HbA_2 and/or slightly reduced red cell indices are observed, indicating the presence of a thalassaemic allele, and thus the need for further investigation.

Table 4.3 Atypical ß-thalassaemia carriers *δß-thalassaemia carriers

Phenotype	Genotype
Normal MCV and MCH	- co-inheritance of α-thalassaemia
Borderline/normal HbA_2	- some mild ß-thalassaemia alleles
	- co-inheritance of δ-thalassaemia
	- εγδß-thalassaemia
	- Corfù δß-thalassaemia
Normal MCV, MCH and normal/borderline HbA_2	- very mild/silent alleles
	- triplicated α-globin gene
	- KLF 1 gene mutations
Significant clinical phenotype	- co-inheritance of α-globin gene defects:
	- triplicated α-genes*, HbH disease genotype (--/-α)
	- hyperunstable globin chains (dominant ß-thalassaemia)
	*or segmental duplication of whole α-gene cluster

4.3.2 THE δß-THALASSAEMIAS

The δß-thalassaemias may be divided into $(δß)^+$ and $(δß)^0$, based on the residual output of δ- and ß-chains from the affected chromosome (7). $(δß)^+$-Thalassaemia includes Hb Lepore determinants and more complex disorders resulting from the presence of two different mutations within the same ß-like gene cluster (Corfu and Chinese δß-thalassaemia determinants). $(δß)^0$-Thalassaemias are due to large deletions involving the εγδß-gene cluster, removing the δ- and ß-genes, but leaving one or both γ-genes intact.

Carriers of $(δß)^0$-thalassaemia deletions are characterised by milder haematological changes than those observed in the ß-thalassaemia trait. The Hb level may be normal or slightly reduced and the red cell changes (i.e. microcytosis, hypochromia, anisopoikilocytosis) are mild (MCV 70 fl and MCH 24 pg). HbA_2 is normal or slightly reduced but HbF is characteristically increased (5-20%) with a heterogeneous distribution amongst the red cells. Globin chain imbalance is mild (α/non-α ratio around 1.5).

Hb Lepore is a haemoglobin variant that results from non-homologous crossing-over between the δ- and ß-globin genes, the product of which is a hybrid δ- and ß-globin chain. Four types of Hb Lepore have been identified to date (Hb Lepore Boston, Hollandia, Leiden and Baltimore), differing at the exact point at which the crossover has occurred. The four types have similar electrophoretic and chromatographic properties, whereby at alkaline pH the electrophoretic mobility is slightly anodal to HbS. In carriers of Hb Lepore, the abnormal fraction constitutes between 5-15% of total haemoglobin, with reduced HbA_2 levels (approximately 2%) and mildly increased HbF (2-5%). The

haematological picture is characterised by mild anaemia (Hb 11-13g/dl), microcytosis (MCV: 70-75fl) and hypochromia (MCH: 20-24pg). The mean α/non-α ratio is approximately 1.5.

Corfu δß-thalassaemia results from the presence of two different mutations in the same chromosome (in cis): partial deletion of the δ-gene and an IVSI-5 (G→A) ß-thalassaemia mutation. Carriers of this form of (δß)+-thalassaemia have haematological findings comparable to the ß-thalassaemia trait, but with normal or slightly reduced HbA_2.

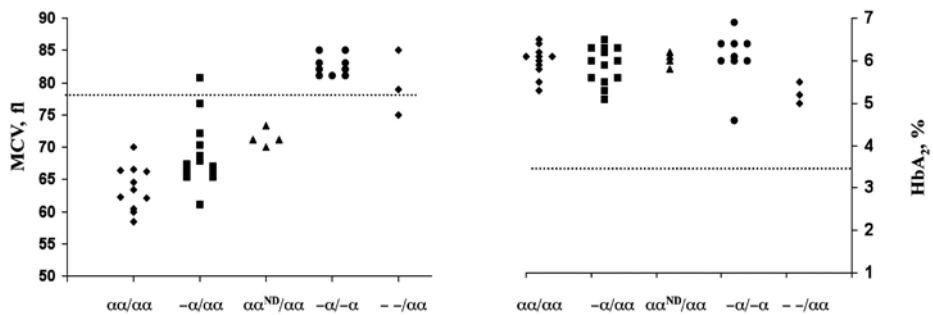
The Sardinian δß-thalassaemia is a non-deletion allele, characterised by a (C →T) substitution at position –196 of the Aγ-globin gene in cis to the common ß° Cd 39 (C →T) nonsense mutation. Sardinian δß-thalassaemia heterozygotes show typical but mild thalassaemic blood changes, normal HbA2 levels (2-3%) and increased HbF levels (10-20%). The α/non-α globin chain synthesis ratio is only mildly imbalanced (approximately 1.5).

4.3.3 ATYPICAL ß-THALASSAEMIA CARRIERS

The classical phenotype of heterozygous ß-thalassaemia, essentially characterised by reduced MCV and MCH and increased HbA_2, may be modified by several genetic determinants, with resulting potential problems in carrier identification. The most common forms of atypical carriers, with the corresponding genotype, are summarised in Table 4.3. The category of atypical ß-thalassaemia carriers includes ß-thalassaemia heterozygotes with an unusually severe haematological and clinical phenotype.

The co-inheritance of heterozygous ß-thalassaemia with homozygous α+-thalassaemia (-α/-α) or heterozygous α°-thalassaemia (--/αα) has a significant effect on the red cell indices, particularly the MCV and MCH, which may be normalised (8). The effect of interacting heterozygous α^+-thalassaemia (-α/αα) is usually less evident (Figure 4.1). Of note is that the HbA_2 levels in these double heterozygotes remain elevated within the range for ß-thalassaemia carriers, the practical consequence of this being that if HbA_2 determination is always performed in screening programmes, double heterozygotes for ß- and α-thalassaemia will not escape diagnosis. On the other hand, a primary screen carried out using red cell indices followed by HbA_2 determination only in those individuals with reduced MCV and MCH may result in failure to identify these double heterozygotes.

Figure 4.1 Effect of co-inheritance of different α-thalassemia alleles an ß-thlassemia carriers



Some ß-thalassaemia carriers have normal or borderline-raised HbA_2 levels but the MCV and MCH values are within the typical carrier range. Such carriers include heterozygotes for some mild mutations, such as IVSI-6 (T→C), double heterozygotes for δ- and ß-thalassaemia (in cis or in trans), carriers of the Corfu δß-thalassaemia allele and, rarely, carriers of εγδß-thalassaemia, which involve large deletions of the ß-like gene cluster but which spare the ß-genes (Spanish, English, Dutch types). To differentiate these atypical ß-thalassaemia carriers from α-thalassaemia heterozygotes, it is necessary to perform family studies and/or globin chain synthesis and/or globin gene analysis.

A third group of atypical carriers are those with normal MCV, MCH and normal/borderline HbA_2 values (silent ß-thalassaemia carriers). Subjects with this phenotype may be carriers of very mild or silent ß-gene mutations (associated with high residual ß-globin chain output), or carriers of the triple α-globin gene arrangement (ααα/αα), in whom the excess α-globin chain synthesis is equivalent to that in carriers of very mild ß-thalassaemia alleles. Recently mutations at KLF1 gene have been associated with borderline HbA levels and normal MCV and MCH (9). Identification of silent carriers is usually retrospective in parent(s) of patients with mild thalassaemia intermedia. However, if a silent carrier is suspected on the basis of borderline red cell indices and/or borderline HbA_2 levels, a definitive diagnosis may be obtained using globin chain synthesis analysis (sometimes slightly imbalanced), or more often in most laboratories, through characterisation of the mutation by DNA analysis. Because of their silent phenotype these carriers may escape identification in general population-screening programmes. However, this will not have critical consequences, since homozygosity for very mild and silent mutations, or compound heterozygosity for mild with even severe ß-thalassaemia mutations, usually results in attenuated forms of thalassaemia intermedia (11).

An extreme, although rare, instance of a complex thalassaemia gene combination is the co-inheritance of α-, δ- and ß-thalassaemia alleles, which may lead to a silent phenotype and pitfalls in carrier diagnosis (10).

In rare instances, ß-thalassaemia carriers may have a significant clinical phenotype. Co-inheritance of heterozygous ß-thalassaemia with the triple or quadruple α-globin gene arrangement generally results in mild thalassaemia intermedia (12-15). On the other hand, the presence of an HbH disease genotype (--/-α or --/$α^{ND}α$) in interaction with heterozygous ß-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 HbA_2 is usually in the ß-thalassaemia carrier range and that HbH inclusion bodies are absent (16, 17).

Some rare molecular lesions of the ß-gene, most commonly in exon 3, produce highly unstable ß-globin chain haemoglobin variants, which precipitate in erythroid bone marrow precursors because they fail to assemble in functional haemoglobin tetramers. This results in ineffective erythropoiesis and a clinical phenotype of thalassaemia intermedia with an increased HbA_2 and an imbalanced α/ß ratio. Because of the precipitation in early red cell precursors, the ß-chain variant is usually undetectable in peripheral blood. Since the inheritance of a single ß-thalassaemia allele results in a clinically evident phenotype, these forms are also known as dominant ß-thalassaemias (14).

4.4 CHARACTERISTICS OF α-THALASSAEMIAS

α-Thalassaemia is usually caused by α-globin gene mutations that either reduce (α+) or completely abolish (α°) the production of α-globin chains by the affected allele. α-thalassaemia is most frequently caused by deletions that remove part or all of the α-globin gene cluster. Less commonly, the mutations are nucleotide changes within either of the duplicated α-globin genes (the so-called non-deletion determinants) and, very rarely, deletions that include the HS-40 region but leave α-genes intact (Annex 3).

4.4.1 α^+-THALASSAEMIA TRAIT (α-THALASSAEMIA-2)

α^+-thalassaemia is an asymptomatic carrier state in which one α-globin gene is dysfunctional. Red cells are often not microcytic, and HbA_2 and HbF levels are always normal. In the neonatal period, small amounts (1-3%) of Hb Bart's (γ_4) may be detected. Reliable diagnosis of the α^+-thalassaemia trait can only be achieved by DNA analysis.

4.4.2 α^0-THALASSAEMIA TRAIT (α-THALASSAEMIA-1)

The α^0-thalassaemia trait results when two α-globin genes are dysfunctional. It is usually associated with a slight reduction in haemoglobin concentration and red cell indices (MCV, MCH), hypochromia, microcytosis and anisopoikilocytosis, with decreased erythrocyte osmotic fragility and HbA_2 levels in the low to low-normal range (1.5-2.5%). The α/ß-globin biosynthetic ratios average 0.7. During the neonatal period, there are moderate amounts of Hb Bart's (3-8%) and cord blood erythrocytes are microcytic. At the DNA level, the phenotypic presentation of α-thalassaemia-1 trait may be caused by deletions that remove both loci from the same chromosome (αo-deletions), or homozygosity for α^+-thalassaemia deletions.

4.4.3 SYMPTOMATIC FORMS

HbH disease occurs when α-globin synthesis is reduced to about one-quarter of normal levels. It is characterised by the presence of the abnormal haemoglobin component, HbH, a homotetramer of ß-globin chains ($ß_4$). HbH is detected on electrophoresis of freshly prepared haemolysate at alkaline or neutral pH, and typically amounts to 3-30% of total haemoglobin. The clinical presentation of HbH 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.

At the DNA level, HbH disease most commonly results from co-inheritance of α^0-thalassaemia with α^+-thalassaemia deletions. Interactions of an α^0-thalassaemia deletion with a non-deletion α-thalassaemia allele or co-inheritance of non-deletion α-thalassaemia mutations may also give rise to HbH disease. Studies that have correlated haematological, biochemical and clinical findings with genotypes indicate that patients with non-deletion HbH disease mutations have more severe clinical expression, and patients with the severest phenotypes usually have α-thalassaemic (hyperunstable) globin variants (18-20). Phenotypic severity is not simply related to the degree of α-globin deficiency; in addition, HbH is unable to deliver oxygen to the tissues and is unstable, tending to pre-

cipitate within the red cells. Thus high HbH levels may exacerbate anaemia by negatively influencing tissue oxygenation, and both HbH and α-thalassaemic hyperunstable haemoglobin variants appear to reduce red cell survival within the bone marrow and circulation (20).

The moderate haemolytic anaemia may be exacerbated in the febrile state (haemolytic crisis). However, compared to patients with ß-thalassaemia major, they have relatively little ineffective erythropoiesis. The very rare forms of unusually severe HbH disease associated with hydrops foetalis, for which prenatal diagnosis can be indicated, are described in Chapter 7.

Hb Bart's hydrops foetalis This is the most severe form of α-thalassaemia and is discussed in Chapter 7.

4.4.4 STRATEGIES FOR DIAGNOSING α-THALASSAEMIA CARRIERS

These should involve an evaluation of the incidence of α^{0}-thalassaemia and α^{+}-thalassaemia in the population group, reflected by the prevalence of symptomatic forms of α-thalassaemia (HbH and/or Hb Bart's).

HbH disease is not considered to be amongst those haemoglobinopathies targeted for prevention, while couples at risk for having a child with Hb Bart's hydrops foetalis should be detected and prenatal diagnosis is always indicated in such cases to avoid the severe toxaemic complications that frequently occur in pregnancies with hydropic foetuses and which are potentially detrimental to the pregnant mother. The approaches for prenatal diagnosis of Hb Bart's hydrops foetalis are described in Chapter 7.

In order to avoid unnecessary (expensive) investigations and the engendering of anxiety, it is recommended that DNA analysis for definitive characterisation of the α^{0}-thalassaemia trait be carried out only when BOTH partners have an MCH < 25pg, after iron deficiency has been excluded.

It should be noted that the presence of the ß-thalassaemia trait may mask the simultaneous presence of the α^{0}-thalassaemia trait. Therefore, in certain ethnic groups (e.g. Chinese), a detailed investigation is indicated if one partner has the ß-thalassaemia trait and the other is a probable carrier of the α^{0}-thalassaemia trait.

4.5 HbS SYNDROMES

The asymptomatic sickling disorders include sickle cell trait (HbAS) and the doubly heterozygous condition of HbS and Hereditary Persistence of Foetal Haemoglobin (HbS/HPFH) (21). The symptomatic sickling disorders can be divided into mild and severe conditions (see Table 4.4). The milder conditions include HbSC disease, HbS/δß-thalassaemia, HbS/$ß^{+}$-thalassaemia and the homozygous condition HbSS (or sickle cell anaemia) associated with the Arab-Indian ß-globin haplotype (22). The severe sickling disorders are HbSS, associated with the Cameroon, Benin, Senegal and Bantu

ß-globin haplotypes, HbS/ß°-thalassaemia and, finally, the HbS trait combined with the ß-globin chain variants Hb D-Punjab, Hb O-Arab and the rare doubly substituted HbS variants.

Table 4.4 Average haematological findings of some sickling disorders.

Severity	Condition	Hb	MCV	HbA_2	HbF	HbS
Asymptomatic	Hb AS	14.3	87	3.6	0.8	38
	HbS / HPFH	14.0	86	2.1	35.9	62
Mild	HbS /δß-thal	11.4	74	2.2	28.8	69
	HbS /$ß^+$-thal	10.3	71	4.8	5.0	67
	Hbss (Arab-Indian)	9.3	70	2.1	20.0	77
Severe	HbS /$ß^0$-thal	8.6	69	5.0	6.5	89
	HbSS (African)	7.8	90	2.8	5.3	92

4.5.1 ASYMPTOMATIC FORMS

HbS trait. HbS heterozygotes are normally asymptomatic, although sickle cell formation leading to vascular occlusion may occur under certain conditions of significant hypoxia, e.g. at high altitude or under anaesthetic. HbAS individuals without interacting α thalassaemia have 35-40% HbS. The interaction of α-thalassaemia reduces the percentage of HbS and the red cell indices (see Table 4.5). The HbA_2 level is often slightly above normal (3.5-4%), but this never signifies the presence of a ß-thalassaemia gene unless the HbS is greater than 50% of total haemoglobin.

Table 4.5 Percentage HbS and MCV values in HbS individuals with and without $α^+$-thalassaemia

	αα/αα	-α/αα	-α/-α
hBS (%)	35 - 39	29 - 34	24 - 28
MCV (fL)	80 - 90	75 - 85	70 - 75

HbS/HPFH. Patients doubly heterozygous for HbS and Hereditary Persistence of Foetal Haemoglobin (HPFH) are either asymptomatic or have an extremely mild form of sickle cell disease. There is usually no anaemia and patients have very few episodes suggesting sickle cell crises, although occasional mild bone pains have been reported. The condition has been reported in Africans with HbS trait and either the black HPFH1 or the Ghanaian HPFH2 deletion, and also in Indians with the Indian HPFH3 deletion. Patients have nearly normal red cells, each with 20-30% HbF in a pancellular distribution. However, this condition may be difficult to diagnose haematologically because many patients have reduced red cell indices due to co-existing $α^+$-thalassaemia.

4.5.2 SYMPTOMATIC FORMS

HbS in the homozygous state or in combination with either HbC, Hb O-Arab, Hb D-Punjab or

ß-thalassaemia causes sickle cell disease. HbS is less soluble than normal haemoglobin during deoxygenation, crystallising out into polymers in the form of long fibres that cause the classical sickle-shaped deformation of the red cell. The sickle-shaped cells are more rigid than normal red cells and tend to block small arteries, resulting in an inadequate oxygen supply to the tissues and organs. In addition, the sickle-shaped cells have a shorter lifespan, resulting in a lifelong haemolytic anaemia. The sickle cell disease genotype interactions are discussed in Chapter 7.

4.6 HbE SYNDROMES

HbE was the fourth abnormal haemoglobin to be identified by haemoglobin electrophoresis, in 1954 (23), and in 1961 it was characterised as having the substitution of lysine for glutamic acid at position 26 of the ß-globin chain (24). Many types of HbE syndromes are observed, due to various interactions with α- thalassaemia, ß-thalassaemia or other haemoglobin variants. The symptomatic and asymptomatic forms are summarised in Tables 4.6 and 4.7. The asymptomatic are discussed below; forms for which prenatal diagnosis may be considered are discussed in Chapter 7.

Table 4.6 Summary of the common HbE syndromes

Phenotype	Genotype	Anaemia	Distinguishing Features
Asymptomatic			
HbE heterozygote	β^N/β^E	No	HbE (25-30%) + HbA
HbE heterozygote & α$^+$-thalassaemiaheterozygote	β^N/β^E & αα/α$^+$-thal	No	HbE (25-30%) + HbA
HbE heterozygote & α°-thalassaemia heterozygote	β^N/β^E & αα/α°-thal	No	HbE (19-21%) + HbA
HbE homozygote	β^N/β^E	No	only HbE
HbE homozygote & α-thalassaemia heterozygote	β^E/β^E & αα/α-thal	No	only HbE
HbE/HbC	β^E/β^C	No	HbE (32%) + HbC (56%)
Symptomatic			
HbE homozygote & Hb CS homozygote	β^E/β^E & Hb CS/Hb CS	Mild	Hbs E: ($\alpha_2\beta_2^E$) + $\alpha_2^{CS}\beta_2^E$
HbE/ßo-thalassaemia	β^o/β^E	Moderate to severe	HbE + HbF
HbE/ß$^+$-thalassaemia	β^+/β^E	Mild	HbE + HbF + HbA
EA Bart's:			
- HbH disease with HbE heterozygote	α°-Thal /α$^+$-thal & β^N/β^E	Moderate	HbE + HbA + Hb Bart's
- HbH-CS disease with HbE heterozygote	α°-Thal /Hb CS & β^N/β^E	Moderate	Hb CS + HbE + HbA + Hb Bart's
EF Bart's:			
- HbH disease with HbE homozygote	α°-Thal /α+-thal & β^E/β^E	Moderate to severe	HbE + HbF + Hb Bart's
- HbH-CS disease with HbE homozygote	α°-Thal /Hb CS & β^E/β^E	Moderate to severe	Hb CS + HbE + HbF + HbBart's
- HbH disease with HbE/ß-thal disease	α°-Thal /α$^+$-thal & β^o/β^E	Moderate to severe	HbE + HbF + Hb Bart's
- HbH-CS disease with HbE/ß-thal disease	α°-Thal /Hb CS & β^o/β^E	Moderate to severe	Hb CS + HbE + HbF + Hb Bart's

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

Table 4.7 Haematologic data in various HbE syndromes

	Hb (g/dl)	MCV (fl)	MCH (pg)	Osmotic fragility	DCIP	Hb typing
Normal	M15.9±0.9	87±6	31±1.1	N	-	A2 (2.5±0.2) + A
	F12.5±2.0					E (29.4±2.3%) + A
HbE trait	12.8±1.5	84±5	30±2.4	N or D	+	E (28.5±1.5%) + A
HbE & α⁺-thalassaemia	13.1±1.4	88±4	ND	N or D	+	E (20.7±1.2%) + A
HbE & α°-thalassaemia	12.5±1.4	77±5	23±1.1	D	+	E (87.7±5.9%)
Homozygous HbE	11.4±1.8	70±4	22±1.9	D	+	E (58±11.5%) + F
HbE/ßo-thalassaemia	7.8±2.6	67±6	19±3.6	D	+	
EA Bart's disease:						
HbE & α°-thal /α⁺-thal	9.1±1.1	60±3	17±2	D	+	E (13.0±2.1%) + A + Bart's (2.2±1.8%)
HbE & α°-thal /Hb CS	8.0±0.9	67±4	19±2	D	+	CS (1.1±0.4%) + E (13.9±1.8%) + A + Bart's (3.9±1.5%) E (80%) + F + Bart's (5%) or
EF Bart's disease	8.0±1.3	63±6	18±2	D	+	CS (1.9±0.9%) + E (86.4±8%) + F + Bart's (3.7±1.9%)

Abbreviations: ND, not determined; N, normal; D, decreased; CS, Constant Spring; thal, thalassaemia

4.6.1 ASYMPTOMATIC FORMS

HbE Trait. HbE heterozygotes are clinically normal, with minimal changes in blood counts and erythrocyte indices. Red cell morphology is similar to that in thalassaemia minor with normocytic or slightly microcytic red cells (MCV 84±5fl). A few target cells may be present in the blood smear. Osmotic fragility curves may be within normal limits or moderately shifted to the right, indicating slightly decreased osmotic fragility. Haemoglobin electrophoresis reveals both HbA and HbE. HbE quantification is crucial for the diagnosis of HbE syndromes arising from the interaction of HbE with other inherited haemoglobin abnormalities or non-genetic factors (see Table 4.6). HbE constitutes 25-30% of the haemolysate in simple HbE trait or compound heterozygosity for HbE and α⁺-thalassaemia, and in general these two conditions cannot be differentiated by haematological screening. HbE levels are reduced by co-inherited α°-thalassaemia to 19-21% (25), and markedly reduced in individuals who co-inherit heterozygous HbE and HbH disease (HbAE Bart's disease syndrome) to 13-15% (26). HbE levels above 39% suggest the interaction of ß-thalassaemia with HbE. HbE heterozygotes deficient in iron also have reduced amounts of HbE and lower MCV and MCH, depending on the degree of iron deficiency.

Homozygous HbE. HbE homozygotes usually have normal haemoglobin levels (although some may be mildly anaemic) and clinical symptoms, such as jaundice and hepatosplenomegaly, are rare. Bone changes are not present. Reticulocyte counts are consistently normal and nucleated red cells are absent from the circulation, but a characteristic finding is 20-80% target cells and osmotic fragility is markedly decreased (see Table 4.7). Haemoglobin analysis reveals about 85-95% HbE with remainder HbF. There is defective ßE-globin chain synthesis in all HbE homozygotes with an average

α/non-α ratio of 2, equivalent to the ratio found in β+-thalassaemia heterozygotes (23). Defective βE-chain synthesis is due to decreased βE mRNA production, a result of abnormal RNA splicing caused by HbE mutation (28,29).

4.7 GENERAL APPROACH FOR CARRIER IDENTIFICATION

Carrier identification strategies should ensure that no carrier eludes detection. There are two possible methodological approaches for β-thalassaemia carrier identification:

a. 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
b. complete screening based on determining red cell indices, haemoglobin pattern analysis and HbA_2 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.

A flow-chart illustrating the strategy used to identify carriers in high risk areas is shown in Figure 4.2, while the recommended methods with cut-off indices are summarised in Table 4.8. These cut-off indices are the 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. In addition, there should be regular quality control programmes in order to monitor the accuracy of laboratory results. The techniques for carrier identification are extensively treated in Prevention Book 2.

4.8 PROBLEMS IN SCREENING FOR THE HAEMOGLOBINOPATHIES

Screening programmes for the haemoglobinopathies have become well-established in many countries over the last 20 years. But despite the large amount of accumulated knowledge, several problems in carrier identification remain. The most common problem is the presence of microcytosis with normal HbA_2 and HbF, which may be due to iron deficiency, the α-thalassaemia trait or δβ+-thalassaemia (see Figure 4.2). Iron deficiency anaemia produces a wide range of red cell abnormalities (reduction of MCV, MCH, and Hb levels, and raised RBC), depending on the severity at the time of haematological analysis. For this reason, iron deficiency anaemia is easily mistaken for some forms of heterozygous thalassaemia. Besides iron deficiency, similar haematological findings are associated with α-thalassaemia and δβ+-thalassaemia. Iron deficiency may be distinguished from forms of α- or δβ+-thalassaemia by an increase in zinc erythrocyte protoporphirin (ZnPP) and a reduction in serum iron, transferrin saturation and serum ferritin. Alternatively, an imbalance in the α/β-globin chain synthesis ratio supports the presence of α-thalassaemia (α/β ratio < 0.9) or δβ+-thalassaemia (α/β ratio > 1.2). Finally, family studies may also be useful for distinguishing the diagnosis.

Figure 4.2 Flow chart for thalassaemia screening

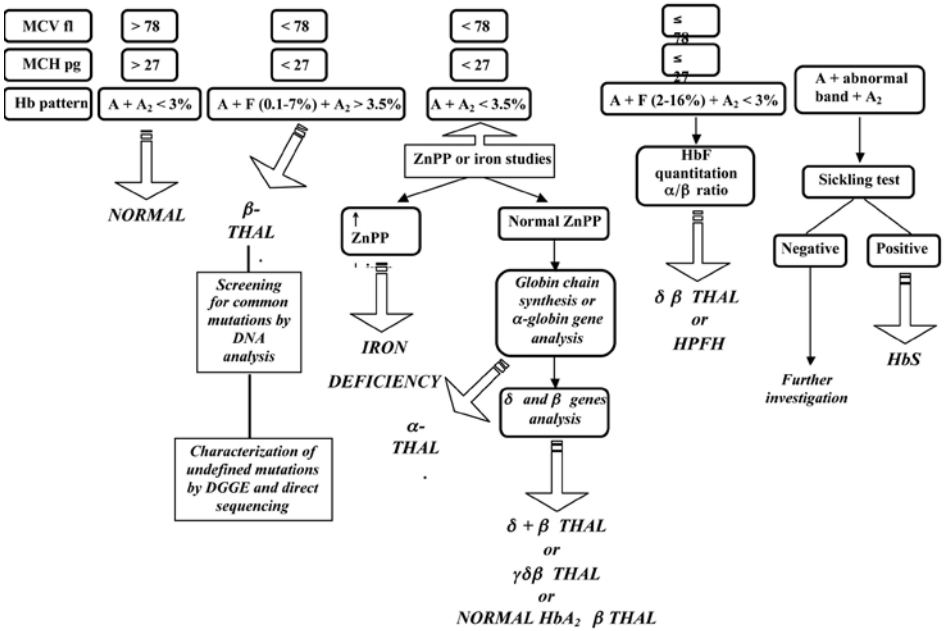


Table 4.8 Screening for ß-thalassaemia carriers: recommended methods and cut-off values (see also Prevention Book 2)

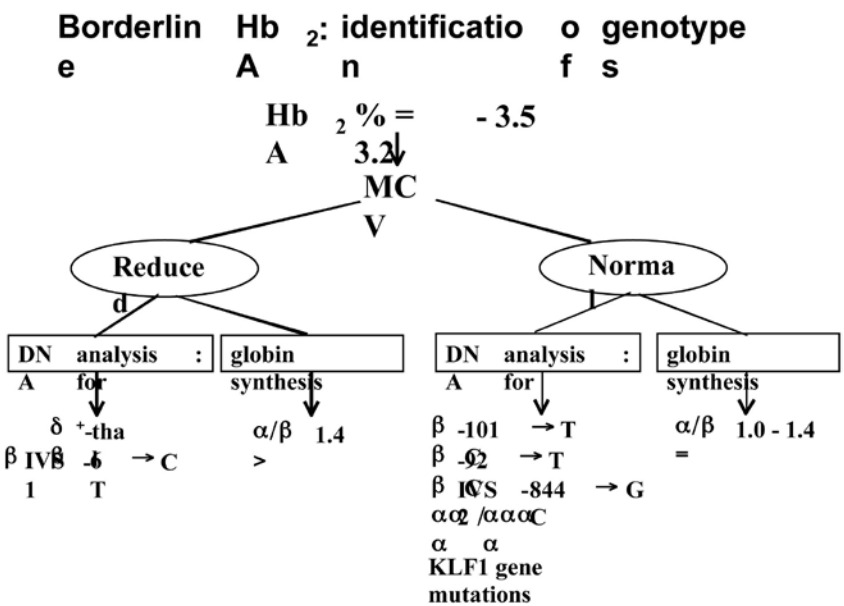
Indices	Method	Cut-off value for carriers
MCV	Electronic determination	< 78 fl
MCH	Electronic determination	< 27 pg
HbA_2	Elution after electrophoresis	> 3.6 %
	Microchromatography on DE-52	
	HPLC	
Hb analysis	Electrophoresis on cellulose acetate	Increased HbF (> 5.0 %)
	HPLC	Presence of abnormal band
	IEF	
	Capillary electrophoresis	
HbF (quantitative)	Alkali denaturation	>5%*
	HPLC	
	Immunologic determination	
	Capillary electrophoresis	
	CMC chromatography	> 1.2
	HPLC	
	Capillary electrophoresis	

In rare cases when ß-thalassaemia carriers have concomitant iron deficiency, HbA_2 levels may be reduced, although they usually remain within the ß-thalassaemia carrier range. In exceptional cases where ß-thalassaemia carriers have very severe iron deficiency, HbA_2 levels may fall to within the normal range. In practice, if an individual has very severe iron deficiency anaemia with normal HbA_2, it is preferable to treat the patient with iron to correct the anaemia before repeating tests to determine HbA_2 (30).

In the course of screening to identify ß-thalassaemia carriers, it is not uncommon to find individuals with borderline HbA_2 levels (3.2–3.5%) and normal or mildly reduced red cell indices (31, 32). The majority of cases with borderline HbA_2 levels and normal red cell indices have normal ß- and α-globin genes, and the borderline HbA2 levels may be explained by the extreme distribution of the normal range of HbA_2. However, these cases are sometimes carriers of silent ß-thalassaemia mutations (-101 C→T, IVSII-844 C→G, +1480 C→G, +33 C→G) or a triplicated α-gene locus. As previously mentioned some cases of borderline HbA2 with normal RBC indices are associated with mutations at KLF1 gene (8). Individuals with borderline HbA_2 values (3.4-3.8%) and reduced MCV and MCH are usually carriers of a mild ß-thalassaemia allele (e.g. IVSI-6 T→C, CAP+1 A→C, polyA T→C), or compound heterozygotes for δ- and ß-thalassaemia.

Diagnosing the atypical carriers involves family studies and/or globin chain synthesis and/or DNA analysis, and it is recommended that the presence of a ß-thalassaemia allele be excluded in any subject with a borderline HbA_2 level, especially if their spouse is a typical ß-thalassaemia carrier (see Figure 4.3).

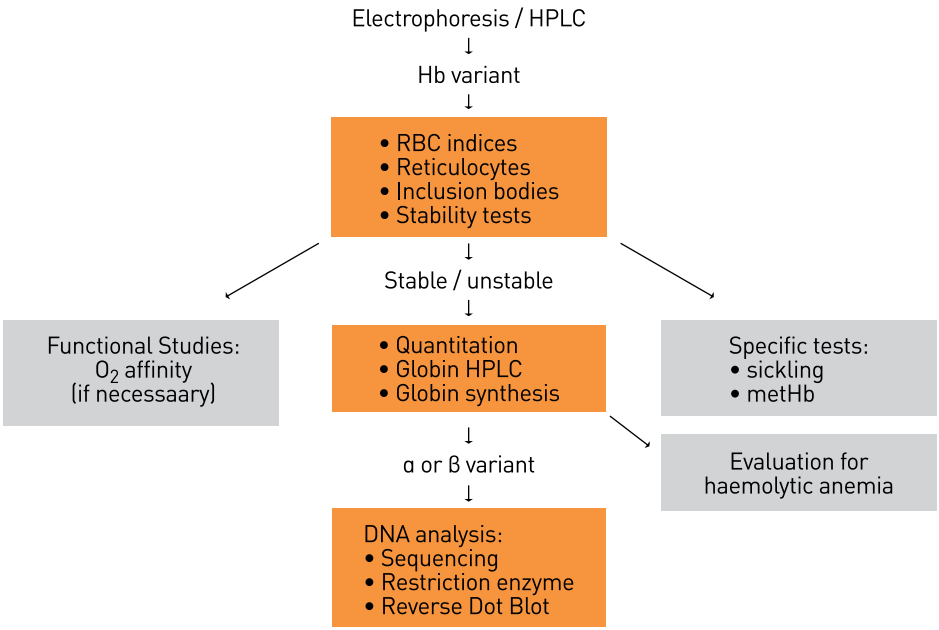
Figure 4.3



The most common forms of δß°-thalassaemia and HPFH are characterised by normal HbA2 and increased HbF levels. Red cell indices in HPFH are normal compared to δß°-thalassaemia carriers, in whom the MCV and MCH are usually slightly reduced. Differentiation between δß°-thalassaemia and HPFH is facilitated either by globin chain synthesis (normal or minimally imbalanced α/ß synthesis ratio in HPFH, mild to moderate imbalance [α/ß ratio 1.4–2] in δß°-thalassaemia), or by DNA analysis. HbF distribution in red cells is reported as homogeneous or pancellular in HPFH and heterocellular or uneven in δß°-thalassaemia, although the distinction is less clear if more sensitive immunological methods are used. It is also important to differentiate between HPFH and δß°-thalassaemia in the context of genetic counselling: genetic compounds with ß-thalassaemia and HPFH result in a silent or very mild clinical phenotype, while δß°-thalassaemia carriers are at risk of producing thalassaemia major in combination with classical ß-thalassaemia.

Carriers of structural haemoglobin variants are usually detected during screening programmes for the haemoglobinopathies. The identification of a variant is based on a series of specific tests, summarised in Figure 4.4. The simple sickling test allows correct diagnosis of HbS. For all the other variants, even if a presumptive diagnosis may be achieved by careful interpretation of several laboratory methods (such as relative electrophoretic mobility, chromatographic elution times, amount and analysis of globin chains), DNA analysis remains the only approach to provide a precise identification. Finally, due to the influence of co-existing α-thalassaemia on haematological parameters it is sometimes difficult to accurately diagnose the phenotypes of HbSS, HbS/ß°-thalassaemia, HbS/δß-thalassaemia and HbS/HPFH based on a haematological assessment of the patient, and family studies are therefore recommended.

Figure 4.4 Flow-chart for Hb variant identification



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CHAPTER 05

Mary Petrou

GENETIC COUNSELLING

Genetic counselling is the most complex aspect of prevention. Genetic counselling is inseparable from genetic diagnosis, aiming to replace misunderstandings about the causes of genetic disease with correct information, and to increase people's control of their own and their family's health by informing them of the resources available for diagnosis, treatment and prevention. Although counselling has a role in many medical consultations, it is particularly important in medical genetics because of the often predictive nature of genetic information, implications for other family members, the difficult choices that sometimes have to be made and the important ethical problems that can be involved.

Genetic counselling has been defined as:

"The process by which patients or relatives at risk of a disorder that may be hereditary are advised of the consequences of the disorder, and the probability of developing and transmitting it and the ways in which this may be prevented or ameliorated" (1).

This definition requires:

- A correct diagnosis in the presenting family member.
- Explanation of the nature and prognosis of the disorder, the treatment available and where to find it.
- 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.

Meeting the above needs requires specialist genetic knowledge, training in counselling skills, time, communication skills, and back-up from a medical geneticist or trained genetic counsellor.

5.1 COMPLEXITIES IN GENETIC COUNSELLING

The haemoglobin disorders are the most common world-wide inherited conditions. They are common in populations in tropical Africa, Asia and the Mediterranean region, and have spread by migration throughout the world (2, 3). It is estimated that 307,900 children are born annually with a severe haemoglobin disorder and 60-70% of births occur in sub-Saharan Africa (4). Thalassaemia is prevalent in the Mediterranean area, the Middle East and Southeast Asia, and the Pacific. The carrier rates range from 2-19% in the different populations (5). The birth prevalence of the haemoglobin disorders in countries affected by migration of populations varies according to the geographic location and the origin of the populations.

Accurate genetic diagnosis is often difficult, in view of the enormous diversity of conditions involved, and the wide cultural, social and religious structures in the countries where the haemoglobin disorders exists makes genetic counselling more challenging.

Thalassaemia mutations and various abnormal haemoglobins interact to produce a wide range of disorders of varying degrees of severity. There are four main categories of interactions associated with severe disease states, for which genetic counselling and prenatal diagnosis is indicated for thalassaemia disorders: 1) co-inheritance of ß- and/or δß-thalassaemia mutations; 2) coinheritance of ß-thalassaemia mutations with Hb E or Hb O-Arab; 3) for alpha thalassaemia: Hb Bart's hydrops foetalis syndrome (α^0/α^0), and much more rarely, HbH hydrops foetalis syndrome (α^0/α^T); 4) for sickle cell disorders: HbSS, HbS/C, HbS/ß-thalassaemia, HbS/D-Punjab, HbS/O-Arab, HbS/Lepore) and HbSE disease.

To date, 1538 variations in the globin gene sequences have been described, including the thalassaemia mutations, which are all listed in the Annexes, and the haemoglobin variants, which are documented on HbVar, a database of human haemoglobin variants and thalassaemias [http://globin.bx.psu.edu].

The phenotypic heterogeneity of thalassaemia requires a genetic counsellor to be knowledgeable in the molecular genetics of thalassaemia, so that he/she can understand the molecular mechanisms involved and communicate this information to families. An example of this is the complexity in the phenotypic variability in patients with ß-thalassaemia. More than 200 ß-thalassaemia mutations have been described wide range of phenotypic severity (from silent to very severe), all of which can potentially interact to produce a ß-thalassaemia disorder ranging from a very mild form of intermedia to the severe form, thalassaemia major. For instance, the mild $ß^+$-type mutation -88 (C→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 $ß^+$-type mutation -88 (C→T), the question arises whether the couple should consider prenatal diagnosis (PND)? This is also the case with a number of other mild ß-thalassaemia mutations in the homozygous state, such as IVSI-6 (T→C), [HBB:c92+6T>C]. However the mild $ß^+$-thalassaemia mutation Cap+1 (A→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 ß-thalassaemia mutations, such as -101 (C→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 ß-thalassaemia mutation, such as IVSI-110 (G→A). PND is not considered in such cases, or where one partner has ß-thalassaemia trait and the other carries a triplicated α-gene allele (ααα) or a hereditary persistence of foetal haemoglobin (HPFH) gene.

The severity of homozygous ß-thalassaemia is also affected by ameliorating factors such as the co-inheritance of α-thalassaemia or gene modifiers that increase the HbF production such as ß-globin locus-linked quantitative trait loci (QTL): Gγ globin Xmn1 polymorphism or trans acting QTL's; BCL11A on chromosome 2 and HBS1L-MYB on chromosome 6 and other rare QTL's (6).

The effect of these modifying factors on the clinical phenotype is becoming more informed, and the counsellor should have sufficient experience and expertise to communicate this information and the likely outcome to patients. Indeed, as knowledge of the genotype mechanisms that modify thalassaemia continues to accumulate, particularly with genome wide association studies, this area will become more complex but will allow us to carry out more accurate predictions of phenotype. But how far should geneticist persevere to obtain more accurate prediction of phenotype? So far it is very rare to test for modifying factors at the time of a foetal diagnosis; this is because the effect of these factors is not so consistent, except perhaps when there is an existing mildly affected child in the family. But as the information accumulates geneticists will need to address this important issue.

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 ß-thalassaemia, the above modifying factors may also influence the severity of sickle cell. A study performed in London showed that decision-making was greatly influenced by the experience of sickle cell disease within the family: a couple with an affected child or who had experience of severe sickle cell, was more likely to undergo PND and selective termination of an affected foetus than one with no such experience. The study also found that uptake of PND was influenced by the gestation at referral: a woman referred in the second trimester was less likely to proceed with PND (7).

By contrast, 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 (8). 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 (9). But more information is needed about the outcomes of such interventions and the best way forward to provide this service. Preferably this information should come through an international collaborative study collecting data on all cases of intra-uterine transfusion, including the foetal treat-

ment provided and outcomes, in order to produce guidelines for the most successful approaches. This information is essential 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 (10). 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 ß-thalassaemia major, such as regular blood transfusions and iron chelation therapy.

5.2 ETHICS AND RELIGION IN GENETIC COUNSELLING

The acceptability or otherwise of prenatal diagnosis and the selective abortion of an affected foetus is influenced by many factors besides the information that couples receive, including their social, cultural and religious backgrounds, personal beliefs and experiences.

Because the choices facing people at genetic risk can be so difficult and can have life-long consequences, experienced genetic counsellors generally consider that informed individuals or the couple themselves are the best judges of what to do. Genetic counselling should therefore 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 morally right for them.

Some reasons why genetic counselling should be non-directive are:

- People at risk often have first-hand experience of the condition in question - unlike most of their advisers.
- They have to learn all the facts, think the issues through and reach a decision that they must live with for the rest of their lives.
- The right choice for a given individual among the options actually available is likely to be determined by many factors, including their social and religious attitudes, personal experiences, economic and educational level, and family and reproductive history.
- Doctors and other professionals are no more or less qualified than their patients to make moral choices of the type generated by awareness of genetic risk.

Therefore, ethical practice in genetic counselling, as it has gradually evolved in the West during the past 20 years, requires the following (11, 12):

1. The autonomy of the individual or couple
2. Their right to complete information
3. The highest standard of confidentiality

This highlights the important role of information in genetics and the need to develop systems for providing and accessing genetic information. Specialist genetic information must be accessible to health workers, patients and the community in order to permit informed choice. Additionally, in-

formed choice should determine outcome at both the individual and the community level, so patient choices should be recorded, collected, reported and used to guide service development (13).

People at-risk, especially those identified by population screening, represent a randomly selected sample of the population. They have to learn in depth about the risk, and often have to use the information in life-determining choices. Each choice represents a complex personal judgement; for example, couples at genetic reproductive risk must balance the burden of the disease, such as thalassaemia against the burden of prenatal diagnosis and termination of a wanted but affected pregnancy. The statistical accumulation of individuals' informed choices represents a collective judgement on what services are available within the health care of the country (13, 14, 15). Since such choices are or should be recorded, registers that aggregate this information and outcomes can be used to listen to the community verdict of their reproductive choices such as the acceptability of prenatal diagnosis.

The outcome of high uptake of prenatal diagnosis by couples at-risk for thalassaemia in the United Kingdom and Mediterranean countries reflects the at risk community's view of the heavy burden of the disease and its treatment. However, things can change as treatment becomes more effective and acceptable and the uptake of prenatal diagnosis may fall. If new approaches like preimplantation genetic diagnosis (16) become easier and more widely available, then the burden of prevention may decline and uptake may rise.

Life-expectancy for thalassaemia has improved significantly with modern medical treatment, and therefore Quality of Life (QoL) should now be considered an important factor of effective health care. The burden of treatment and QoL should be discussed and considered within genetic counselling. An assessment of QoL differs from other forms of medical assessment in that it focuses on the individuals' own views of their well-being and assesses other aspects of life, thus giving a more holistic view of well-being. Within countries that provide optimal treatment, thalassaemia major patients survive into their 50's and beyond, however some do not. How does QoL have an impact on patient survival?

QoL is defined as "...individuals' perceptions of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns" WHO 1997 (http://www.who.int/mental_health/media/68.pdf). QoL is a concept affected in a complex way by the person's physical health, psychological state, personal beliefs, social relationships and their relationship to salient features of their environment (17).

However at a conference organised by the United Kingdom Thalassaemia Society, QoL was defined as: 'graduating, having a suitable job, getting married, having children' (18). What constitutes an acceptable QoL depends also on cultural context, age, and a complex interplay of the social, environmental and spiritual background of the patient. To achieve these goals it is necessary for patients to be intergrated within society and treated as equal members of society. QoL is dependent on factors such as making a success of: education, profession, relationships with their partner and family and parenthood. However, these are all related to treatment and the creative ways treatment is offered

that consequently has an impact on the burden of the disease. Life can be improved by introducing effective therapy, which is easy and convenient, such as the availability of evening and weekend transfusions, evening clinics, easy effective iron chelation treatment, psychological support and very importantly a more tolerant, informed and accepting society towards affected patients. However do society's social and cultural beliefs allow patients to integrate into society? Is it possible for quality treatment encompassing an optimal QoL to be integrated into the health system to reduce the burden of the disease? By denying patients this holistic approach the burden of the disease remains high and consequently uptake of prenatal diagnosis remains high. Genetic counsellors will always need to bear these issues in mind when counselling individuals and couples.

Medical training rarely equips doctors to provide adequate genetic counselling and to discuss complex issues with their patients in order to help patients reach their own decisions. The responsibility involved in genetic counselling should not be underestimated. There are ethical problems faced by the counsellor as there is no universal model for genetic counselling; counselling is an understanding of a set of facts according to the counsellor's frame of reference, background in science and genetics, and their previous training and experience in effectively communicating with the individual/couple. In order to communicate effectively the counsellor must take into account the educational, social, cultural and religious background of the individual/couple. Other obstacles to effective genetic counselling are emotional conflicts and lack of knowledge of genetics by the counsellor (19). Genetic counselling should be non-directive and the counsellors main role is to provide information in a non-biased manner and support decisions that are morally right for the individual, couple or family. When the decision is genuinely their own, the individuals are more likely to be able to live with it. It is often indeed difficult for a counsellor to impart this information in an unbiased manner because of his/her patients' family history including parental age, ethnic background and reproductive history.

A counsellor may be faced with an at-risk couple, where the woman is in her 40's pregnant with no living children. This couple has the option of prenatal diagnosis, with a 25% chance of an affected fetus and the option of selective termination for an affected fetus, but also a small risk of miscarriage following prenatal diagnosis. But, there is also the prospect, that this may be the last chance for this couple to have a child. Situations such as this may lead the counsellor to adopt a directive rather than a non-directive approach to genetic counselling (21). The major difference between directive and non-directive counselling is whether or not the counsellor helps the couple to make a decision that is right for them. Directive counselling can have a positive influence on the couple's decision; the non-directive approach involves presenting the facts in an unbiased manner and helping the couple come to the decision that is right for them and leaving the responsibility of the decision making to the couple. However can the counsellor completely disassociate the couple from their own values and present the information in such a way that they are completely free to make their own decision? Another example of this is the tone or manner that the counsellor uses to provide the information to a couple, which is dependent on their level of personal involvement in a particular case. The counsellor may change the manner of speech and body gestures, which in turn may influence how a couple interprets the information. For instance, in cases where the counsellor feels it is better for the couple to continue a pregnancy without prenatal diagnosis, they could say to

the couple "Your chances of a healthy baby are really quite high at 75%" However for another couple the counsellor may say "Your chances of an affected baby with thalassaemia major are really quite high at 25%". Therefore the information being imparted to both couples is correct but presented in different ways.

Medical ethics is also based on the moral, religious and philosophical ideals and principles of the society in which they are practiced. It is therefore not surprising to find that what is considered ethical in one society might not be considered ethical in another. The wide range of family and social structures, religious and legal conventions and economic resources within, countries where the haemoglobin disorders are found, may also lead to conclusions that differ between countries. Sensitive services like genetic counselling cannot be transported from one social context to another, they need to be developed within the framework of each country.

Truly ethical conduct consists of a personal search for relevant values that lead to an ethically inspired decision. Chapter 9 on "Surveillance" shows how information on parental informed choice is obtained, and how this reflects what is ethically acceptable in a particular society. Practitioners and critics of conduct must be sensitive to such information before they make their judgements on what is acceptable. The ethical attitude of an individual, whether it is a patient or a genetic counsellor, is often coloured by the attitude of their society. Social attitudes are influenced by theologians, demographers, family planning administrators, doctors, policymakers, sociologists, economists and legislators. However it is important all these groups to also consider the fundamental ethical principles of genetic counselling, particularly the autonomy of the individual, and place primary emphasis on informed parental choice for at-risk individuals, before making judgements on behalf of society.

All medical programmes, including genetic prevention programmes, must operate within existing legal and social frameworks. However, technology can develop rapidly while legal, social and religious attitudes evolve more slowly. Most religions incorporate a range of opinions and dilemmas. The high uptake of prenatal diagnosis in Italy, Cyprus and Greece clearly shows that people make their own choices for what is appropriate to them (21, 22, 23).

Several Muslim countries are also already offering prenatal diagnosis and selective abortion to at-risk couples, or are at various stages of developing these services (24, 25, 26). It is therefore important to comment on information so far available on the acceptability of such services in these countries.

For many Muslims, religion is central to daily life and influences a lot of behaviours, attitudes and practices, including policy-making (27). Instructions that Muslims are expected to follow regulating everyday activities are called Sharia. The Sharia is not rigid or fixed, except for a few rules such as those concerning worship, rituals and codes of morality. Islamic Sharia accommodates different, honest opinions, as long as they do not conflict with the spirit of its primary sources and are directed toward the benefit of humanity. In formulating rules and guidelines in Muslim societies, account should first be taken of the provisions and spirit of Islamic Sharia, as well as prevailing local social conditions.

Muslim theologians regard foetal development as occurring in three stages, each lasting 40 days: the sperm cell and ovum, the clump resembling a blood clot and the lump of flesh (foetus). At the end of these stages, the foetus is ensouled. However, the belief that ensoulment occurs only after 120 days does not change the fact that life starts at a much earlier stage of the embryo's development.

From a Muslim perspective, it is considered ethical to perform an abortion to protect the mother's life or health, or because of a foetal anomaly incompatible with life (17, 28, 29). However, the stage at which termination is deemed permissible seems to vary. Some Muslim jurists do not allow abortion in any circumstances, while others would permit abortion in the first 120 days of foetal life, where there is a reason such as danger to the mother or the foetus. The time when an abortion is allowed also varies: some jurists would only allow an abortion at 40 days and others at 90 days.

As an example, we have reproduced below a fatwa issued by two renowned religious scholars prior to prenatal diagnosis being implemented in Pakistan (30):

1. Termination of pregnancy is absolutely prohibited after the soul is breathed into the foetus (after 120 days of gestation) even if test shows the presence of a serious disorder. Termination after the soul is breathed into the foetus is like killing a live child. Killing a child, no matter how seriously ill that may be, is not permissible under any circumstances. Similarly, to do termination of pregnancy or to get a pregnancy terminated after the soul is breathed into the foetus is also not permissible.

2. If the test done before the soul is breathed into the foetus (before 120 days of gestation) shows that the foetus has a serious disorder and an honest physician suggests to terminate the pregnancy then the mother has the permission to get the pregnancy terminated.

Mohammad Taqi Othmani, Mufti, Jamia Dar ul-Uloom, Karachi

Most Muslims accept that life does not begin at conception, believing that human life requiring protection commences some weeks, perhaps two weeks or so, after the development of the primitive streak (19, 31, 32). Therefore, preimplantation genetic diagnosis (PGD) is encouraged where feasible, as an option to avoid termination of pregnancy (31, 32). Indeed, the importance of PGD was recognised at the international workshop on Ethical Issues in Assisted Reproductive Technology, held at the International Islamic Centre for Population Studies and Research of Cairo's Al-Azhar University.

However, it should be recognised that ethical and religious reasoning on the same issue can justify different conclusions. It must therefore be accepted that adherents of one preferred outcome may well acknowledge that adherents of an alternative preferred outcome are applying approaches that result in different but equally ethical conclusions.

5.3 OPTIONS AVAILABLE TO PEOPLE WITH A GENETIC REPRODUCTIVE RISK

Peoples' options are greatly influenced by the stage at which they learn of their genetic risk and by whether prenatal diagnosis is available. If the risk is found before marriage, the options are to remain single, not to marry another carrier, or to marry irrespective of carrier status. If the risk is found after marriage, the options are to separate and find a non-carrier partner, to have a few or no children, to take a chance and have children as usual, or to use PND and selective termination of pregnancy if it is acceptable and available.

Unless an active carrier-screening programme is in place, it is unusual for a couple to learn they are at risk of having children with a haemoglobin disorder before marriage or before starting a family. In countries where premarital or antenatal screening is not offered, most couples currently learn of their risk only after the diagnosis of an affected child, and this limits their choices.

In most Western countries, prenatal diagnosis for couples at risk for haemoglobin disorders is available, with the option of selective abortion. It is recognised that selective abortion is not an optimal or an easy solution, that early prenatal diagnosis is preferable to later prenatal diagnosis, and that not all couples at risk for having children with thalassaemia feel that prenatal diagnosis is the right choice for them. However, these attitudes have evolved only gradually over the decades since prenatal diagnosis first became available. In the early days, the acceptance or otherwise of such techniques in different Western countries depended largely on the status of family planning and the existing abortion law in each country.

It is difficult for people to deal with the idea of prenatal diagnosis unless they are already used to the concept of controlling their own reproduction, a social change that takes time. When genetic diagnosis began to be developed in the 1950s, the options available to couples at reproductive risk were to ignore the information and hope for the best, to remain unmarried or to separate, or to limit their reproduction using family planning (i.e. the same choices as those now faced by couples in countries where PND is not available). Family planning was already becoming widely accepted in the West at the time, and it soon became clear that when options were so limited, most people chose either to avoid or ignore information on risk, or to limit the size of their family (33, 34, 35, 36).

There are therefore challenges involved in genetic counselling because all the available choices involve difficult moral and social problems, and in most cases there appears to be no "right answer". But on the other hand, once people understand their risk, they cannot escape from making a choice. Even the decision not to choose is a choice.

It is useful to examine in more detail the options available to people with a genetic reproductive risk. The fact that carriers of thalassaemia can be detected and advised of reproductive risk either before or after marriage opens up a wide range of choices to couples and there is extensive experience of the choices that people make in practice.

But how can information on which approaches are acceptable and which unacceptable to a particular population be obtained? One possibility is through public meetings, discussions and questionnaires addressed to relevant professionals and the general public. However, this presupposes an informed public – a prerequisite found in very few countries at present - and therefore requires a substantial component of public education. The most extensive of such consultation exercises was carried out in the early 1990s by the Canadian Royal Commission on New Reproductive Technologies (37). The results of that exercise, like many other similar surveys, showed that the majority of people, including many who might not use the service themselves for religious or other reasons, approved of the availability of prenatal diagnosis and the termination of pregnancy for serious genetic disorders. Another possibility is to seek the views of informed people who are themselves at genetic risk. However, there can be a considerable difference between what people think they would do and what they actually do when they have to make a choice. A more objective approach is to observe and report the choices that people at risk actually make.

Community-based programmes for the prevention of haemoglobin disorders in Europe (including Cyprus) now include the option of prenatal diagnosis and selective abortion, but carrier screening and genetic counselling were introduced in some countries either before prenatal diagnosis was possible (38, 39, 40) or before it was legal (40). This early experience produced useful information on the acceptability of some alternative approaches in Europe and Cyprus.

5.4 PREMARITAL SCREENING

It is often thought that affected births can be prevented if at-risk couples are identified prior to marriage, on the assumption that they will then decide to separate and each find another, non-carrier partner. However, we understand too little of how most marriages come about to be able to make any valid assumptions about how choice of partner might be affected by genetic information. In many societies, marriage is a complex social phenomenon that involves many other family members besides the prospective couple, and marriage partners are usually selected either because of a strong personal preference, or for valid family or traditional reasons, or a mixture of the two.

When a planned marriage is called off, it can cause social embarrassment or stigma to the young people and their families – and there is a risk that the problem will recur if a new partner is also found to be a carrier. (For example, if population carrier frequency is 6%, the chance that one or other of the new partners will be a carrier is 12%, or even higher if the new potential partner is a relative.)

A second possibility is to marry as planned, but avoid having children altogether. This is always a difficult choice and is unrealistic in a strongly family-centred society. It is also technically difficult unless high quality family planning is readily available. A more realistic option for married at-risk couples is to limit their family size. If they limit themselves to two healthy children, 56% of them will never have an affected child. Where this is the outcome, such couples could well feel they made a good decision.

Other options for having children while avoiding a known genetic risk, such as artificial insemination by donor, egg donation or adoption, have so far proved unpopular in all societies, and are unacceptable in many communities.

Another possibility is to marry and wait to have a family until appropriate methods for prevention become available. For example, when at-risk Cypriot couples were informed that prenatal diagnosis would become available in the foreseeable future, many postponed conceiving until they could use the service (38). A final possibility is to marry and have a family as usual, trusting in God. This often seems to be a common choice in the absence of acceptable alternatives that are acceptable to families..

Before prenatal diagnosis was feasible, research into the effect of informing young people of their carrier status on choice of marriage partner was carried out in the Arta area of Greece, where 20% of the population carry either the thalassaemia or sickle cell trait. All young people of marriageable age were screened and counselled, after which counselling contact was maintained for a two-year period. When the pattern of marriages was assessed at the end of this period, screening was found to have had no measurable effect on choice of partner (39).

In the same period, a similar approach was tried in Cyprus much earlier when marriages between carriers was actively discouraged. This approach proved unacceptable to the general public and was soon abandoned "because of evasions" (38). Once prenatal diagnosis became possible for thalassaemia, it was made available within the Cypriot health service. Soon afterwards, confidential premarital screening became mandatory among Greek Cypriots by the Greek Orthodox Church, and among Turkish Cypriots by the civil authorities. It was then found that 98% of at-risk couples detected just prior to marriage proceed to marry, even though Cypriot parents often have considerable influence on their children's choice of partner. Nevertheless, the annual number of new births of thalassaemia major children has fallen to almost zero in Cyprus because couples use the information on genetic risk in a variety of ways to obtain a healthy family. Less than 5% of the fall in thalassaemia major births is due to separation of engaged couples, while about 80% is due to prenatal diagnosis and selective abortion. The remainder is the result of at-risk couples having fewer children, on average, than couples not at risk (35). In both Cyprus and Sardinia, the population is now very well-informed and has gained confidence that local thalassaemia control programmes involve little if any coercion. There is popular demand for carrier testing in high schools, so that young people and their families can take information on carrier status into account at an earlier stage in settling on their choice of marriage partner (36). It will be of great interest to continue to follow the results, to find out if earlier screening does influence partner choice, in the Mediterranean area so far this has not seemed to have influenced choice.

More recently, mandatory premaritial screening for thalassaemia and sickle cell has been conducted in Saudi Arabia with the objective of decreasing at risk marriages. However, following counselling almost 90% of couples married despite being aware of their risk and being actively discouraged from marrying (41). The option of prenatal diagnosis is usually unavailable in the current Saudi Health System. Similarly mandatory screening is also carried out in the Palestinian territories

(42), where couples are also actively discouraged from marrying, even though couples have a right to marry if they wish. Iran has a programme of premaritial screening, but this follows 'informed choice' (see section 5.5). Mandatory screening does raise ethical questions as couples do not have a right to decide for themselves if they wish to be screened. However these countries believe that this method of screening is the most effective to decrease the incidence of thalassaemia.

In Canada, a programme of information and screening for carriers of Tay Sachs disease or thalassaemia in high schools in Montreal has proved highly acceptable, as well as highly informative about the pitfalls in attempting to foresee how people will use genetic information (43). A sample of the young people screened was followed up with a questionnaire that included questions such as, "Do you think that a couple planning to marry who found they were both carriers would change their marriage plans? Would you change your own marriage plans?" Interestingly, almost 80% thought that other couples would change their marriage plans but only 10% thought they would change their own plans. Clearly, it is all too easy to underestimate the importance of other peoples' inner life. Practical experience is the only reliable guide to how people are prepared to use information on genetic risk.

There therefore seems to be a strong case for early carrier diagnosis and genetic counselling, so that at-risk couples and their families can make an informed choice whether to separate or stay together. However, knowledge of a genetic risk seems to be insufficient to change the choice of a partner.

5.5 PRENATAL DIAGNOSIS

Services aimed at the prevention of haemoglobin disorders are not yet comprehensive, even in the most highly resourced countries. The follow-up of thalassaemia major children born despite the existence of prenatal diagnosis indicates that most births are not due to a rejection of prenatal diagnosis, but rather patients' and doctors' unawareness of risk and of the services available. Inadequate service delivery or no service availability is therefore a key ethical issue because it can deprive couples of the ability to make an informed choice.

Prenatal screening is steadily spreading around the world and is now available in several low resourced countries, some of which have also introduced carrier-screening programmes (44, 45). Experience of a number of large countries that have gone a long way towards implementing screening and prevention programmes, but which first had to understand and solve many practical as well as religious and ethical dilemmas.

5.5.1 IRAN

Iran, a large country in the Eastern Mediterranean region, has a comprehensive primary health care system capable of reaching every family, which incorporates regular in-service staff training. In 1996 the population of Iran was over 60 million, the majority of whom are Muslim. Depending on

the region, between 1.5-12% of the population carry ß-thalassaemia; consanguineous marriage is common and there are over 1,200 affected births per year.

The majority of affected children are diagnosed and every effort is made to provide the best possible treatment, significantly improving survival rates. As a result, the number of thalassaemic patients under treatment was increasing by about 1,200 per year with over 20,000 attending dedicated treatment centres. The present cost of optimal treatment (46) is about $150 million a year, equivalent to almost 8% of total national health expenditure (47). If the trend continued, future costs could rise to over half current national health expenditure. As this is obviously unfeasible, a national programme providing genetic screening and counselling was developed.

Following a careful pilot study, premarital thalassaemia screening was introduced in 1997 (48). The results were considered at the highest medical and political levels, where they were understood to represent the verdict of the people, and a fatwa was issued permitting termination of pregnancy up to 120 days foetal life, when a foetus is confirmed as having a serious disorder. For reasons of cost efficiency and to reduce the possible stigma for women, many centres test the man first, only testing the woman if his result is positive. Prospective couples that are both carriers see a trained health worker (usually a doctor) for counselling. The programme is conducted according to the ethical principles of autonomy, full information and confidentiality and allows informed choice.

Annual statistics on outcomes returned by district health centres to the Ministry of Health indicated that by the end of 2005 over 5,663,000 couples had been tested and over 27,514 at-risk couples have been identified and counselled. In the early years of the programme when prenatal diagnosis was only available through a private clinic in Tehran, 50% of couples identified at risk proceeded to marry. However since 2000 the figure has increased steadily to around 75%. This is thought to reflect the spread of the policy of non-directive counselling and knowledge of the availability of prenatal diagnosis. A national network of DNA diagnostic laboratories is available making prenatal diagnosis accessible to all within the national health system (25).

5.3.2 PAKISTAN

Where a primary health care system is well organised, as in Iran, it is possible to deliver genetic screening and counselling through primary care. Equally, however, it is still possible to develop imaginative and economical approaches to community-based genetic counselling in the absence of a strong primary health care system, as in Pakistan, by using techniques such as targeted screening of individuals in the extended family (49).

Thalassaemia is the most common single gene disorder in Pakistan, with more than 4000 births of affected children each year. Prenatal diagnosis was first introduced in Pakistan in Rawalpindi in 1994, by Dr Suhaib Ahmed (50). Before initiating the service, it was considered important to seek the views of religious scholars. Two renowned religious scholars in Pakistan gave a clear verdict permitting the termination of pregnancy before 120 days (i.e. at 17 weeks of foetal life or 19 weeks gestation), where the foetus is indicated as having a serious disorder (see above). Whether the time period specified refers to gestational or embryonic age is not clear, but in practice the upper limit

is recognised as the foetal age determined by ultrasound examination. Both scholars based their opinion on Quranic verse and the Hadith.

Since 1994 over 4000 prenatal diagnoses have been carried out for ß-thalassaemia (24) and personal communication), with 90% of women carrying an affected foetus terminating the pregnancy. Most couples opting against termination do so on religious grounds. However, a small proportion do not terminate because they cannot afford the cost of hospital admission, etc (50).

5.5.3 INDIA

India is a large Southeast Asian country with a population of over one billion. Most Indians are Hindu, with a large number of Muslims, as well as Buddhists, Sikhs, Christians and Parsees. An estimated 1-3% of the population are carriers of ß-thalassaemia, a figure rising to up to 17% in some ethnic groups (51, 52). About 6,000 children are born with thalassaemia major each year – more than 30% of total annual thalassaemia major syndrome births in the region (Prof Bernadette Modell, personal communication).

The treatment of thalassaemia in India was until recently a picture of despair. A lack of planning compounded by an unwillingness to face the problem means there is no national prevention programme and a large number of patients face difficulties in securing long-term management. Families face the burden of trying to meet the high cost of treatment, often amounting to 20-30% of family incomes (53), while inadequate treatment has led to the early death of many affected children.

In recent years, both government agencies and non-governmental organisations (NGOs) have initiated programmes to deal with the problem, although there is still no coordinated national thalassaemia control policy. In 1988 a thalassaemia control programme was established at the Bai Jerbai Wadia Hospital for Children, a public charitable hospital in Bombay, in collaboration with the Perinatal Haemoglbinopathy Genetics Centre of University College Hospital, London, with financial assistance from the UK government's Overseas Development Administration (ODA, now DfID). This programme has conducted prenatal diagnostic procedures – mostly retrospective – by chorionic villus sampling and foetal blood sampling, using both molecular diagnosis techniques and traditional technology of globin chain synthesis and column chromatography (54). It is estimated that in India, over 34,000 prenatal diagnoses performed over 18 years by several centres around the country providing the service on a private basis. (Dr. PG Natrajan, personal communication).

Non-governmental organisations such as the "We Care Trust" have also started working exclusively in the area of screening, offering tests at very low cost. Efforts such as these have helped produce a gradual but definite shift in medical practitioners' attitudes towards screening for thalassaemia, particularly amongst obstetricians. This in turn is likely to improve the chances of identifying at-risk couples in their first pregnancies.

A strategy aimed at preventing marriages on the basis of premarital screening in not considered a realistic ethical option in India because of the stigma such a policy would impose on carrier women, therefore parents are less inclined to test their daughters because of the possibility of not being

able to find a suitable partner. Premarital testing for thalassaemia has been carried out in extended family members identified via an affected index case, unmarried adult cases of anaemia attending the outpatient department, and adult college students (55). 99% of at risk couples married even after having the knowledge of their risk status and opted for prenatal diagnosis. Therefore premarital screening seems not to be a viable option in India. The most successful policy should aim at screening prior to reproduction or even very early in pregnancy.

For much of the population, illiteracy, limited access to information, social and religious taboos and family influences still hamper couples from seeking PND, even when they already have children with thalassaemia major. It is in this context that genetic counselling was found to be important for couples selecting options for prenatal diagnosis (53). A survey conducted during the 1998 thalassaemia control programme showed that once a couple was counselled and understood their risks, and prenatal diagnosis was discussed with them, they accepted selective termination of an affected pregnancy – a finding that held across social, religious and economic groups. This indicates that it is possible to establish an effective prevention programme if a message can be extended to the relevant population, and that education, counselling and the availability of facilities for prenatal diagnosis are key elements in any such programme.

Creating awareness through popular media such as television, which reaches a large proportion of the Indian population in both urban and rural areas, could play a key role in any future control strategies, as well as establishing counselling centres and a few – well run – prenatal diagnostic centres. In time, efforts to control the constantly rising burden on an already over-stretched infrastructure would also begin to improve the treatment of patients with thalassaemia currently seeking help.

5.6 CONSANGUINEOUS MARRIAGE

The question of how to provide genetic counselling in societies that favour consanguineous marriage can pose a dilemma for families and health workers alike. The ideal genetics programme must include a sensitive and realistic approach to the issue.

Over 20% of the world's population live in communities that favour cousin marriage. Worldwide, at least 1 in 12 children are born to parents who are related (56). Cousin marriage has been customary in many parts of the world for thousands of years. Although the practice is often associated with the Muslim religion, the custom is also prevalent in other cultures. Indeed, a practice that has been so common for so long obviously has important social functions. Currently in Northern European populations about 0.5% of marriages are between first cousins.

The great majority of families where the parents are related suffer no adverse effect. The reported increase in average childhood mortality and morbidity in such populations is largely due to relatively severe effects in a limited number of families, which shift the average figures for the group as

a whole. Therefore medical attempts to help families to reduce genetic problems should focus on identifying families at particularly high risk and providing them with genetic counselling and access to appropriate services.

Nevertheless, efforts to discourage consanguineous marriage have been made in some countries, using public information programmes that emphasise associated genetic risks. Such programmes tend to arise from the perception that recessively inherited diseases (especially rare and unusual types) are unusually common in these populations and that the need for prevention is urgent, while appropriate genetic counselling services are rudimentary or non-existent. It may then seem that altering the behaviour of the population is the only possible way to reduce the incidence of genetic disease.

In view of our ignorance of the social causes and consequences of customary consanguineous marriage, attempts to reduce its frequency on genetic grounds run the risk of doing more harm than good, disturbing customary marriage arrangements when the majority of families would have suffered no ill-effects from such pairings. Furthermore, the concept of non-directive genetic counselling is incompatible with a campaign against consanguineous marriage. Policies relating to consanguineous marriage should be firmly grounded in an understanding of the social role of the practice, and the possible consequences of attempts to disturb it.

Discussions with families in the UK who have faced pressure against consanguineous marriage (56, 57)), led to the following conclusions:

- Pressure against cousin marriage rarely alters what people actually do, although it can make them feel uncomfortable about it
- If people are told their children are sick because they are related, it causes great unnecessary distress, may alienate them and makes it more difficult for them to understand the real explanation
- Where cousin marriage is common, people are aware that most couples of cousins have perfectly healthy children. If they are told not to marry a cousin because their children may be sick, they may become confused and could lose confidence in medical advice
- Avoiding cousin marriage does not guarantee that children will not have a congenital disorder. Unrelated couples who have affected children may lose confidence in medical advice
- People do not attend for genetic counselling if they think they will be criticised and their cultural conventions attacked

Why is cousin marriage favoured in some communities?

Many non-European communities have a patrilineal kinship pattern (family name and property are inherited in the male line). The men and their descendants tend to stay together, especially when the family owns land and members of the extended family share responsibilities for each other. At marriage, women leave their family to enter their husband's family. Cousin marriage can soften the implications of this transition and contribute to family well-being by strengthening the woman's position within the family and promoting female networks. The following are conclusions presented by A. Darr and B. Modell at a meeting on 'Consanguineous marriage in the UK: A multidisciplinary strategy', held at the Royal Society of Medicine:

- Parents are able to remain closer to their children, particularly their daughters, when they marry a relative
- A woman is likely to be comfortable with a mother-in-law who is also an aunt she has known since childhood
- There is increased financial security, because if one partner dies the remaining partner is still a member of the family in their own right
- Equal numbers of sons and daughters are needed within the extended family, so a daughter is not seen as a burden
- In societies that practice segregation between the sexes, young people can get to know each other before marriage more easily if they are related
- Expenses and exchange of property associated with marriage may be reduced

A convention of cousin marriage makes family-oriented genetic counselling particularly effective, as unusually large numbers of carriers of the presenting disorder may be detected within the family. Thus carrier testing may permit early detection of many individuals at risk for conditions such as thalassaemia and sickle cell, as well as identifying newly-affected individuals where surveillance and early treatment could be beneficial. Additionally when cousin marriage is common within the family, carriers are at particularly high risk of making an at-risk marriage. Family studies following the diagnosis of a child with a disorder will identify many carriers, who should then be offered counselling about their reproductive risk. Many will already be married, and some of these may be at-risk couples, identified in time for prospective reproductive counselling. Many will be children or not yet married, and early information might permit carrier status to be taken into account to avoid further at-risk marriages within the family. This approach has been tested in Pakistan (49).

5.7 GENERAL POINTS AND CONCLUSIONS

The moral problem of genetics is based on the fact that since what is known cannot be unknown. This fact places a responsibility on parents and the medical services. The medical profession's responsibility starts from the time carrier screening, genetic counselling and prenatal diagnosis becomes available, and from then on a new social responsibility is generated by every birth of an affected child. This responsibility can only be discharged by handing it on to the parents through adequate information and genetic counselling.

However the ethical principles governing carrier screening, genetic counselling and prenatal diagnosis need to be assessed for each country, taking into account local social and religious structures and the views of the at risk population.

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CHAPTER 06

Mary Petrou and Eric Jauniaux

FOETAL SAMPLING FOR THE PRENATAL MANAGEMENT OF HAEMOGLOBINOPATHIES

Couples identified as being at risk should be counselled by a specialised genetic counsellor well-versed in the genetic and molecular aspects of the haemoglobinopathies and their haematological management. Counselling should be non-directive, aiming to provide enough information to the individual or couple for them to make their own informed reproductive choices (see Chapter 5). Details of prenatal diagnosis, including foetal sampling, and the risk of miscarriage following the procedure or of failure and misdiagnosis should be clearly discussed with the individual or couple concerned.

The most commonly used methods of foetal sampling for prenatal diagnosis are chorionic villus sampling (CVS), amniocentesis and foetal blood sampling, each of which requires expert ultrasound guidance. Currently, only CVS gives reliable results during the first trimester of pregnancy and it is the preferred sampling method for DNA analysis of the haemoglobinopathies.

6.1 CHORIONIC VILLUS SAMPLING (CVS)

The aim of CVS is to obtain a small sample of the developing placenta, which has the same genetic make-up as the foetus. CVS was originally carried out using either forceps or a catheter passed through the uterine cervix, from 10-11 weeks' gestation (1).

More recently, transabdominal CVS has been introduced in clinical practice. This method involves obtaining a biopsy of the placenta by means of a needle inserted through the abdominal wall under ultrasound guidance (Figure 6.1). A small sample of chorionic villus material is then aspirated. The technique can be used at any stage of pregnancy from 11 weeks onwards.

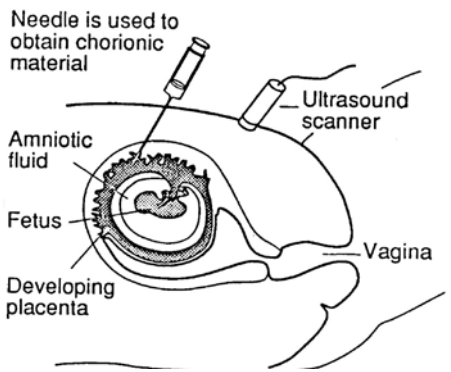

Figure 6.1: Transabdominal CVS: The needle may be inserted through the abdominal wall and into the placenta under ultrasound guidance

The transabdominal method of sampling is in increasing use at prenatal diagnosis centres throughout the world. With the advent of high-resolution ultrasound, transabdominal CVS has been routinely performed from 6 weeks of gestation until the third trimester, using the freehand ultrasound-guided single needle aspiration technique. Several refinements have been proposed in the last decade, including the use of biopsy forceps or a double needle system.

CVS has great advantages because it can be done in the first trimester of pregnancy, i.e. within the limits laid down by abortion law in many countries. This also reduces the emotional stress associated with late prenatal diagnosis and the risk of complications where a late termination of pregnancy is required. In addition, parents' privacy is protected as the entire procedure can be carried out before others are aware of the pregnancy. CVS also has technical advantages, as the technique obtains many more foetal cells than are obtained through other sampling procedures. DNA methods usually produce a result within a few days.

Studies of the additional risk of foetal loss associated with CVS have given divergent results, ranging from 0.5-4.5%. However, there is little doubt that CVS procedure-related accident is related to operator experience, in particular the number of attempts needed to obtain a sufficient villus sample (2, 3). In short, CVS requires an expert team and in experienced hands the foetal loss rate appears to be 0.5-1% (4). There is no evidence of an increase in obstetric complications. Vaginal spotting or bleeding is the most common immediate complication following a CVS procedure (1-4% of cases) and is often observed after a transcervical sampling (in up to 20% of cases) (1). Direct vascular injury of small branches of the utero-placental or umbilico-placental circulation may also lead to a retro-placental haematoma and/or a subchorionic haemorrhage and subsequently to a miscarriage. The formation of an haematoma inside the gestational sac often becomes apparent during or within a few minutes after the procedure. Because CVS involves puncturing the amniotic sac, oligohydramnios is another, albeit rare, complication associated with the technique. Intrauterine infection and chronic amniotic fluid leakage are two other possible complications, occurring a few days to 3 weeks after the procedure. Although intrauterine infection after CVS is extremely rare (<0.1% of cases), it is a serious complication that can lead to maternal septic shock and requires immediate evacuation of the uterine contents. Active vaginal infection is a contra-indication for transcervical invasive procedure. In early series, up to 30% of catheters used for the procedure were reported to be colonised by bacteria and there was some concern about secondary intrauterine infection (5, 6). However, the rate of foetal loss is not directly associated with bacterial colonisation of the cervix and life-threatening infections have not been encountered since single catheters ceased to be used for repeated insertions.

The possibility of an increased risk of limb malformation has been widely discussed (7, 8). The background frequency of limb reduction defects in the general population is low, between 0.04-0.06% of births (9). The findings of several pieces of research indicate that very early CVS may be associated with a 10-fold increase in the overall incidence of limb reduction defects and oromandibular-limb hypogenesis. The spectrum of limb defects after CVS reported by Firth et al. (10) seems to be more severe than the limb defects seen in the general population and directly related to the timing of CVS during pregnancy. It must be stressed, however, that the larger the series the lower the risk of limb

defect identified (see Table 1). A World Health Organisation (WHO) international register of post-CVS complications, including limb defects, indicated that from May 1992 to May 1994, 77 foetuses or infants were reported to have defects of the lower or upper limbs, out of a total 138,996 pregnancies involving CVS (9). These figures correspond to the distribution of limb defects found in several large population-based studies, suggesting that there is no difference in the overall frequency or distribution pattern of limb defects between the background population and after CVS. There was also no correlation between gestational age at CVS and severity of defects. Any increased risk after the beginning of the 9th week of gestation has been virtually excluded (10, 11), but there is evidence of an association between some foetal malformations and very early sampling (before the 8th completed week from the LMP) (10, 11, 12). It is therefore recommended that CVS be carried out after the beginning of the 9th week of gestation at the earliest, and preferably from 101/2 weeks' gestation.

6.2 AMNIOTIC FLUID SAMPLING

Mid-second trimester (15-19 weeks) amniocentesis was introduced in the 1960s and is still the most commonly used invasive technique for the prenatal diagnosis of genetic defects of the foetus. Worldwide acceptance of the technique has rapidly increased with the improved safety of so-called "classical amniocentesis" when performed under ultrasound guidance. Amniocentesis is conventionally done from 16 weeks' gestation onwards. The procedure involves withdrawing 15-20ml of amniotic fluid from the amniotic cavity surrounding the foetus, using a small needle inserted through the abdomen under continuous ultrasound guidance (Figure 6.2).

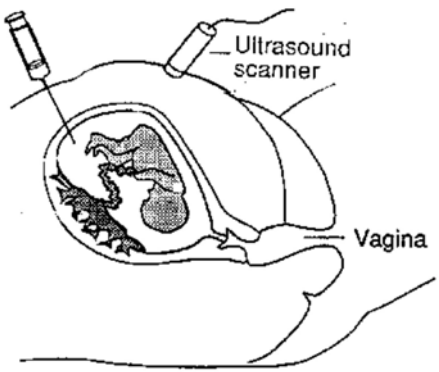

Figure 6.2: Amniotic fluid is obtained by transabdominal needling

The procedure-related risks of amniotic fluid sampling at 16 weeks have been reported to range between 0.5-1%, but are probably much lower in experienced centres. The introduction of real-time ultrasonography, enabling the operator to continuously visualise the needle tip to avoid direct damage to the foetus, should now be standard practice (13). A Danish randomised controlled trial conducted at a centre with considerable experience in ultrasound-guided procedures demonstrated that mid-trimester amniocentesis was associated with a 1% risk of foetal loss (14). However, most data on the risks associated with amniocentesis were published before the era of evidence-based medicine and randomised trials. Furthermore, centres of excellence providing these data had considerable experience in performing ultrasound-guided invasive procedures. So far there has been little information on the risk of routine amniocentesis by operators with limited ultrasound experience performing a small number of procedures each year.

A recent cohort study of more than 2,000 amniocentesis procedures performed at 15-16 weeks by experienced operators using continuous ultrasound guidance showed no difference in foetal loss rates and premature delivery compared to gestational age-matched controls who did not have amniocentesis (15). This indicates that the risks of complications after amniocentesis can be minimal when the procedure is carried out by competent and experienced staff. If the placenta can be avoided during the procedure, the risk of complication is mainly linked to the risk of premature rupture of membranes. Compared to spontaneous rupture of the membranes, pregnancies complicated by rupture of the sac following amniocentesis have a better perinatal outcome (16).

First trimester amniocentesis is considered to carry a higher risk of miscarriage than mid-trimester amniocentesis. Early amniocentesis is also associated with a slight increase in the risk of respiratory distress and common mild, correctable orthopaedic deformities such as clubfoot (17).

The main disadvantage of amniocentesis is the lateness of diagnosis. Amniotic fluid samples also often do not yield a sufficient amount of DNA for diagnosis, requiring culturing of cells to obtain sufficient cells for analysis, which delays the foetal diagnosis result. Such delays are unacceptable for most patients, particularly if the foetus is affected and a termination of pregnancy is considered, as diagnosis may not be reached until the 20th week or even later.

6.3 FOETAL BLOOD SAMPLING (FBS)

This procedure can be safely carried out after 20 weeks of pregnancy. A needle is passed through the abdominal wall under continuous ultrasound monitoring and inserted into the placental insertion of the umbilical cord, and a small sample of foetal blood withdrawn (see Figure 6.3).

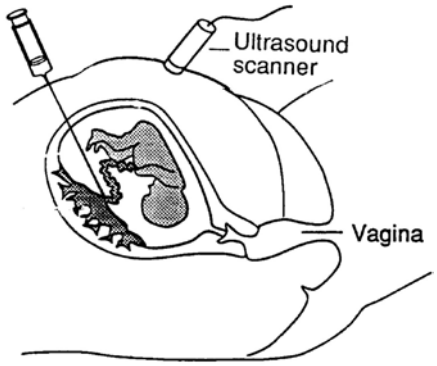

Figure 6.3: Foetal blood sampling

Nearly two decades ago Daffos et al (18) introduced a new sonographically-guided technique for obtaining pure foetal blood, which rapidly superseded foetoscopy or foetal scalp sampling. Due to the small size of the umbilical vessels earlier in gestation, cordocentesis or percutaneous umbilical cord sampling are currently performed during the second half of gestation, with a foetal loss rate of 1-2% (19). Foetal blood sampling is associated with late diagnosis and requires a high level of expertise. Foetal blood sampling is now rarely used to obtain a sample of foetal blood at a relatively late stage of pregnancy for rapid DNA analysis or globin chain synthesis.

6.4 TECHNICAL CONSIDERATIONS

From 10 weeks' gestation, the uterus is more stable and tends to rotate less on attempting to insert the needle through the myometrium. The double needle system produces samples of better quality, larger quantity and is probably less traumatic than single needle or trancervical catheter aspiration. Biopsy forceps produce clean atraumatic samples and have been used increasingly for transcervical CVS. For both amniocentesis and CVS, the transabdominal route is more practical than the transcervical route and is associated with less secondary bleeding. In cases of a posterior placenta or an obese patient, the transcervical approach is probably safer and should be considered for a CVS. Conversely, if an amniocentesis is planned and the placenta is anterior and obstructing, a transabdominal CVS would be more appropriate and probably safer. Operator experience and/or the expert use of ultrasound imaging should be evaluated for each individual centre offering foetal sampling, in order to precisely determine the risks associated with each technique at each centre.

6.4.1 SAFETY OF CVS COMPARED AMNIOCENTESIS

Most clinical trials on the safety of invasive procedures during pregnancy have compared the outcome of CVS in the first trimester with second trimester amniocentesis; it is only recently that data on early amniocentesis have been incorporated. According to the first analyses (20, 21), CVS resulted in a 0.5-4% increase in the risk of post-procedure pregnancy loss compared to late amniocentesis. Considerable differences in study design and, in particular, in the number and experience of operators involved, size of study groups and sampling approaches make comparison and interpretation of these data difficult. In most of these studies, the sampling procedure failure rate is at least three times higher for CVS than for amniocentesis, which is directly related to operators' greater experience in performing mid-trimester amniocentesis than CVS. Furthermore, the spontaneous miscarriage rate between the CVS period (9-12 weeks) and the amniocentesis period (15-17 weeks) is at least 3% and should be clearly indicated in the final statistics and correlated not only with gestational age but also maternal age. Other major bias includes the definition of post-procedure foetal loss, which includes complicated cases from 3 weeks after the procedure to any lethal complications up to 28 weeks of gestation. Partially randomised trials comparing early amniocentesis with CVS performed at the same gestational age period have demonstrated that early amniocentesis is associated with approximately 3% higher pregnancy loss than CVS performed at the same gestational age (22, 23).

6.4.2 MULTIPLE GESTATIONS

Twin pregnancies are more common in certain areas of West Africa where there is also a high incidence of haemoglobinopathies. In the absence of randomised studies, it is not possible to estimate accurately the excess risk following CVS in twins. Currently available data show similar overall pregnancy loss rates for both amniocentesis and CVS, with the excess risk of around 1% above the background risk (24). The foetal loss rate after amniocentesis in twins was evaluated by a 16- year retrospective cohort study of women with twin pregnancies who underwent mid-trimester amniocentesis compared to controls who did not have an invasive procedure (25). The authors found a post-procedure pregnancy loss before 24 weeks of gestation of 1.8% (1 in 56) and they concluded that the procedure –related risk is higher than what one would empirically estimate by doubling

the associated risk rate in singleton pregnancies. A recent analysis of published data has demonstrated a pooled amniocentesis-related loss rate of 3.5% in twin pregnancies at <24 weeks (26). There is no statistically significant difference in foetal loss between the single versus double uterine entry amniocentesis (24). Few obstetricians are trained or reluctant to offer a selective reduction after 14 weeks as the risk of post-procedure pregnancy loss increases with advancing gestational age. Thus an early diagnosis with CVS is a major advantage in cases of twins discordant for an haemoglobinopathy.

6.4.3 ADVANTAGES AND DISADVANTAGES OF FOETAL SAMPLING TECHNIQUES

The advantages and disadvantages of techniques that are commonly used to obtain samples of foetal cells for prenatal diagnosis are summarised in Table 6.1 below.

Table 6.1: A summary of the main advantages and disadvantages of current foetal sampling methods

Method	Success rate, %	Timing (weeks from LMP)	Time to DNA diagnosis	Foetal Loss Rate[1]
CVS	>99	10 onwards	48 hours	1%
Amniocentesis	>99	15 onwards	2-16 days[2]	0.5-1%
Foetal Blood Sampling	>95	20 onwards	72 hours	2%

Note [1] : At expert centre

Note [2] : May require cell culture

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CHAPTER 07

John Old

CONVENTIONAL PRENATAL DIAGNOSIS

The haemoglobinopathies were the first genetic diseases to be characterised at the molecular level and for more than 30 years have been used as a prototype for the development of new techniques of mutation detection and approaches for the prenatal diagnosis of single gene disorders. Prenatal diagnosis of thalassaemia was first achieved by the measurement of the ratio of newly synthesised α-globin to ß-globin chains in foetal blood. This approach was widely used from 1975 to 1980, with more than 13,000 cases being performed successfully worldwide, despite the disadvantages that foetal blood sampling carried a high risk of foetal loss if not performed at an expert centre, and that the procedure could not be carried out until 18 weeks of gestation. However once the common ß-thalassemia mutations had been characterised by DNA cloning and sequencing of ß-globin genes for thalassaemic patients, the prenatal diagnosis of ß-thalassaemia switched from globin chain synthesis to the molecular analysis of foetal mutations, first in amniotic fluid DNA, and then in chorionic villus DNA following the development of the procedure of chorionic villus sampling. The discovery of the polymerase chain reaction technique led to the rapid development of a number of molecular diagnostic techniques which were applied for prenatal diagnosis, including allele specific oligonucleotide (ASO) hybridisation or dot blot analysis, reverse dot blot analysis (RDB), the amplification refractory mutation system (ARMS), gap-PCR, restriction endonuclease analysis and direct DNA sequencing (1).

These techniques are not discussed here as each of the above molecular diagnostic methods are described in some detail in the accompanying volume to this one (Prevention of Thalassaemia and other Haemoglobin Disorders, 2nd edition - Volume 2: Laboratory Protocols. This chapter concentrates on the different haemoglobin disorders that feature in prevention programmes and the strategies for the conventional prenatal diagnosis of these disorders by mutation detection in foetal DNA. Although a brief description of the different types of globin gene mutations is presented here, a full list of all the known α- and ß-thalassaemia point mutations is contained in tables in the Annexe to this book. Each at risk population has its own particular spectrum of ß-thalassaemia mutations, and therefore as an aid to prenatal diagnosis, the Annexe also contains tables listing the frequencies of the ß-thalassaemia mutations observed in countries in the Mediterranean, Middle Eastern, Asian and Southeast Asian regions.

7.1 WHICH MOLECULAR DIAGNOSTIC TECHNIQUES ARE BEST FOR PRENATAL DIAGNOSIS?

The variety of techniques for the DNA-based diagnosis of the haemoglobinopathies creates a problem for laboratories wishing to start molecular analysis of the globin gene mutations: what is the best method to adopt for the diagnosis of ß-thalassaemia? For the more common non-deletional mutations, the RDB hybridisation and the ARMS techniques are still widely practised methods despite being twenty years old. Both offer a rapid, cheap and convenient method to test for multiple mutations. Whether one chooses the reverse dot blot method or the ARMS approach will depend on a number of factors – for example, the range of mutations to be diagnosed, the ability to develop one's own ARMS primers, or the need to need to use a certified procedure available in a commercial kit, such as the RDB approach. The laboratory also needs to become proficient in a number of other techniques such as gap-PCR and MLPA for large deletion mutations.

For the identification and diagnosis of rare or novel point mutations, automated DNA sequencing is the gold standard choice, and DNA sequencing provides the second method of mutation identification required for the confirmation of a prenatal diagnosis. A foetal diagnosis result should be confirmed by a different diagnostic approach whenever possible according to best practice guidelines (see section 7.6), and therefore a prenatal diagnostic laboratory needs to have several different mutation detection techniques available, including techniques to check for maternal contamination of foetal DNA samples. Finally a haemoglobinopathy diagnostic laboratory should also network with reliable haematology laboratories to have access to certain haematological procedures such as the quantification and characterisation of Hb variants by HPLC and other relevant electrophoretic techniques, such as isoelectric focussing, in order to fully interpret the genotype/phenotype relationship in complex diagnostic cases.

Therefore, the answer to the question is that each laboratory must carry out preliminary studies utilising a number of these approaches and work out which techniques are best suited to its needs and the mutations that need to identified in the target population.

7.2 HAEMOGLOBIN DISORDERS REQUIRING PRENATAL DIAGNOSIS

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

7.2.1 THALASSAEMIA MAJOR

The majority of individuals homozygous for ß-thalassaemia have the transfusion-dependent condition called ß-thalassaemia major. At birth, ß-thalassaemia homozygotes are asymptomatic because of the high production of HbF but as this declines, affected infants present with severe anaemia during the first or second year of life. Treatment 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 ß-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 progress at last with research into gene therapy and a clinical trial is now underway (2), bone marrow transplantation remains the only cure for ß-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.

ß-thalassaemia is caused by more than 180 different point mutations or small insertions/deletions of DNA sequence in and around the ß-globin gene, plus more than 28 known gene deletions ranging from 25bp to 67kb in size (see Annex II). A description of each mutation, can be found on the Globin Gene Server database (3) at http://globin.cse.psu.edu. The mutations either reduce the expression of the ß-globin gene ($ß^+$-type) or result in the complete absence of ß-globin ($ß^0$ -type).

ß-thalassaemia major results from either the homozygous state for a $ß^0$ -type or severe $ß^+$-type mutation or, more commonly, from the compound heterozygous state for two different $ß^0$ or severe ß+ mutations. The mutations are regionally specific; Annex II lists the ß-thalassaemia mutations found in countries from the four main regions – the Mediterranean, Middle East, Asia and Southeast Asia. Each country within a region has its own characteristic spectrum of mutations, with a small number of mutations usually accounting for more than 90% of the total.

7.2.2 ß-THALASSAEMIA INTERMEDIA

Some individuals homozygous for ß-thalassaemia have a milder clinical condition called thalassaemia intermedia.Such patients present later in life relative to those with thalassaemia major 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 (4). 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 with Hb levels of 8-10g/dl. However, even these milder patients tend to accumulate iron with age and many thalassaemia intermedia patients develop clinical problems relating to iron overload after the third decade (5). Prenatal diagnosis is often requested by couples at risk of having a child with thalassaemia intermedia due to the unpredictability of the phenotype, especially in cases in which one partner carries a severe ß-thalassaemia mutation.

Such patients may present later in life relative to those with thalassaemia major and are able to survive without transfusions. 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 ß-thalassaemia mutation, but also in some cases where both partners carry mild mutations.

A few $ß^{+}$-thalassaemia mutations that are associated with an unusually mild phenotype are sometimes designated $ß^{++}$-type (see Annex II). Thalassaemia intermedia patients that are homozygous for a $ß^{++}$-type mutation usually have a very mild disorder and prenatal diagnosis is not normally indicated for this genotype. However, the combination of a mild $ß^{++}$ and a severe $ß^{+}$ or $ß^{0}$ mutation results in a severe disorder in some cases and predicting the phenotype of thalassaemia intermedia on the basis of a known genotype remains a challenge. Other genotypes causing thalassaemia intermedia include the compound heterozygosity for a ß-thalassaemia mutation with either δß-thalassaemia trait, Hb Lepore trait or a triple /quadruple α-gene locus. It also results from the heterozygous state for one of the rare ß-thalassaemia mutations in exon 3 that cause inclusion-body haemolytic anaemia, sometimes called dominantly inherited ß-thalassaemia. These mutations are listed in Annex II. Finally, co-inheritance of $α^{0}$-thalassaemia trait, homozygous $α^{+}$-thalassaemia, or the HPFH determinant, the Gγ –158 (C→T) mutation, or the more recently identified genetic modifiers (see Chapter 5 this volume) can ameliorate a thalassaemia major genotype to the phenotype of thalassaemia intermedia.

Table 7.1 Beta thalassaemias and beta globin gene disorders - interactions and indications for prenatal diagnosis and preimplantation genetic diagnosis.

Genotype interaction	Clinical Phenotype	DNA Diagnosis	PND indicated
homozygous			
$ß^{0}$ or severe $ß^{+}$-thal	Thal major	ASO, ARMS, sequencing	Yes
Mild $ß^{+}$-thal	Thal intermedia	ASO, ARMS, sequencing	Occasionally*
Mild $ß^{++}$-thal (silent)	Very mild thal intermedia	ASO, ARMS, sequencing	No
δßo -thalassaemia	Thal intermedia	Gap-PCR, MLPA	Occasionally*
Hb Lepore	Thal intermedia to major (variable)	Gap-PCR, MLPA	Occasionally*
HPFH	Not clinically relevant	Gap-PCR, MLPA	No
HbC	Not clinically relevant	ASO, ARMS	No
Hb D-Punjab	Not clinically relevant	RE-PCR, ASO, ARMS	No
HbE	Not clinically relevant	ASO, ARMS	No
Hb O-Arab	Not clinically relevant	RE-PCR, ASO, ARMS	No
Compound heterozygous			
ßo/severe $ß^{+}$-thal	Thal major	ASO, ARMS, sequencing	Yes
Mild $ß^{+}/ß^{0}$ or severe $ß^{+}$-thal	Thal intermedia to major (variable)	ASO, ARMS, sequencing	Occasionally*
Mild $ß^{++}/ß^{0}$ or severe $ß^{+}$-thal	Mild thal intermedia (variable)	ASO, ARMS, sequencing	Occasionally*

Genotype interaction	Clinical Phenotype	DNA Diagnosis	PND indicated
Mild β^{++}/β^{0} or severe β^{+}-thal	Mild thal intermedia (variable)	ASO, ARMS, sequencing	Occasionally*
$\delta\beta^{0}/\beta^{0}$ or severe β^{+}-thal	Thal intermedia to major (variable)	Gap-PCR, MLPA / as for β+/βo-thal	Occasionally*
$\delta\beta^{0}$/mild β^{+}-thal	Mild thal intermedia	Gap-PCR, MLPA, as for β+/βo-thal	Occasionally*
$\delta\beta^{0}$/Hb Lepore	Thal intermedia	Gap-PCR, MLPA,	Occasionally*
Hb Lepore/β^{0} or severe	Thal major	Gap-PCR, MLPA / as for β+/βo-thal	Yes
β^{+}-thal			
HbC/β^{0} or severe β^{+}-thal	β-thal trait to intermedia (variable)	ASO, ARMS, sequencing	Occasionally*
HbC/mild β^{+}-thal	Not clinically relevant	ASO, ARMS, sequencing	No
Hb D-Punjab/β^{0} or severe β^{+}-thal	Not clinically relevant	RE-PCR, ASO, ARMS, sequencing	No
HbE/β^{0} or severe β^{+}-thal	Thal intermedia to major (variable)	ASO, ARMS, sequencing	Yes
Hb O-Arab/β^{0}-thal	Severe thal intermedia	RE-PCR, ASO, ARMS, sequencing	Yes
αα/β^{0} or severe β^{+}-thal	Mild thal intermedia	Gap-PCR, MLPA / as for β+/βo-thal	No
αααα/β^{0} and αααααα/β^{0}-thal	Mild to severe thal intermedia (variable)	Gap-PCR, MLPA / as for β+/βo-thal	Occasionally*

* Occasionally, depending upon patient choice following genetic counselling

7.2.3 Hb BART'S HYDROPS FETALIS SYNDROME

The most severe form of α-thalassaemia is the homozygous state for αo-thalassaemia, known as Hb Bart's hydrops fetalis syndrome. This condition results from a deletion of all four globin genes and an affected fetus cannot synthesise any α-globin chains to make HbF or HbA. Fetal blood contains only the abnormal haemoglobin Bart's (γ_4) and a small amount of Hb Portland (which can facilitate the application of prenatal diagnosis based on HPLC analysis of foetal blood). Without intrauterine 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 toxaemic complications that occur frequently in pregnancy with hydropic fetuses, which endanger the pregnant mother (6).

α^{0}-Thalassaemia results from large deletions involving both α-globin genes in the α-globin gene cluster. At least 32 different deletions have been described, although many are extremely rare or have been observed on just one occasion. The deletions that have attained the highest gene frequencies are those found in individuals from Southeast Asia and South China (the $--^{SEA}$ deletion mutation), the Philippine Islands ($--^{FIL}$), Thailand ($--^{THAI}$) and some Mediterranean countries such as Greece and Cyprus (the $--^{MED}$ and $-(\alpha)^{20.5}$ deletions). Although one α^{0}-thalassaemia mutation ($--^{SA}$) has been described in Indians, it is extremely uncommon. No α^{0}-thalassaemia deletions have been reported in individuals from sub-Saharan Africa. In Northern Europe α-thalassaemia has been observed only sporadically because of the lack of natural selection, although one particular α^{0}-thalassaemia mutation ($--^{BRIT}$) has been reported in a number of British families, probably through a founder effect.

7.2.4 HbH HYDROPS FETALIS SYNDROME

HbH patients with nondeletional α+-thalassemia exhibit more severe symptoms than those with deletional α+-thalassemia, and may require recurrent blood transfusions and splenectomy (7). In rare cases, the interaction of α°-thalassemia with a severe type of non-deletion α+-thalassemia allele can lead to HbH hydrops fetalis syndrome, eg the interaction of the $--^{SEA}$ allele with the hyperunstable α-globin variant Hb Adana (8). In these families prenatal diagnosis is indicated.

Table 7.2 Alpha thalassaemias - interactions and indications for prenatal diagnosis and preimplantation genetic diagnosis.

Genotype interaction	Clinical Phenotype	DNA Diagnosis	PND indicated
homozygous			
α°-thalassemia (--/--)	Hb Bart's hydrops fetalis	Gap-PCR, MLPA	Yes
α+-thalassemia (-α/-α)	Not clinically relevant	Gap-PCR, MLPA	No
α+-thalassemia ($\alpha^T\alpha/\alpha^T\alpha$)	Severe α-thal carrier to severe HbH disease	ASO, sequencing	Occasionally*
Compound heterozygous			
α°-thal/α+-thal (--/-α)	HbH disease	Gap-PCR, MLPA	No
α°-thal/α+-thal ($--/\alpha^T\alpha$)	Severe Hb disease to HbH hydrops fetalis	Gap-PCR, MLPA	Occasionally*

* Occasionally, depending upon patient choice following genetic counselling

7.2.5 SICKLE CELL DISEASE

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% (see Fig 1.2). The course of the illness is very variable, even within individual sibships let alone different racial groups. Sickle cell disease results not only from homozygosity for the sickle cell gene, HbS/S, but also from a number of different compound genotypes, most commonly HbS/ß° thalassemia, HbS/ß+ thalassemia, HbS/δß thalassemia, HbS/HbC, HbS/Hb D-Punjab and HbS/Hb O-Arab. A few rare ß-chain variants have also been found to cause sickle cell disease in the compound heterozygous state with HbS in one or two reported cases, namely Hb Quebec-Chori, Hb C-Njamena and Hb O-Tibesti.

There are also 11 other sickling Hb variants (listed in Table 2.1b in Volume 2: Laboratory Protocols), consisting of the Hb S mutation and an additional amino acid substitution in the ß-globin chain, and which potentially will result in sickle cell disease in the homozygous state, although no such cases have been reported. Two of these, Hb S-Antilles and Hb S-Oman, having 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 HbS or HbC: namely Hb C-Harlem, Hb S-Southend and Hb S-Antilles.

HbSS. In patients homozygous for HbS, the variable clinical expression is partly explained by the interaction of other factors, including genetic factors that can be co-inherited with the sickle cell gene, although it is not possible to predict accurately how the disease will be manifest in any one

individual. The main modifying genetic factors include α-thalassaemia and genetic determinants that increase HbF, such as the –158Gγ C→T polymorphism linked to the Arab-Indian and Senegal haplotypes. The HbF level is usually around 5-10% but can approach up to 30%. Different HbF levels are associated with homozygotes for different ß-globin gene haplotypes: Cameroon (5-6%), Benin and Bantu (6-7%), Senegal (7-10%) and Arab-Indian (10-25%).

HbS/ß thalassaemia. The clinical course of sickle cell ß-thalassaemia is very variable, ranging from a disorder identical to sickle cell anaemia to a completely asymptomatic condition. The Hb concentration varies from 5g/dl to within the normal range. The heterogeneity is mostly due to the type of ß-thalassaemia mutation that is co-inherited. The disorder tends to be very mild in Africans because of the likelihood of the co-inheritance of one of the three mild $ß^+$ mutations commonly found in this racial group: -88 (C→T); -29 (A→G); Cd24 (T→A). However, those patients who inherit a $ß^0$-thalassaemia allele exhibit a clinical disorder very similar to sickle cell anaemia. HbS/ß thalassaemia is characterised by microcytic red and target cells with occasionally sickled forms. Haemoglobin electrophoresis reveals 60-90% HbS, 0 30% HbA, 1 20% HbF, and above normal HbA_2 levels. The percentages of HbS and HbA vary depending on whether the ß thalassaemia gene is $ß^+$- or $ß^0$-type. Co-existing α-thalassaemia increases Hb concentration, MCV and MCH.

HbS/δß thalassaemia. The phenotype of HbS/$(δß)^0$-thalassaemia is milder than sickle cell anaemia because the high percentage of HbF (15-25%) produced due to the presence of the $(δß)^0$-thalassaemia allele protects against sickling. HbS/δß thalassaemia has been characterised in Sicilian, Italian, Greek, Arab and Afro-American individuals. Patients have a mild anaemia with an Hb concentration in the range of 10-12g/dl, a significantly reduced MCH and MCV, HbS and HbF and a normal or low HbA_2 level. Vasocclusive problems can occur, but are less frequent than in sickle cell anaemia of HbS/ß-thalassaemia.

HbS/HbC. Hb SC disease is usually a milder form of sickle cell disease with a variable course. Most complications occur less frequently than in SS disease. HbC is found at frequencies of up to 0.15% in parts of West Africa, where it co-exists with HbS. The HbC mutation, ß6 Glu → Lys, (GAG → AAG), causes a decrease in solubility of both the oxygenated and the deoxy forms of the haemoglobin, resulting in the formation of crystals instead of long polymers. In individuals homozygous for HbC, the red cells become dehydrated and rigid, causing a haemolytic anaemia but such patients do not develop any sickling symptoms. The clinical importance of HbC lies in its interaction with Hb S.

HbS/Hb D-Punjab. Hb D-Punjab (ß121, Glu→Gln) interacts with HbS to produce a moderately severe sickle cell disorder. This compound heterozygous state has been observed in patients of African origin and from Central and South America and India, as well as in individuals with only Mediterranean or northern European ancestry. Patients have a mild to moderate haemolytic anaemia (Hb of 5-10g/dl) with sickling crises.

HbS/Hb O-Arab. Hb O-Arab (ß121, Glu→ Lys) interacts with HbS to result in a severe sickle cell disorder. This compound heterozygous genotype has been observed in Arabs, Africans, Afro-Caribbeans and Afro-Americans, with patients having a haematological mand clinical findings indististi-

guishable from those with sickle cell anaemia. The Hb concentration varies from 6-10g/dl and the blood film shows target and sickled cells.

HbS/Hb C-Harlem. The compound heterozygous state for HbS and Hb C-Harlem (ß6 Glu→Val and ß73 Asp→Asn) results in a severe sickle cell disorder. Hb C-Harlem has two amino acid substitutions, which make the haemoglobin move like HbC in electrophoresis at alkaline pH and like HbS at acidic pH. The haematological and clinical findings are similar to those in sickle cell anaemia.

HbS/Hb S-Southend. This compound heterozygous condition is reported to result in severe sickle cell disease. Hb S-Southend (ß6 Glu→Val and ß132 Lys→Asn) has only been described in the compound heterozygous state with HbS, and comigrates with Hb S on electrophoresis and HPLC.

Hb S-Antilles. Hb S-Antilles (ß6 Glu→ Val and ß23 Val→ Ile) has been described in one family from Martinique in the French West Indies. Hb S-Antilles is more prone to sickling than HbS and results in a mild anaemia and a moderate sickling disorder in heterozygotes. In the compound heterozygous state with HbS, it is reported to produce a very severe form of sickle cell disease with a severe chronic haemolytic anaemia. Compound heterozygosity for Hb S-Antilles with HbC is also reported to result in a severe form of sickle cell disease.

Hb S-Oman. The variant Hb S-Oman (ß6 Glu →Val and ß121 Glu→ Lys) exhibits two different phenotypes in the heterozygous state, depending on whether the patients are heterozygous or homozygous for α-thalassaemia (to date, all individuals identified with HbS Oman have been found to have either co-inherited α^{+}-thalassaemia trait or homozygous α^{+}-thalassaemia). Individuals with α^{+}-thalassaemia trait have about 20% Hb S-Oman and a moderate sickling disorder. The blood film shows a unique form of irreversibly sickled cell called a 'Napoleon Hat cell' or 'yarn and knitting needle cell'. In contrast, individuals with Hb S-Oman trait and homozygous α^{+}-thalassaemia have about 14% Hb S-Oman and are asymptomatic.

The compound heterozygous state for HbS and Hb S-Oman has been described in a few Omani patients. Patients have 25% HbS, 11% Hb S-Oman and the blood film shows Napoleon Hat cells. Patients have very severe sickle cell disease, with an Hb level of 7g/dl.

HbS/Other ß-chain variants. A number of ß-chain variants have now been observed in the compound heterozygous state with HbS, including HbE. The majority of these variants are not associated with any haematological and clinical features of sickle cell disease when co-inherited with HbS. A few have been reported to be associated with a haemolytic anaemia in the compound heterozygous state, but this appears to be due to the phenotype of the variant rather than the variant affecting HbS polymerisation. In particular, Hb Shelby and Hb Hope have been found to interact with HbS to produce a haemolytic anaemia most probably due to their mild instability, and similarly for Hb North Shore due to its association with a mild ß-thalassaemia phenotype. Hb I-Toulouse has also been observed to result in a haemolytic anaemia in the compound heterozygous state with HbS, but the variant is also associated with a mild chronic haemolytic anaemia in the heterozygotes. The compound heterozygous state for HbS and HbE is described as HbSE disease, and while

uncommon due to the different ethnic backgrounds of the variants, is expected to be encountered more often due to population migrations and increasing racial intermarriage. HbSE disease is reported to have a variable phenotype, with a review of the reported cases revealing that about half were generally asymptomatic and the other half of patients aged 20 and exhibiting a mild form of sickle cell disease (9). The symptomatic patients developed sickling-related complications and had similar hematological features and clinical course to those with HbS/mild ß$^{+}$-thalassemia.

However there are three reported cases of a rare ß-chain variant being associated with severe sickle cell disease in the compound heterozygous state with HbS, for which prenatal diagnosis would be indicated: Hb C-Ndjamena/HbS, Hb O-Tibesti/HbS and Hb Quebec-Chori/HbS. Hb Quebec-Chori (ß87, Thr → Ile) results in a mild to moderately severe form of sickle cell disease due to the variant participating with Hb S in the polymerisation process. The ß-chain variant Hb C-Ndjamena (ß6 Glu → Lys and ß37 Trp →Gly) contains the HbC mutation and Hb O-Tibesti (ß11, Val → Ile and ß121, Glu → Lys) contains the Hb O-Arab mutation, so a sickle cell disease phenotype is not unexpected when these variants are co-inherited with HbS.

Table 7.3 Sickle cell disorders - interactions and indications for prenatal diagnosis and preimplantation genetic diagnosis.

Genotype interaction	Clinical Phenotype	DNA Diagnosis	PND indicated
homozygous			
HbS	Sickle cell disease	RE-PCR, ASO, ARMS	Yes
Compound heterozygous			
HbS/ß^{0} or severe ß$^{+}$-thal	Sickle cell disease	RE-PCR, ASO, ARMS	Yes
HbS/mild ß$^{+}$-thal	Mild sickle cell disease	RE-PCR, ASO, ARMS	Occasionally*
HbS/δß^{0}-thal	Mild sickle cell disease	RE-PCR, ASO, ARMS /	Occasionally*
		Gap-PCR, MLPA	
HbS/Hb Lepore	Mild sickle cell disease	RE-PCR, ASO, ARMS /	Occasionally*
		Gap-PCR, MLPA	
HbS/HbC	Sickle cell disease (variable severity)	RE-PCR, ASO, ARMS	Yes
HbS/Hb D-Punjab	Sickle cell disease	RE-PCR, ASO, ARMS	Yes
HbS/Hb O-Arab	Sickle cell disease	RE-PCR, ASO, ARMS	Yes
HbS/Hbs C-Harlem, S-Southend, S-Antilles	Sickle cell disease	RE-PCR, ASO, ARMS, sequencing	Yes
HbC/Hb S-Antilles	Sickle cell disease	RE-PCR, ASO, ARMS, sequencing	Yes
HbS/Hbs Quebec-Chori, C-Ndjamena, O-Tibesi	Sickle cell disease	RE-PCR, ASO, ARMS, sequencing	Yes
HbS/Hbs I-Toulouse, Shelby, Hope, North Shore.	Haemolytic anaemia	RE-PCR, ASO, ARMS, sequencing	No
HbS/HbE	Mild to severe sickle cell disease	RE-PCR, ASO, ARMS	Occasionally*
HbS/HPFH	Sickle cell trait	RE-PCR, ASO, ARMS / Gap-PCR, MLPA	No

* Occasionally, depending upon patient choice following genetic counselling

7.2.6 HbE/ß-THALASSAEMIA

HbE thalassaemia, the compound heterozygous state of HbE and ß thalassaemia, is a common disease in Thailand and parts of Southeast Asia. It results in a variable clinical picture similar to that of homozygous ß thalassaemia, ranging from a condition indistinguishable from thalassaemia major to a mild form of thalassaemia intermedia. The severest conditions are found in individuals with ß0 thalassaemia who usually have about 40-60% HbF, the remainder being HbE. Compound heterozygotes for HbE and ß$^{+}$ thalassaemia usually have a milder disorder and produce variable amounts of HbA. As with homozygous ß-thalassemia, the genetic factors that account for a mild phenotype in some, but not all patients, are mild ß$^{+}$- type mutations, the co-inheritance of α-thalassemia, and the homozygosity for the XmnI restriction site due to the C→T polymorphism at position -158 5'to the Gγ-globin gene.

7.2.7 OTHER HbE INTERACTIONS

Many types of HbE syndromes are observed due to the interaction of HbE with α-thalassaemia or α-thalassaemic haemoglobin variants such as Hb Constant Spring. These have been summarised in Tables 4.6 and 4.7. The two most commonly observed symptomatic disorders are described below, although prenatal diagnosis for these conditions is not normally indicated.

HbAE Bart's Disease, characterised by the presence of HbA, HbE and Hb Bart's, results from the interaction of HbH disease with heterozygous HbE. Two common subtypes of HbAE Bart's disease have been observed: α°-thalassaemia/α^{+}-thalassaemia & ßA/ßE, and α°-thalassaemia/Hb Constant Spring & ßA/ßE. The latter disorder was found to have a more severe clinical syndrome than the former type of HbAE Bart's disease. Usually the HbE levels ranged from 13-15% (see Table 4.8). The presence of α-thalassaemia leads to less availability of α-globin chains for HbE production. Small amounts of Hb Bart's are always present in this genotype. Intraerythrocytic inclusion bodies (HbH inclusions) can be demonstrated in 5-6% of the erythrocytes, indicating the presence of small amounts of HbH (β4). This amount is insufficient to be resolved by electrophoresis.

HbEF Bart's disease is characterised by HbE, HbF and Hb Bart's. HbE constitutes 80% and HbF 10% of the haemolysate, with the remainder being Hb Bart's. The presence of Hb Bart's indicates an excess of γ-globin chains. However, no inclusion bodies or HbH are present, probably because the abnormal ßE-globin chains cannot form tetramers. Four genotypes of HbEF Bart's disease can be found: HbH disease, due to either α°/α^{+}-thalassaemia or α°-thalassaemia/Hb Constant Spring, in combination with either homozygous HbE or HbE/ß-thalassaemia (10). Hb Constant Spring and small amounts of HbA may be observed in patients with the αo -thalassaemia/Hb Constant Spring and HbE/ß$^{+}$-thalassaemia genotype. To differentiate among these genotypes, family studies and further investigation by DNA analysis are required.

Individuals homozygous for HbE and homozygous for Hb Constant Spring have been reported to have mild thalassaemia intermedia. Their MCV ranged from 75-80fl and MCH was between 23-25pg (see Table 4.8). Compared with homozygous HbE alone, there were minimal red cell changes. This may be due to the interaction of α-thalassaemia with the ß-thalassaemia-like reduced globin synthesis of HbE.

7.3 GENOTYPE-PHENOTYPE PREDICTIONS

In the last 20 years tremendous progress has been made both in molecular diagnostic methods and in the understanding of the mechanisms of gene regulation and expression. This progress has made it possible to relate the phenotype with the genotype and to partly understand the wide clinical variability of the haemoglobinopathies. It is beyond the aim of this work to deal with this fascinating problem, which has been reported in excellent reviews and books (11). However, some aspects should be discussed because the prediction of phenotype from genotype is important for genetic counselling. We should clearly say that despite the above-mentioned progress, the prediction of phenotype from genotype is not perfect. Genetic counsellors should carefully refer to articles dealing with the genotype-phenotype correlation or seek advice from experts in the field in all difficult and unusual cases.

There are several layers of complexity to consider when in trying to predict phenotypes from genotypes, in order to offer genetic counselling to at-risk couples so they can make a decision about prenatal diagnosis. The first is that the phenotypes of some individual mutations are not characterised very well and it is difficult to classify the severity of the mutation. The second complexity is that the interaction between two inherited mutations can be of variable severity, making it difficult to predict the clinical outcome of the combination. The third layer of complexity is the interaction of other genetic factors such as α-thalassaemia and HPFH genes. Finally, a lack of knowledge about the natural history of some forms of thalassaemia intermedia makes providing accurate information for counselling complex.

These complexities mean that it is not possible to predict the clinical outcome in any individual case with absolute certainty. However, we have a reasonable idea about the likely degree of severity for most of the different molecular forms of thalassaemia, or can predict the likely outcome, as summarised below:

7.3.1 α–THALASSAEMIA

All α°-thalassaemia alleles result in Hb Bart's hydrops fetalis syndrome in the homozygous state and compound heterozygous state. In the last few years, a small number of hydropic infants have been kept alive with regular blood transfusions following premature delivery or after receiving transfusions in utero from 25 weeks onwards. Although long-term survival of these children may allow later bone marrow transplantation, the high risk of complications in the mothers and of congenital malformations and developmental abnormalities in the infants strongly suggests that prenatal diagnosis followed by termination of pregnancy should be considered by at-risk couples (12).

HbH disease has a wide phenotypic diversity and the majority of those affected do not have major clinical problems. Some have thalassaemia intermedia, however prenatal diagnosis is considered appropriate only for the most severe form, HbH hydrops fetalis, described in section 7.2.

7.3.2 ß-THALASSAEMIA

ß-thalassaemia mutations are classified as either ß$^+$- or ß°-type, the majority having a severe phenotype and resulting in thalassaemia major in the homozygous or compound heterozygous state. However, some of the ß$^+$-types have a mild phenotype and these pose problems for genetic counselling. In many cases involving rare mutations, there is very little or no published data on their haematological characteristics, the clinical phenotype of the homozygous state, and the results of interactions with other ß-thalassaemia mutations.

We know that some of these mild ß+ mutations, especially the few silent mutations (those with a normal HbA_2 value and a normal MCH in the heterozygous state), produce mild or very mild clinical phenotypes in the homozygous state or in interaction with severe ß-thalassaemia mutations. One of the most common is the –101 C→T mutation, which even in the genetic compounds with the severe mutations Cd39 T→C or IVSI-110 G→A, results in a very mild clinical picture. Therefore, prenatal diagnosis in at-risk couples where this silent allele is present should not be considered. The same applies to the coinheritance of the triple α-gene (ααα) or of the forms of HPFH (deletion and non-deletion) with high levels of HbF with a severe ß-thalassaemia allele. However, the position for the other silent mutations (-92 C→T, the 5' UTR mutations, IVSII-844 C→G, +1480 C→G and the 3' UTR mutations) is less clear. Because these mild mutations are very uncommon, homozygotes are non-existent and there is a general lack of data on cases where these mutations are co-inherited with other ß- thalassaemia alleles.

For the mild ß$^+$-thalassaemia alleles, such as the promoter mutations, CAP+1 A → C and IVSI-6 T→C, the picture is more complicated. In the homozygous form they usually result in mild thalassaemia intermedia. However, there are some exceptions such as IVSI-6 T → C, which has been shown in the homozygous state to have a phenotypic diversity in some populations. They also result in a variable and unpredictable phenotype in combination with a severe ß-thalassaemia allele. For example, CAP+1 A → C interacts with severe ß-thalassaemia alleles to produce a phenotype ranging from thalassaemia intermedia to major.

For other ameliorating genetic factors, such the co-inheritance of α-thalassaemia or determinants increasing HbF production, their effect on the clinical phenotype is less consistent in each individual case. Therefore genetic counselling in these cases is much more difficult. As a general rule, studies have shown that patients homozygous for a severe ß-thalassaemia allele with two α-globin genes missing are much more likely to have thalassaemia intermedia than patients with α+-thalassaemia trait (13). The loss of only one α-globin gene has minimal effect and patients are much more likely to have thalassaemia major, although the onset of blood transfusion requirement may be delayed (14). A few ß-thalassaemia deletion mutations, namely the larger deletions that remove the promoter region, seem to elevate the HbF level sufficiently by themselves to produce a mild phenotype, in the same manner as the δß-thalassaemia deletion mutations. Thus one would predict homozygotes to have a mild phenotype, but again there are only reports of homozygous or double heterozygote cases for one or two mutations. A few severe mutations, e.g. the ß° mutations IVSII-1 (G→A) and Cd8 (-AA), may be linked to a ß-globin gene haplotype that includes a genetic determinant that increases HbF, usually the –158 Gγ (C → T) mutation that results in an XmnI (+) polymorphism.

In these cases, homozygotes may have thalassaemia intermedia instead of the expected thalassaemia major observed in homozygotes for these mutations linked to a different ß-globin gene haplotype with a XmnI (-) polymorphism. However, there are some patients without the XmnI (+) polymorphism who have enhanced HbF production and thus it is not the only factor involved, making prediction of a mild phenotype difficult unless it can be inferred from family studies. Some of these genetic factors are now beginning to be understood, such as mutations in the KLF1gene (15).

7.4 SOURCES OF DNA

7.4.1 BLOOD

Parental DNA is normally prepared from 5-10ml of peripheral blood that is anticoagulated with heparin, or preferably EDTA because heparin may interfere with the PCR process. The DNA can be isolated by the standard method of phenol-chloroform extraction and ethanol precipitation, or by using one of several kits on the market based on salt extraction, protein precipitation, etc. Sufficient DNA is obtained for molecular analysis and subsequent storage in a DNA bank at –20oC. If DNA banking is not required, a much smaller quantity of blood may be used for PCR diagnosis of the globin gene disorders. Mutation analysis can be carried out using just 1ml of whole blood, by simply boiling and adding it to the PCR reaction mixture (16).

7.4.2 AMNIOTIC FLUID

DNA can be prepared from amniotic fluid cells directly or after culturing. It takes approximately 2 weeks to grow amniocytes to confluence in a 25ml flask, but culturing has the advantage that a larger amount of DNA is obtained (in our experience, the yield from such a flask has varied from 15-45µg, compared to 5-10µg from 10-15ml of amniotic fluid). However the use of cultured cell DNA delays the diagnosis and therefore a diagnosis is usually attempted using DNA prepared directly from amniotic fluid in most cases, with cultured cells grown up as a backup in case of diagnostic failure. The method of DNA preparation for both cultured and non-cultivated cells is essentially the same as that for chorionic villi (17).

7.4.3 CHORIONIC VILLI

The two main approaches developed for chorionic villus sampling – ultrasound-guided transcervical aspiration and ultrasound-guided transabdominal sampling – both provide good quality samples of chorionic villi for foetal DNA diagnosis, although the latter method is most commonly used today (see also Chapter 6 of this volume). Sufficient DNA is normally obtained for both sets of PCR reactions for the diagnosis of a mutation by two different methods to run in duplicate or triplicate for each method, and for the analysis of STR polymorphic markers for checking maternal contamination.

The main technical problem with this source of foetal DNA is the risk of contamination with maternal DNA, which arises from the maternal decidua that is sometimes obtained along with the cho-

rionic villi at foetal sampling. However, by careful dissection and removal of the maternal decidua with the aid of a phase-contrast microscope, pure foetal DNA samples can be obtained, as shown by Rosatelli et al (18) who reported no misdiagnoses in a total of 457 first trimester diagnoses for ß-thalassaemia in the Italian population. However there is always the possibility that some maternal tissue may remain with villi, so a check needs to be made for the presence of any maternal DNA in the foetal DNA preparation. This is done in most laboratories by the analysis of a panel of short tandem repeat polymorphisms, and contamination can be ruled out in most cases by the presence of just one out of two possible maternal alleles for more than one of the polymorphic markers, as described in detail in chapter 6 of Volume 2.

7.5 DISEASE-SPECIFIC STRATEGIES

Antenatal screening programmes are designed to obtain a reliable but essentially a presumptive diagnosis of a haemoglobinopathy phenotype in haematology laboratories, with referral to a DNA laboratory to obtain a definitive diagnosis by molecular analysis in appropriate cases (19). The DNA diagnosis is often required within a very short turnaround time, so it is important that molecular diagnostic laboratories develop strategies for mutation analysis that deliver a correct diagnosis in timely and inexpensive manner (20).

7.5.1 CHECKING HAEMATOLOGICAL RESULTS

A very important part of any diagnostic strategy for the molecular diagnosis of thalassaemia mutations is checking haematological results. This is involves two steps, the first being the repeating of the basic haematological tests on any blood sample referred from another laboratory in order to confirm the previous haematological results and provide an accurate phenotype. The second step is the interpretation of the haematological results with regard to which haemoglobinopathy alleles need to be targeted for mutation analysis, especially in individuals presenting with an atypical phenotype.

The key to identifying the globin gene mutation/s in a carrier or affected patient is an expert knowledge of the possible mutations and their phenotypes, and also experience of how various haemoglobinopathy mutations can interact to produce complex phenotypes. A patient's α- and ß-genotype may be very complicated, eg a patient with HbS/ß-thalassaemia may also be homozygous for α+-thalassaemia and be homozygous for the alpha chain variant Hb G-Philadelphia. Such a patient will exhibit no HbS or Hb G-Philadelphia, only the hybrid variant (αG/ßS) is visible by HPLC or electrophoresis.

7.5.2 ß-THALASSAEMIA POINT MUTATIONS

The strategy for identifying ß-thalassaemia mutations in carriers in most diagnostic laboratories depends on the spectrum of common and rare mutations in the ethnic group of the individual being screened. North European countries, such as the UK and the Netherlands, have a significantly larger number of thalassaemia mutations which need to be detected for prenatal diagnosis due to the impact of immigration of ethnic minority populations, and the simplest strategy for ß-thalassaemia

mutation identification is by sequence analysis of the whole of the ß-globin gene. For other countries with a more limited spectrum of mutations, the common mutations may be analysed using PCR techniques that allow the simultaneous detection of a small number of targeted mutations. This approach is cheaper than DNA sequencing and will often identify the mutation in more than 90% of cases, while a further screening for the possible known rare mutations will identify the mutation in most of the remaining cases, leaving just a very small number of cases for which the more expensive method of direct DNA sequencing is necessary for mutation identification. Once a rare mutation has been identified in an antenatal patient by DNA sequencing, other less expensive diagnostic techniques may be employed to detect it for prenatal diagnosis, eg. ARMS, or pyrosequencing (21).

7.5.3 ß-THALASSAEMIA DELETION MUTATIONS, δß-THALASSAEMIA, Hb LEPORE AND HPFH DELETIONS.

The (δß)o-thalassaemia, Hb Lepore and HPFH deletion mutations were originally characterised by restriction enzyme mapping and Southern blotting, but are now diagnosed by gap-PCR and/or MLPA. Selecting the correct gap-PCR primers depends on identifying the ethnic origin of the individual to be studied and characterisation of the phenotype in the heterozygous state. Gap-PCR can only be used if the deletion breakpoints have been sequenced, and a list of such deletion mutations is listed in table 5.2 of Volume 2. Traditionally, the identification of the breakpoint sequences was achieved by cloning and sequencing of the deletion allele, eg for Hb Lepore, six δß-thalassaemia alleles and three HPFH deletion mutations. However the technique of MLPA may be used to identify the breakpoint regions and then a fine-tiling array used to delineate the sequences, so that gap-PCR primers can be designed for sequencing analysis of the breakpoints to characterise the deletion to provide a simple method for screening and diagnosis, as described in chapter 7 of Volume 2.

7.5.4 α-THALASSAEMIA MUTATIONS

The diagnostic strategy for the identification of α-thalassaemia alleles again depends on the types of alpha thalassaemia mutations prevalent in the target population, but also involves a differential diagnosis to distinguish between alpha plus and alpha zero thalassaemia, especially in cases of homozygous α+-thalassaemia and α°-thalassaemia trait. In the UK, knowledge of the ethnic origin of the individual is requested as part of the national antenatal sickle cell and thalassaemia screening programme, as α°-thalassaemia is so uncommon in many ethnic groups residing in the UK particularly Indians and Africans,, the screening programme algorithm does not require partner screening for suspected α-thalassaemia in these ethnic groups.

Deletion mutations. The two common α+-thalassaemia deletion genes, the -α3.7 and -α4.2 alleles, together with five α°-thalassaemia deletion genes, the $--^{SEA}$, $--^{THAI}$ $--^{FIL}$, $--^{MED}$, and the -(α)20.5 alleles, are diagnosed most simply and economically by gap-PCR, as detailed in chapter 5, Vol 2. These deletions can be conveniently identified using three multiplex reactions: one for the two α+ deletions, one for the two Mediterranean α° deletions, and one for the three Southeast Asian α° deletions. Most laboratories use the more sophisticated method of MLPA to diagnose rare/unknown mutations after negative results using gap-PCR, and MLPA is used routinely as the second method for the prenatal diagnosis of the common α°-thalassaemia deletions.

Point mutations. The non-deletion α+-thalassaemia mutations are identified in most laboratories by DNA sequencing following the technique of selective amplification of the α-globin genes. Thus the strategy is usually to screen first by gap-PCR, and then to sequence potential α+-thalassaemia samples that test negative for two common deletions. Several of the non-deletion mutations alter a restriction enzyme site and may be analysed for by restriction enzyme digestion, eg Hb Constant Spring, and the techniques of allele specific oligonucleotide hybridisation or allele specific priming have been used for the diagnosis of more common non-deletion α+-thalassaemia alleles. Similarly, pyrosequencing has been developed recently as an alternative technique for identification of some of the more common alleles (22).

7.5.5 Hb VARIANTS

Hb Variants are first detected by HPLC and/or electrophoresis methods and given a presumed diagnosis. Although more than 700 haemoglobin variants have been described to date (6), the most clinically important ones requiring routine diagnosis by DNA analysis methods are HbS, HbC, HbE, Hb D-Punjab and Hb O-Arab. Hb S can be confirmed by a sickling test, while the other four variants must be confirmed by an alternative method (e.g. an electrophorectic or chromatographic method, mass spectrometry or DNA). For prenatal diagnosis, the mutations for these five abnormal haemoglobins can be diagnosed by a variety of techniques, as described below.

HbS (β Glu → Val) is caused by an A → T substitution in the second nucleotide of the 6th codon of the β-globin gene. The mutation destroys the recognition site for three restriction enzymes, MnI I, Dde I and Mst II. For PCR diagnosis the enzyme Dde I is used as it is a frequent cutter and several constant sites can be included in the amplified β-gene fragment to act as a control for the complete digestion of the amplified product. The βS mutation can also be detected by a variety of other PCR-based techniques, such as ASO/dot blotting, ARMS, pyrosequencing and DNA sequencing.

HbC (β6 Glu → Lys), a G → A substitution at codon 6, also occurs inside the recognition sites for MnI I, Dde I and Mst II, but in this case for the latter two enzymes it affects a non-specific nucleotide in the recognition sequence. Thus Dde I or Mst II cannot be used to detect the βC mutation and thus ASO/dot blotting, ARMS, pyrosequencing or DNA sequencing are used.

Hb D-Punjab and H O-Arab. The mutations giving rise to the abnormal variants Hb D-Punjab (β121 Glu → Gln) and Hb O-Arab (β121 Glu → Lys) both abolish an EcoR I site at codon 121. Their detection is carried out very simply by amplification of a fragment containing the codon 121 site and digesting the product with EcoR I, combined with HPLC or electrophoresis data to distinguish the two codon 121 variants. As there are no other EcoR I sites within several kilobases of the β-globin gene site, care should be taken to always run appropriate control DNA samples.

HbE, a G → A mutation at codon 26, abolishes a MnI I site and may be diagnosed by PCR amplification and restriction enzyme analysis of the product. However, the digestion products are quite small in size due to nearby MnI I sites and the HbE mutation is better diagnosed by the use of ASO/dot blotting, ARMS, pyrosequencing or DNA sequencing.

7.6 GUIDELINES FOR BEST PRACTICE

Best Practice Guidelines for prenatal diagnosis by foetal DNA analysis were initially published by the European Molecular Genetics Quality Network (EMQN) (web site: http//www.emqn.org/emqn.php) following a best practice meeting held in September 2002. Revised guidelines are now under preparation following a second best practice meeting in 2012 and will be published by EMQN on their web site in 2013.

The main technical problem associated with the use of PCR techniques for prenatal diagnosis is the very high sensitivity of PCR to the presence of maternal DNA contamination. However, a study of the accuracy of 3,254 prenatal diagnoses in the UK (23) revealed a total of ten non-laboratory errors as well as technical problems associated with the foetal blood sampling (8 diagnostic errors), Southern blotting (5 errors) and PCR techniques (2 errors). The diagnostic error rate for prenatal diagnosis by PCR methods including both non-laboratory and technical errors was calculated to be 0.41%, confirming it to be a more reliable method than the previous technologies of Southern blotting (0.73% error rate) and globin chain synthesis (1.55% error rate).

Non-laboratory errors occurred from misdiagnosis in parents, non-paternity and clerical error. Misdiagnosis in parents were observed when parental genotypes could not be confirmed by haematological and DNA analysis, usually in couples at risk for sickle cell anaemia due to the unavailability of the father at the time of prenatal diagnosis. Haematological results reported from other laboratories on the carrier status should **not be relied upon with certainty** in such instances, and it is recommended that in the absence of a partner, if the prenatal analysis is positive for the maternal mutation, then the entire ß- globin gene should be sequenced.

Laboratory errors such as partial digestion or allele drop out are minimised by performing duplicate tests, and by using two independent diagnostic methods on each sample whenever possible. Short tandem repeat (STR) polymorphism analysis by PCR is routinely used to exclude error due to maternal DNA contamination which also may identify non-paternity. These precautions increase laboratory costs but are essential to avoid the serious consequences of prenatal misdiagnosis.
The above precautions form the basis of a best code of practice (to be published on the EMQN web site: http://www.emqn.org/emqn/Best+Practice) for minimising errors in prenatal genetic testing for any genetic disorder. The guidelines for best practice are:

1. Ensure that fresh parental blood samples are obtained with the foetal sample in order to check the parental phenotypes and to provide fresh control DNA samples.

2. Ensure that the chorionic villus sample has undergone careful microscopic dissection to remove any contaminating maternal decidua.

3. Always analyse parental and the appropriate control DNAs with the foetal DNA and always repeat the foetal DNA analysis to double-check the result.

4. Whenever possible, use an alternative diagnostic method to confirm the diagnosis.

5. Use a limited number of amplification cycles to minimise any co-amplification of maternal DNA sequences.

6. Check for maternal DNA contamination in every case.

7. The foetal DNA diagnosis report should detail the types of DNA analysis used and clearly state the risk of misdiagnosis due to technical errors based on current data.

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CHAPTER 08

Joanne Traeger-Synodinos

NEW DEVELOPMENTS IN PRENATAL DIAGNOSIS

In couples at risk for transmitting a severe disorder, such as the haemoglobinopathies, prenatal diagnosis represents a means by which they can have healthy off-spring. Currently the most common approach involves invasive biopsy of foetal material, either a trophoblast sample from 11 weeks of pregnancy, or amniotc fluid from 15 weeks. Both such approaches have a small risk of pregnancy loss following the biopsy procedure (1, 2), and additionally involve the need to terminate affected pregnancies. New developments in prenatal diagnosis are directed towards improving both timing and safety of the procedure. The need to terminate on-going pregnancies may be overcome through preimplantation genetic diagnosis (PGD), which with over 20 years of clinical application, has evolved from an experimental procedure to an established reproductive alternative (3). The other approach precludes the need of invasive foetal sampling and is thus termed 'non-invasive' prenatal diagnosis (NIPD) (4). It is based on the analysis of foetal cells or free foetal DNA present in the circulation of the pregnant mother. Clinical applications are currently limited to those in which the foetus is likely to inherit genomic DNA from the father that has different characteristics relative to those inherited from the mother, including foetal sexing or detection of a Rhesus positive pregnancy in a Rhesus negative mother. For monogenic diseases such as the haemoglobinopathies, clinical application still awaits the development of robust protocols, the development of which constitute a focus of many research efforts worldwide.

8.1 PREIMPLANTATION GENETIC DIAGNOSIS (PGD)

PGD represents a means to avoid the termination of affected pregnancies, through the identification and selective transfer of unaffected embryos created by assisted reproductive techniques (ART) (5). PGD is now widely considered an established reproductive alternative for couples with a high-risk of transmitting an inherited disorder, although regulatory frameworks may vary between countries (6). However, as a technically challenging, multi-step procedure which requires the close collaboration of experts from several medical specialities, PGD is generally reserved for couples with a difficult or unsuccessful reproductive history, including fertility problems (in which case they will undergo ART procedures anyway) or an objection to pregnancy termination (either ethical/moral objection, or following experience of a previous termination for medical reasons) (5). Since the first clinical cycles applied over 20 years ago (7), PGD is currently offered in a substantial number of specialized centres throughout the world (http://www.eshre.com) for a huge range of

monogenic diseases, as long as there is a definitive molecular diagnosis and/or clear linkage defined within a family.

PGD is a multi-step procedure which requires close collaboration between experts in ART (gynaecologists and embryologists) and genetics. The many stages include evaluation and counselling of the couple on aspects of genetics and reproductive potential, application of all stages of ART, biopsy of genetic material representing each embryo, the genetic analysis, and, if implantation occurs subsequent to embryo transfer, follow-up of pregnancy and baby (or babies) delivered (8, 9).

ART procedures for PGD are the same as for infertility treatment, (with the exception of the step for oocyte/zygote or embryo biopsy), irrespective of whether the couple is fertile or not. Biopsy of material for genetic analysis may be performed at various stages post-fertilization, including polar body biopsy on the first day post-insemination, blastomere biopsy (1-2 cells) from cleavage-stage embryos on the third day post-insemination, or trophectoderm (5-10 cells) from blastocysts on the fifth day post-insemination. Each stage has relative advantages and limitations (not discussed here), but most PGD cycles reported to date have been based on blastomere biopsy (10, 11).

For all types of biopsy, the quantity of sample available for genetic analysis is very limited, and is usually only a single cell. The limited sample is considered the most technically challenging aspect of PGD, often compounded by the sub-optimal quality of the embryo and/or embryo cell biopsied. For monogenic diseases PGD protocols always require an initial amplification step, usually based on the polymerase chain reaction (PCR), although some protocols use whole genome amplification (WGA). Either way, prerequisites of PGD protocols include: speed (to produce a result within 24-72 hours), sensitivity (to genotype a single cell), robustness (to always produce a result from good quality samples) and accuracy (with results approaching 100% accuracy), so that affected embryos are never transferred. All PGD protocols require a stringent work-up prior to clinical application, to address innate limitations of single-cell PCR, including total PCR failure, failure to detect both alleles (allelic drop-out, or ADO), and sample contamination, the latter potentially occurring during any stage of the PGD procedure (ART, embryology and genetic analysis). PCR failure, although undesirable, will not lead to an unacceptable misdiagnosis. On the other hand, ADO and contamination may lead to serious misdiagnosis. Based on experience it is recommended that optimized PGD-PCR protocols be based on multiplex and fluorescent PCR, to facilitate analysis of several linked markers across the disease-associated locus, addressing the aspects of ADO and monitoring contamination. This, along with stringent laboratory procedures applied during PGD analysis, should ensure optimal efficiency and accuracy of the PGD result, supporting the identification of unaffected embryos for transfer (12).

8.2 PGD – EXAMPLES FROM THE HAEMOGLOBINOPATHIES.

According to the annual PGD data collections published by the European Society of Human Reproduction and Embryology (ESHRE), PGD for haemoglobinopathies is one of the most common applications for monogenic diseases (10, 13).

The first PGD cycles for ß-thalassaemia were reported in 1998. They were based on analysis of polar bodies and genotyping was achieved for the presence/absence of the globin gene mutation(s), using nested-PCR with restriction enzyme analysis (14). With the development of improved reagents and more sophisticated analytical tools, methods have evolved, and currently involve highly multiplexed, fluorescent PCR (F-PCR) protocols (15, 16). As with classic PND, PGD applications are restricted to the haemoglobinopathies with severe clinical expression, mainly the ß-thalassaemia and sickle cell syndromes (see Tables 7.1 and 7.3).

Although almost 200 clinically relevant mutations have been described associated with the ß-haemoglobinopathies, the small size of the gene means many of the more recent PGD protocols described are generic in as much as they are applicable for a wide range of genotype interactions. This precludes the need to develop patient-specific protocols each time, and is particularly appropriate for the haemoglobinopathies since PGD centres can expect a relatively high number of cycles. More recent methods described for PGD of haemoglobinopathies involve minisequencing or real-time PCR, along with multiplexed analysis of polymorphic sites (17, 18, 19).

PGD to preclude the severe forms of α-thalassaemia are more relevant for populations of South-east Asia and China, where deletions of both functional HBA genes from the α-globin gene cluster are common (Hb Bart's hydrops foetalis, see Table 7.2). Several protocols have been described, based on either multiplex fluorescent gap PCR (20) or linkage analysis of polymorphic sites within the disease-associated locus (21). Recently a generic protocol based on multiplex linkage analysis of polymorphic STRs located within the alpha-globin gene cluster has been described which is suitable for PGD for preclude severe forms of HbH disease and HbH-Bart's disease encountered in the Mediterranean populations (22).

Many of the disadvantages of testing single cells with PCR-based protocols can be overcome by using an initial step of whole-genome amplification (WGA) on the single (minimal) cell(s) prior to a genotyping step which can then be applied under standard laboratory conditions (23). Some WGA protocols are PCR-based, such as Degenerated Oligonoucleotide Primed PCR (DOP-PCR) or Primer Extension PCR (PEP), although the first descriptions of such methods had limited success (24). More recent developments include the use of bacteriophage φ 29 polymerase for multiple displacement amplification (MDA), which provides more efficient and faster DNA amplification from single cells (25, 26). Alternatively PCR-based methods have been developed involving adaptor-mediated amplification of a library of short, overlapping amplimers. WGA protocols are not ideal; to date none are capable of amplifying the entire genome of a single cell with homogeneous efficiency, and the levels of ADO post-WGA are observed to be high (up to 30%) (24, 25, 26, Georgia Kakourou, personal communication). Consequently, WGA is only worthwhile when PGD protocols are targeted for diseases associated with large genes and many potential mutations (for example Cystic Fibrosis or Duchenne Muscular Dystrophy), supporting more generic and highly multiplexed protocols, such as Preimplantation Genetic Haplotyping (PGH) (26).

For the haemoglobinopathies, WGA could be considered when the PGD is applied for human leukocyte antigen (HLA) testing in order to obtain compatible offspring to affected siblings who require

haematopoietic stem cell transplantation (HSCT), and was first described in 2001 (27). HLA-typing in addition to the selection of embryos unaffected for a haemoglobinopathy requires the analysis of many amplicons both across the 3.6Mb of the HLA locus on chromosome 6p21 and the HBB locus. Although WGA is appropriate, it has been observed that direct single cell analysis without prior WGA gives robust results for up to around 20 multiplexed loci (28, 29, Georgia Kakourou, personal communication). Although potentially ethically controversial, the selection of a histocompatible sibling to facilitate a bone marrow transplant in a thalassaemia major patient is considered acceptable in a number of countries, where many of the ethical controversies associated with donor-sibling selection have been resolved (30, 31). Of course the chance of ultimate success i.e. the birth of an unaffected histocompatible baby, is very limited in practice. Firstly, the likelihood that two siblings are HLA-matched is 25%. This has to be combined with the chance that only 75% of embryos will be unaffected for the haemoglobinopathy, meaning that potentially only 18.8% of all embryos fertilized will be genetically suitable for transfer in each cycle. Combined with overall success of implantation and pregnancy delivery rates of approximately 30% on average, the overall success rate for PGD-HLA matching rarely surpasses about 10-15% for any cycle initiated, and all couples should be clearly counselled and informed about this before embarking on this reproductive option. Despite this, it is estimated that over 500 clinical cycles have been performed to date (32, 33, 34).

During the twenty years during which PGD has been applied in a clinical context, continuous technical improvements have supported progression from an experimental procedure to a widely acceptable alternative to conventional PND. Most of the technical, practical and ethical issues have been addressed, and extremely reliable and accurate single-cell genetic diagnostic protocols are now available (16).

However, despite many advances, PGD remains for the most part a technically challenging, multistep and labour-intensive procedure, requiring close collaboration between many specialists. Although the genetic analysis can be stringently optimized, limitations to wider application of clinical PGD cycles include the requirement to involve ART, even if the couple are fertile, the high cost of a complete PGD cycle and the fact that pregnancy and birth rates rarely surpass 30-35% in most cases. The latter can be partly attributed to chromosomal mosaicism and chaotic cell division frequently observed in preimplantation embryos, even from normal fertile couples (35, 36). With respect to improving ART outcome there is currently much research directed at identifying biomarkers and characteristics that can be used to select the best quality preimplantation embryos to potentially improve also the outcomes of PGD cycles (37, 38).

Finally, it must be noted that the application of PGD requires the highest standards in laboratory and clinical practice, and strict guidelines should be adhered too for all stages of the procedure (8, 9, 11). To support these high standards the emerging trend is that all PGD laboratories should work towards accreditation (39).

8.2 NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

Several years ago it was discovered that foetal cells circulate in the blood of pregnant women (40), while more recently the molecular analysis of plasma DNA during human pregnancy led to the discovery that maternal plasma contains both free foetal and maternal DNA (41). Non-invasive prenatal diagnosis simply requires a peripheral blood sample from the mother, and can be based on the analysis of either DNA in foetal cells circulating in maternal blood during pregnancy, and/or on the direct analysis of cell-free foetal DNA circulating in maternal plasma. The analysis of foetal cells is more ideal, since they offer the potential of achieving a complete genotype. On the other hand, with most current methods, the analysis of cell free foetal DNA can only be analysed for alleles that have been inherited from the father when they differ from the analogous alleles transmitted from the mother. Overall, there are many technical challenges associated with both approaches and so far, despite over two decades of intense research, only a few applications have been transferred into clinical practice. This section will present a summary of the current state of the field, focusing mainly on applications relevant to single gene disorders such as the haemoglobinopathies.

8.3.1 FOETAL CELLS IN MATERNAL BLOOD

Foetal cells have long been known to be present in the maternal circulation and they provide an attractive non-invasive approach to prenatal diagnosis. Their isolation for foetal DNA analysis must fulfil two conditions: a) the foetal cells must be specific to the on-going pregnancy, since some types of foetal cells may be detected many years post-partum, and b) a pure population of (foetal) cells must be secured for analysis.

Three types of foetal cells have been investigated in the context of NIPD, including leukocytes, trophoblasts and erythroblasts (nucleated red blood cells or NRBCs). All of the cell types have some drawbacks: trophoblast cells may be anucleate or even multinucleate, with additionally a 1% risk of placental mosaicism; foetal leukocytes may persist in the maternal circulation after the baby has been delivered, a disadvantage in women who have had previous pregnancies; and finally, NRBC's may be of both foetal and maternal origin (42). However, the greatest limitation is that foetal cells are very rare, with an estimated single foetal cell per 1millilitre of maternal blood, although more recent studies indicate that this may be an underestimate (43).

Attempts to isolate pure foetal cells have tried to exploit their distinct features relative to the maternal cells, such as their size or cell-surface markers or even magnetic properties (44). More recent approaches have been based on more automated cell sorting techniques, such as automated microscopy (45) microfluidics (46), and light scattering spectroscopy (47). One approach described used a combination of automatic screening for enriched target cells, based on immunofluorescence labelling, with isolation of single candidate microchimeric cells by laser microdissection and laser catapulting, and finally low-volume on-chip multiplex PCR for DNA fingerprint analysis (48). Another approach proposes ex vivo expansion by culture of the rare foetal cells in the maternal circulation (49, 50) Recently novel membrane proteins have been identified on the foetal mesenchymal stem cells (51) constituting promising candidate biomarkers for positively isolating foetal MSCs from maternal blood for noninvasive prenatal diagnosis. However, despite more encouraging

results from recent studies, no method to date has provided a yield and/or stringency acceptable for routine clinical applications. Furthermore, none adequately address the issues of efficiency, effectiveness and acceptable cost (52). Finally a major drawback of targeting foetal cells as a source of NIPD is the often poor quality of genetic material that they contain (53, 54)

8.3.2 FOETAL NUCLEIC ACIDS IN MATERNAL PLASMA

The isolated of cell-free foetal DNA from maternal plasma is a simpler procedure compared to the isolation of foetal cells. The presence of cell free foetal DNA in maternal plasma was first described in 1997 (41). Studies indicate that it is mainly derived from trophoblastic cells and is cleared rapidly after birth (55, 56). It comprises a minority fraction of total cell-free DNA in the maternal circulation, estimated to reach levels of up to 20%, depending upon the stage and state of pregnancy, and the method used for quantification (57). Furthermore, foetal DNA is very fragmented (less than 300bp) compared to the maternal cell-free DNA (of which only half is less than 300bp). Thus simple size fractionation techniques may be employed for the selective enrichment of the foetal DNA. (58, 59)

However, the detection cell-free foetal DNA against a background of a larger amount of maternal DNA is a significant technical challenge, compounded by the fact that half of the foetus' alleles are shared by the mother. Protocols to detect paternally derived genetic loci in cell free foetal DNA, not shared by the maternal genome, are relatively uncomplicated. These include analysis for the rhesus D locus, Y-chromosome sequences, and dominant diseases, when transmitted by the father. Many protocols have been described and some applications for testing the Rhesus status or the sex of an on-going pregnancy have already been transferred into clinical practice (60, 61, 62). However, progress has been slow for the more demanding NIPD applications which aim to distinguish the status of the pregnancy relative to single gene disorders, especially those that have a mode of autosomal recessive hereditary. The detection of paternally transmitted alleles is limited to those with nucleotide differences relative to those of the mother. These sequence differences may be either the pathological mutation directly, or single nucleotide polymorphisms, (SNPs) with known linkage and phase relative to the paternal alleles. In fact, analysis of paternally transmitted linked SNPs is the only option when a couple share identical disease-causing mutations. When the sequence difference(s) are limited to a single nucleotide within a genome region which is otherwise completely identical to that of the mother, then during PCR amplification, the excess maternal alleles can be expected to compete with amplification of the cell free foetal DNA, making detection of the paternally transmitted sequence(s) extremely difficult. To address the very low and minority levels of cell free foetal DNA, methods for NIPD must combine high sensitivity with high specificity in order to fulfil the criteria of a clinically robust assay (4).

8.4 NIPD – EXAMPLES FROM THE HAEMOGLOBINOPATHIES

The haemoglobinopathies, particularly the ß-thalassaemias, constitute one of the main model monogenic disease for which methodologies are being investigated for NIPD, and the first reports of NIPD were reported over 20 years ago (63).

There are only a few reports describing approaches for NIPD of haemoglobinopathies based on analysis of foetal cells. The first described the application of NIPD was for haemoglobin Bart's hydrops foetalis (see Table 7.2) The protocol involved the isolation of NRBC's from at-risk pregnancies (10-26 weeks), enriched using magnetic cell sorting (MACS) with an anti-CD71 antibody, followed by staining of foetal NRBCs with rabbit antihuman alpha-globin antibody and immunofluorescent microscopic evaluation of samples (64). This single report reflects the limitation of this approach generally due to the absence of appropriate target cells and antibodies to distinguish disease versus unaffected status of on-going pregnancies. Two other protocols for NIPD of ß-haemoglobinopathies have also involved the analysis of CD71 MACS-enriched NRBC's, which were then individually isolated from slides by manual microdissection (65) or PALM microbeam laser microdissection and pressure catapulting (65), and then subject to PCR-based genotyping of the HBB gene. One of these studies (65) additionally included the analysis of polymorphic microsatellite loci to differentiate maternal versus foetal NRBCs. Finally another study applied PCR-based analysis of the mutation-containing region of the HBB gene in individual NRBC's enriched by high-speed gradient centrifugation and immunofluorescent staining with anti-ζ-globin (66). As previously mentioned the results of all studies so far do not meet criteria acceptable for clinical application, highlighting the limitations of isolating sufficient quantity of foetal NRBC's, coupled with their unsatisfactory quality for accurate genotype analysis.

Most reports for NIPD based on analysis of cell free foetal DNA, have aimed at the detection or exclusion of the paternal ß-thalassaemia allele. Various different approaches have been described. One of the more simple protocols used Real Time PCR for the paternally inherited codon 41/42 (-CTTT) ß-thalassaemia mutation (67), although of note is that this mutation is a 4 base-pair deletion rather than a single nucleotide substitution, somewhat facilitating its more robust detection in the context of NIPD. Another simple approach describes restriction enzyme analysis of PCR products to detect the paternally transmitted HbE mutation (68).

More sophisticated approaches have used matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) either based on conventional homogeneous MassEXTEND (hME) assay, or for the analysis following the nucleotide-specific single-allele base extension reaction (SABER) or the allele-specific base extension reaction (ASBER) (69, 70). These methods were applied to detect/exclude the foetal inheritance of paternally transmitted alleles, or, when couples shared identical ß-thalassaemia mutations, to exclude informative SNPs linked to the HBB locus. These MS-based methods demonstrated high sensitivity and specificity, with potential for high-throughput automated analysis, but the use of sophisticated and expensive equipment has limited a wider application of this approach in diagnostic laboratories.

Other approaches have involved enrichment of cell-free foetal DNA by exploiting its smaller size (<200-330 bp) compared to circulatory maternal DNA sequences (>500 bp) (71, 72). Even using separation based simply on agarose gel electrophoresis, cell-free foetal DNA could be enriched over 5-6 times (73). For subsequent analysis of enriched paternally-derived alleles, one study used PCR reactions in the presence of peptide-nucleic-acid (PNA) sequences which were designed to selectively hybridize to the normal maternal allele, suppressing its efficient amplification. PNAs are DNA

analogues which have a peptide backbone instead of a ribose-phosphate backbone. Totally complementary PNA-DNA hybrids have much higher thermal stability than corresponding DNA-DNA hybrids, but are more destabilized by single-pair mismatches, whereby when PNAs are designed to hybridize to a specific DNA sequence, they will selectively impede amplification of that allele target (e.g. the wild-type allele), while allowing amplification of the analogous trans allele which may differs by as little as a single nucleotide. In size fractionated cell-free foetal DNA, the paternal mutant allele could be detected by an allele-specific PCR/PNA protocol with an overall sensitivity of 100% and specificity of 93.8% (71). Although this approach does not require sophisticated equipment, the step to enrich the foetal sequences by gel electrophoresis/elution is technically exacting, time-consuming and susceptible to contamination. As a principle, enrichment of cell-free foetal DNA has also been demonstrated with downstream analysis based on real-time PCR and MALDI-TOF MS, with apparently robust results (72).

PNAs have also been used to impede amplification of wild-type sequences equivalent to ß-thalassaemia mutations, without prior enrichment, using subsequent mutation detection based either on a microelectronic microchip or Sanger sequencing or pyrosequencing. Although the results showed complete concordance with classical PND results, each PCR/PNA assay-required separate optimization and overall the approach appears not very robust (73).

Another approach for NIPD applied to the haemoglobinopathies used an arrayed primer extension (APEX) microchip assay, based on enzymatic extension of allele-specific probes (74). It was tested in pregnancies from couples sharing identical ß-thalassaemia mutations to detect more than 10 different paternally inherited SNPs within the HBB locus (75). Through linkage, the paternally-transmitted foetal allele (mutant or normal) was indirectly determined in six of seven maternal plasma samples. However, further optimization of the microarray assay is required, and a major disadvantage is that the microarray analysis involves the use of expensive, specialized equipment (74).

Finally a method was recently described which used a novel chemistry approach for detecting paternally transmitted alleles in maternal plasma. The method described the use of a pyrophosphorolysis-activated polymerization (PAP) assay that was optimized to to detect 12 SNPs within the HBB locus. The optimized method was capable of detecting,<3% target genomic DNA in a background of >97% wildtype genomic DNA. Subsequent application of the optimized protocol in 13 on-going pregnancies (not at risk for transmitting ß-haemoglobinopathies) demonstrated the detection of paternally transmitted alleles in maternal plasma between 10 to 18 weeks of gestation. The "proof-of-principle" for the PAP assay was demonstrated by application in a couple at risk for transmitting sickle-cell disease. DNA analysis in their first (unaffected) child supported identification of informative SNPs linked to the paternal alleles, enabling the application of NIPD and the characterization of an unaffected pregnancy status which was subsequently confirmed by conventional CVS prenatal diagnosis. Although this is the only report applying the PAP assay for NIPD, the method has the potential of being accessible for use by routine genetic laboratories with a standard infrastructure and instrumentation, as it does not require the use of using sophisticated equipment. The only prerequisite, as for most current approaches for NIPD, is the need of family studies to determine the linkage to the paternal SNPs (76).

8.5 FUTURE DEVELOPMENTS IN PGD AND NIPD

With continual developments in available technologies, we can expect further improvements in prenatal diagnosis for inherited disorders, including haemoglobinopathies.

With respect to PGD, one of the limitations of a positive outcome (i.e. the birth of a healthy baby), is posed by the couples response to ART. Even for fertile couples, the chance of delivery after PGD rarely surpasses 30-40%, and one reason could be the high rate of aneuploidy observed in human preimplantation embryos (35, 36). The fast emerging technologies of microarrays and next-generation sequencing both potentially offer approaches for simultaneous diagnosis of monogenic diseases along with chromosome aneuploidy analysis, the latter to select those embryos with a normal chromosome compliment and potentially higher implantation success. One such approach includes a method called 'karyomapping', which is based on the use of single nucleotide polymorphism (SNP)-arrays for embryo fingerprinting, enabling, through family linkage (or 'parental support'), analysis of single-gene disorders along with aneuploidy testing (77). Next-generation sequencing in PGD may facilitate multiple-gene testing, the detection of single nucleotide polymorphisms, copy number variants and chromosomal aneuploidies, as well as epigenetic profiling. Its use at a single-cell level has been described in cancer and for PGD is currently being explored (78).

Despite considerable progress in the field of NIPD, there are many technical challenges to resolve before it can be applied as a reliable alternative for conventional PND, especially for autosomal recessive inherited disorders (including haemoglobinopathies). For NIPD based on analysis of foetal cells, protocols have yet to achieve selective isolation and/or enrichment with acceptable yield and purity. With respect to NIPD based on analysis of cell-free foetal DNA, new technologies such as digital-PCR and next-generation sequencing (79, 80, 81, 82, 83) demonstrate enormous analytical potential, supporting the possibility to determine the "full" foetal genotype in cell-free foetal DNA associated with a monogenic disorder, including the detection and characterization of both paternally and maternally derived alleles.

Digital PCR requires dilution of the extracted DNA to a concentration such that, on average, one template molecule is present per reaction well. The PCR based mutation detection method is then set up (in multi-well plates) so that a large number of single molecule PCRs can be analysed per sample. The results can then be analysed mathematically to ascertain the relative frequency of each allele. This approach has already been used for the detection of foetal aneuploidy (84). The main limitation of digital PCR is that performing the hundreds to thousands of reactions required for each sample is very labour intensive. Recently digital PCR has been used for the analysis of maternal plasma for the NIPD of HbSS, but a correct answer was obtained for only 82% of male foetuses and 75% of female fetuses (85). However recent advances in microfluidics mean this process can be automated (86). A promising avenue is the recent development of an automated digital PCR platform which utilises microfluidics and real time PCR.

Next generation sequencing is another methodology that may provide the solution for NIPD (81, 82, 83). A particular application of this that could be appropriate for haemogloninopathy diagnosis is

ultra-deep amplicon sequencing, producing hundreds of thousands of sequencing "reads" which are then analysed and sorted by sophisticated software. The large amount of data produced and analysed means that the technique is potentially sensitive enough to detect subtle allelic imbalances in the maternal plasma.

These methods require expensive equipment and reagents, and substantial optimization and improvements before they can be considered for clinical application, but they certainly represent a breakthrough that may potentially allow NIPD for all pregnancies and monogenic diseases, irrespective of the mutations involved.

Alternative approaches under investigation seek to enrich the free-foetal DNA by exploiting certain properties which differ from the free maternal DNA. Maternal DNA fragments are on average larger that the foetal DNA fragments, thus enrichment of foetal DNA could be achieved by size separation, although results to date indicate that enriched samples will still contain significant amount of maternal DNA, impeding simple diagnosis by conventional means (87). Another difference between foetal and maternal DNA is methylation patterns at differentially methylated regions (DMRs) in the genome (88). This approach has been successfully demonstrated for NIPD applied for diagnosing trisomy 21 (89) and it may be possible to exploit these differences to isolate foetal DNA from maternal DNA for the subsequent NIPD for single gene disorders.

It could be expected that future prenatal diagnosis strategies will simultaneously include an overall evaluation of foetal well-being, providing couples with wider and even safer reproductive options when planning a family. In addition some advances may also be through approaches other than genetic testing, such as proteomics or metabolomics. Finally, it should also be considered that advances in genetic and/or molecular therapies may eventually reduce the wider need for PND of haemoglobinopathies and even other diseases (90).

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CHAPTER 09

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SURVEILLANCE

A WHO Working Group defined a control programme for haemoglobin disorders as an integrated programme combining best possible patient care with prevention through community information, carrier screening, genetic counselling and the offer of prenatal diagnosis (1). In an integrated programme it is not possible truly to separate surveillance of treatment and prevention, or to separate screening for carriers of thalassaemias from screening for carriers of unusual haemoglobins, e.g. haemoglobin S or E. The recommendations in this chapter apply equally to surveillance of screening for all haemoglobin disorders.

9.1 SOME DEFINITIONS

Prevention is a shorthand term for a programme of risk identification and risk management. The risk to be identified is the risk of having a child with a serious haemoglobin disorder. Risk can be identified prospectively by screening a population for carriers, or retrospectively after a couple has had an affected child.

Risk management in genetics involves providing individuals and/or couples with information on their risk, in time for them to make a choice among available options for reducing or avoiding that risk-that is, the objective is informed choice. Choice presupposes the availability of services both for avoiding and for treating the disorder concerned.

Surveillance as used by the WHO, means obtaining organised information on the effects of a programme on the target problem, at the population level. Alternative terms are monitoring and audit. Surveillance is an integral component of a public health programme-indeed, it is the programme's eyes. It is an ongoing activity that aims to identify weaknesses in the programme so that they can be corrected, and to measure the approach of the programme to its target.

9.2 SURVEILLANCE OF SCREENING FOR HAEMOGLOBIN DISORDERS - BASIC PRINCIPLES

The implementation of population screening for carriers of haemoglobin disorders depends on public health authorities recognising thalassaemia and/or sickle cell disorders as a public health prob-

lem, and on their commitment to planning, co-ordination and surveillance. It is essential for authorities to provide the necessary resources for ongoing surveillance of the programme. Carrier screening is a complex activity, including four main components at both the local and the national level:

1. Ongoing education for professionals and the community.

2. Carrier screening through routine haematology laboratories with agreed standards and quality control.

3. Genetic counselling, including risk assessment. This needs to be provided locally, and so should be integrated into routine medical services. Couple risk assessment can be complex and requires strong links to specialist DNA laboratories.

4. Availability of prenatal diagnosis. This involves the collaboration of obstetricians and specialist DNA laboratories.

The question then arises, what is the most cost-effective approach for comprehensive surveillance of such a complex system? Clearly each component requires its own education programme, service standards and methods for quality control. Comprehensive surveillance requires methods for assessing the integrated functioning of the whole programme at a local and national level. A key requirement for surveillance is a good understanding of the epidemiology of the disorder in question (see Chapter 2).

When the WHO Working Group was first asked how to measure the effects of a carrier screening programme, they noted that once a programme is underway, an affected child can only be born as a result of either informed parental choice, failure to detect parents' risk or failure to inform parents of that risk. The group therefore suggested two surveillance activities: (a) to observe the birth prevalence of affected children and (b) to examine the extent to which new affected births represent parental choice as opposed to a failure in the programme (1). In practice, this recommendation translates into a requirement to establish and maintain registers of patients and prenatal diagnoses, and to examine the circumstances surrounding new affected births.

Registers of patients and prenatal diagnoses are integrally related because they deal with individual members of the same group of families at high genetic risk. Although they differ considerably in the methods required to collect data, the combined data provides a single national "diagnosis register" that can be used to assess:

- Completeness, timeliness and accuracy of carrier diagnosis and risk assessment.
- Safety, timeliness and accuracy of prenatal diagnosis.
- Extent of informed choice provided to at-risk couples.
- Total impact of the programme on the reproductive life of at-risk couples.

The rest of this chapter deals with the practicalities of maintaining these registers, and the information that can be obtained from them.

9.3 A REGISTER OF PRENATAL DIAGNOSES

A prenatal diagnosis register is laboratory-based. Prenatal diagnosis of haemoglobin disorders now depends primarily on DNA analysis. As clinical molecular genetic laboratories keep good records and relatively few are involved in each country, a national register is, in principle, relatively simple to organise. However it requires that (a) all laboratories involved in prenatal diagnosis, whether in the public or the private sector, collaborate in reporting, (b) all pregnancy outcomes following prenatal diagnosis are followed up and (c) a specific laboratory or individual is given responsibility for regular data collection and reporting. These activities should be formalised, mandated and funded by health authorities.

The following is the minimum data set required for a prenatal diagnosis register. All the data required should be included in the laboratory record, whether in paper or electronic format.

- Laboratory responsible
- Mother's unique identification number, date of birth, ethnic origin, area of residence
- Condition for which the foetus is at risk
- Gestation at foetal sampling, obstetric sampling method used and date
- Laboratory diagnostic methods used
- Diagnosis in the foetus and date
- Outcome of the pregnancy and date
- Confirmation of prenatal diagnosis. In the newborn at risk for thalassaemia this requires DNA analysis. Haemoglobin analysis alone may suffice at or after 6 months of age

The aggregated data allows assessment of the following indicators of service quality at the national and local levels. The data should be extracted and reported annually or biennially, preferably at a national meeting.

- Timeliness of risk detection and referral (gestation at sampling)
- Safety of the obstetric procedure (rate of pregnancy loss following sampling)
- Accuracy of laboratory diagnosis (mainly assessed from the number of affected children born following diagnosis of an unaffected foetus)
- Other causes of misdiagnosis (e.g. mis-referral, maternal contamination, non-paternity) (2)
- Potential reduction in affected births due to prenatal diagnosis (by comparing register data with epidemiological data)

A prenatal diagnosis register can assess service quality only for those that actually have a prenatal diagnosis. It can give no information on timeliness and accuracy of risk detection for at-risk couples that did not have a prenatal diagnosis. This is one of many reasons for combining prenatal diagnosis and patient registers.

9.4 A PATIENT REGISTER

It is more difficult to maintain a patient register because it requires the collaboration of all clinicians who treat patients and this can be hard to achieve, especially in large countries where patients are treated at many centres. In order to be successful, a two-way system should be established that helps participating doctors provide best possible care—e.g. the patient register can send out regular reports, treatment protocols and patient information materials. A patient register requires the support of a team including a dedicated data manager, who can become known to treating doctors and who can phone and/or visit to help with data extraction where necessary, as well as appropriate IT support and expert clinical supervision. The requirements are summarised in reference (3). A patient register has two main functions: (a) assessing the quality of patient care, including providing early warning of serious clinical problems (e.g. through study of survival and causes of death) and (b) assessing the quality of carrier screening and counselling by observing the number of affected children born and identifying the relative roles of informed parental choice and programme failures in these births.

9.4.1 MONITORING THE AFFECTED BIRTH PREVALENCE

This requires reliable data on the number of affected children diagnosed each year. The following is the minimum data set required. The last two items are needed to assess differences in the effect of the programme by region or ethnic group.

- Treatment centre
- Patient's identification number, date of birth
- Ethnic origin
- Area of residence

The best example of an application of aggregated patient data is provided by a WHO collaboration, whose results are summarised in Figure 9.1.

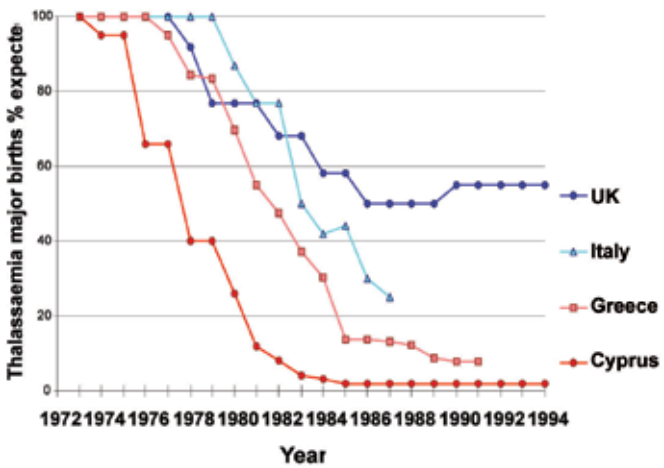

Figure 9.1: The fall in thalassaemia major birth rate in several national thalassaemia prevention programmes (12, 13, 19 and Angastiniotis M. personal communication).

9.4.2 ASSESSING INFORMED PARENTAL CHOICE

A patient register can collect the following data at the time each new patient is registered:

- Were the parents aware of their risk before the birth of the affected child? (If not, what was the reason?)
- Were they offered prenatal diagnosis? (If not, what was the reason?)
- Gestation at the time prenatal diagnosis was offered.

When the WHO study described above first got underway, an enquiry into the circumstances of new births of affected children in Italy, Greece and Cyprus showed that most were associated with lack of awareness of the potential problem and doctors' failure to screen and inform parents, rather than parents' refusing prenatal diagnosis (4). The approach has been used more recently in Sicily (5) and the UK, with similar results (6).

9.5 A COMBINED "DIAGNOSIS" REGISTER

Used together, a patient register and a prenatal diagnosis register identify all known affected conceptions and their outcomes. This provides a very powerful approach for comprehensive evaluation of a screening programme (see Fig 9.2).

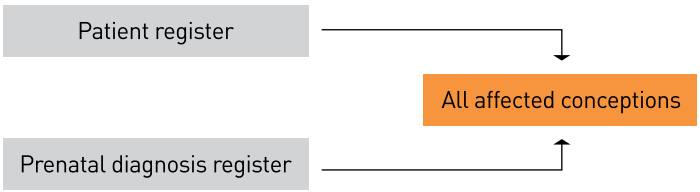

Figure 9.2: Two registers can be used as a "pincer" identifying all known affected conceptions and their outcomes.

The methods for programme evaluation discussed so far are based on measuring total affected births and prenatal diagnoses in a population. However, the objective of a genetic programme is to identify and inform families at risk (at risk couples, patients, other relatives) and to make services for treatment and prevention available to them. The extent to which this target is achieved can be assessed by linking individual data from the prenatal diagnosis and patient registers, using the unique identifier of the mother. It should also be noted that as patients' fertility improves, an increasing number with carrier partners are requesting prenatal diagnosis.

To date, this integrated approach has only been used in the UK, in order to assess the long-term effect of prospective risk identification on couples' reproductive life (7) and to measure the extent of informed choice in a cohort of couples identified in a national confidential enquiry into genetic counselling (6). Figures 9.3 and 9.4 compare the reproductive outcomes for retrospectively and prospectively identified couples at risk for thalassaemia who received prenatal diagnosis at University College Hospital, London.

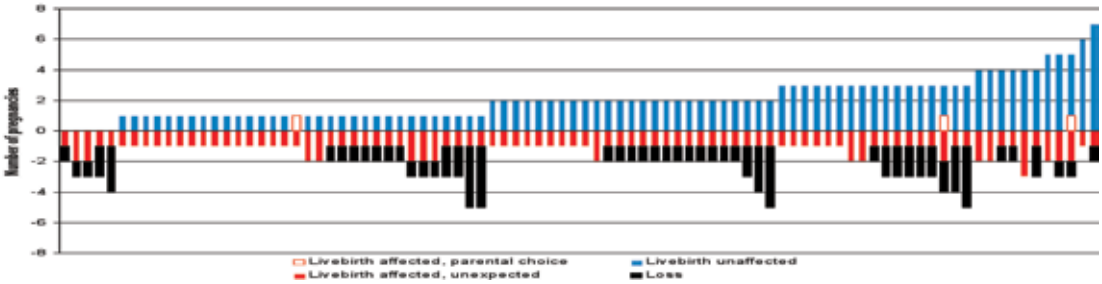

Figure 9.3: Pregnancy outcomes for 90 couples at risk for thalassaemia detected retrospectively (370 pregnancies). Each vertical bar represents one woman's reproductive history. Only continuing pregnancies are included: social abortions, miscarriages and other early losses are excluded. Favourable outcomes are shown above the X-axis, and unfavourable outcomes below it. All couples (by definition) had at least one unexpected affected live-birth. Five couples never had an unaffected child, and five had five unfavourable outcomes.

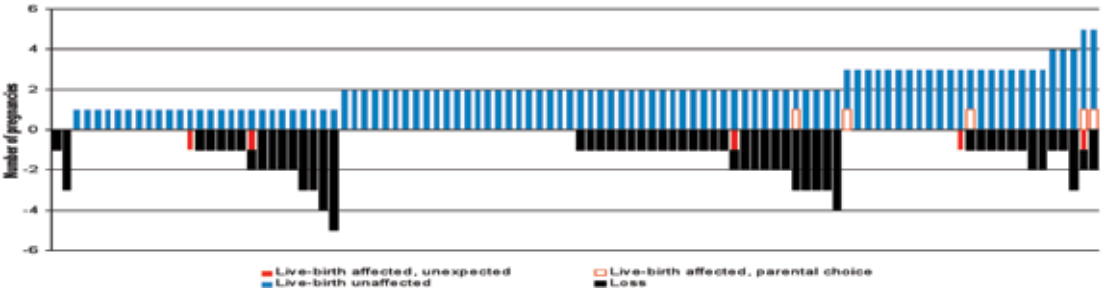

Figure 9.4: Pregnancy outcomes for 102 couples whose thalassaemia risk was detected prospectively by antenatal screening (300 continuing pregnancies). Explanation as above. Favourable outcomes - unaffected live-births and affected live-births following parental choice - are shown above the X-axis. Unfavourable outcomes - pregnancy losses and unexpected affected live-births – are shown below the X-axis. Two particularly unlucky couples became infertile, and have no living child.

9.6 EXAMPLES OF THE USE OF REGISTERS FOR NATIONAL SURVEILLANCE

The above principles of surveillance apply for all communities where carrier screening is practised. However, each country needs to apply them in a way that takes account of local epidemiology, service structure, economic resources, and social and cultural attitudes. It is relatively simple to apply the recommended methods in small populations where thalassaemia is common and there is a disease-oriented programme. It is far harder but much more important to apply such methods in large countries where screening and counselling must be integrated into the general health system

and where a wide range of health professionals are involved (1). Reports have been published from Mediterranean countries where thalassaemia is a recognised public health problem—in ascending order of population size, Cyprus (8, 9), Sardinia (10), Sicily (11), Greece (12) and mainland Italy (13). The larger the country and the greater the number of units involved in treatment and prevention, the more difficult reporting becomes. This strategy has also been adopted in Iran with a population of 74.8 million (14, 15), and in the smallest Asian country, the Maldives (16)

9.6.1 THE EXAMPLE OF THE UK

The rationale for researching the implementation of WHO recommendations in the context of the UK is as follows. The UK is a large country with a population over 56 million. About 7% of residents and 13% of births are in minority ethnic groups at risk for haemoglobin disorders. On the basis of the 2001 census approximately 88,000 women in these groups become pregnant each year, including about 9,000 carriers. About 1,260 of the carriers' partners are also carriers, leading to about 314 affected foetuses per year. About 84% of those foetuses have a sickle cell disorder (including sickle cell/ß-thalassaemia), while a 16% sub-set (about 50/year) has a major thalassaemia (homozygous ß-thalassaemia, homozygous α°-thalassaemia, or HbE/ ß-thalassaemia). However those couples at risk for sickle cell have been increased by immigration from Africa. The figures published by the NHS Sickle Cell and Thalassaemia Screening programme reflect this increase, 361 babies affected with a sickle cell disorder were indentified in 2009/10 (17). When the 2011 census figures become, available more accurate estimates of the at-risk population, and number of expected affected babies, will be possible.

The UK is typical of much of Northern Europe, where thalassaemia is a rare disorder that affects diverse minority ethnic groups scattered among a majority North European population that is not at risk (18). Such communities represent the ultimate challenge for surveliillance. If the WHO recommendations could be implemented in this context, the methods developed would be applicable for surveillance of genetic carrier screening in almost any health system. Additionally, the UK offers a favourable research environment because it has played a leading role in the development of antenatal and neonatal screening for congenital and genetic disorders and so has extensive experience in this arena as well as boasting a tradition of co-operative research.

The three UK laboratories providing prenatal diagnosis for thalassaemia established an ongoing collaborative national register of prenatal diagnosis for haemoglobin disorders (sickle cell disorders and thalassaemias) (19), including every case since the service was initiated in 1974. A second register-the UK Thalassaemia (patient) Register-was effectively initiated in 1966 as a study aimed at defining the natural history of thalassaemia, drawing in all known patients resident in the country (20). The study was subsequently continued as a national patient register including all known patients since thalassaemia started to appear in the UK in the 1950s (21). The register was intended as a model for a future national register of haemoglobin disorders (sickle cell disorders as well as thalassaemia) Both registers were considered to be over 97% complete, and consequently could identify almost all pregnancies involving a foetus with a major thalassaemia ever to have occurred in the UK. The register became a valuable tool, provided an early warning sign to clinicians of risks for thalassaemia. It showed a decline in deaths among thalassaemia patients with use of

the oral iron chelator deferrioprone (22) . It showed the declining efficacy of desferioxamine at the population level, considered largely due to non-compliance due to the burdensome iron chelation regime.. The register identified an increased risk of Klebsiella infections. Like most other registers maintained for surveillance purposes, these two registers were supported by research funding and voluntary effort, rather than by central health service funding.

A UK national sickle cell and thalassaemia screening programme was initiated in 2001, partly based on evidence from these two registers. This was the first, and still is the only, such national screening programme in Northern Europe. The programme has established universal newborn screening for sickle cell disorders, and antenatal screening, counselling and availability of prenatal diagnosis for sickle cell disorders and thalassaemias (23). The surveillance arm of the national programme has taken over the prenatal diagnosis register. The thalassaemia patient register was discontinued in 2003 due to lack of political support. The annual affected births and existing affected patients are now collected by the National Haemoglobinopathy Register (24) but the information is still incomplete and swamped by the large number of sickle cell patients and hence largely sickle cell focused.

9.6.2 CARRIER SCREENING IN THE UK

A fundamental concept in population screening is the "turnstile"—a relevant stage at which members of a community routinely come into contact with the health system and can be offered a screening test. The turnstiles currently used for screening for haemoglobin disorders in the UK are pregnancy (for reproductive risk) and birth (for personal risk of a sickle cell disorder) (20).

Antenatal screening for haemoglobin disorders is a complex, multi-professional activity that involves different groups of health workers and crossing and re-crossing administrative boundaries (see Table 9.1).

Table 9.1. Antenatal screening cascade for haemoglobin disorders in the UK (turnstile = pregnancy)

Action	Professional responsible
Inform population	No defined professional responsibility
Offer screening	Midwife/obstetrician (mandatory) General practitioner, practice nurse (optional)
Screen	Laboratory (algorithm) (23)
Carrier: inform and offer partner test	Sickle cell & thalassaemia counsellor (when available) midwife/obstetrician, General practitioner, practice nurse (optional)
Test partner	Laboratory (algorithm) (23)
Assess couple risk	Expert haematologist and/or expert specialist geneticist
At risk couple: inform and offer PND	Trained counsellor in haemoglobin disorders, haematologist, genetic counsellors
Parents' informed choice	

The sequence of steps in antenatal screening for haemoglobin disorders is shown on the left, and the professional groups involved are shown on the right.

Antenatal screening for haemoglobin disorders had long been considered standard practice in the UK. However, before the national policy on screening programme was established: over 140 district health authorities with widely different at-risk populations were (rather unrealistically) expected to develop and implement local policies (20). The fact that the 1998 confidential enquiry (6) showed that only half of all at-risk couples actually obtain the informed choice, that is the objective of screening reflects a lack of commitment on the part of public health authorities up to that time. Since the national screening programme was established most of the responsibilities of the different health professionals in Table 9.1 have been defined at the national level (23). However the extent to which the objective of informed choice for at risk couples has been achieved remains to be established.

9.7 THE USE OF REGISTERS FOR NATIONAL AUDIT

When prenatal diagnosis is offered to people of Mediterranean origin in the UK, uptake is over 90% (25). However, in both 1985 and 1997, data from the combined national registers indicated a nationwide uptake of only 50% (26, 27). This was because while most affected conceptions of parents of British Cypriot origin end in termination of pregnancy, most affected conceptions of parents of British Pakistani origin (who predominate in the Midlands and the North) end in an affected live-birth (6). This was widely interpreted to mean that Muslim couples declined prenatal diagnosis because of religious objections to termination of pregnancy. However, more precise information was needed through an enquiry into the circumstances surrounding affected births. Fortunately, the UK Ministry of Health funded a National Confidential Enquiry into Counselling for Genetic Disorders, conducted by Professor Rodney Harris, a geneticist not involved with haemoglobin disorders and therefore perceived as truly objective (28). Since cases could be identified through the patient and prenatal diagnosis registers, a Thalassaemia Module was included in the Confidential Enquiry (6). This aimed to audit risk detection and provision of risk information for thalassaemia during pregnancy.

The Confidential Enquiry team reviewed the clinical records of 136 women, 88% of the 156 women with a pregnancy affected by a major form β-thalassaemia during the period of 1990-1994. The results are representative of the national situation in antenatal risk detection and counselling for thalassaemia. The women's records were assessed against a minimum standard of care. The offer of prenatal diagnosis (a precondition for choice) was the selected basic indicator of service adequacy. For a truly informed choice, at-risk couples should be identified before they start a pregnancy or in the early stages of their first pregnancy. However, when antenatal screening is the chosen policy, the risk is often identified before the second trimester of the first pregnancy. The selected standard of care was therefore: (a) risk identification and offer of prenatal diagnosis before 23 weeks in the first pregnancy and (b) offer of prenatal diagnosis in the first trimester in all subsequent pregnancies (assuming the woman presented in time).

The cohort of 136 women had 485 pregnancies. The enquiry found that in the first pregnancy only 50% of at-risk couples were identified and informed of their risk in time for the offer of prenatal diagnosis. Risk was identified too late to consider prenatal diagnosis in 11% of cases, and was not recognised at all in 38%. As failure to identify risk is recurrent, currently 28% of at-risk couples discover their risk only through the diagnosis of an affected child.

Analysis of the clinical records showed that service failure occurred in 126 of the 485 pregnancies (26%), and revealed contributing reasons at many different points in the screening cascade. Most of the failures reflected an inadequate local screening policy (see Table 9.2).

Table 9.2 Causes of service failure.

Cause of failure	Number	%
Woman not screened	35	27.8
Partner not tested	29	23.0
Laboratory error	15	11.9
Undue delay	13	10.3
Unavoidable	10	7.9
Known risk "lost"	9	7.1
Unknown	6	4.8
Failed communication	5	4.0
Not counselled	4	3.2
Total	**126**	**100**

9.8 THE NEED FOR EARLY RISK DETECTION AND EARLY PRENATAL DIAGNOSIS

The Confidential Enquiry findings do not necessarily mean that use of prenatal diagnosis would increase if all couples were identified and informed. However, the Enquiry also revealed that over 70% of British Pakistanis requested prenatal diagnosis when it was offered in the first trimester, while less than 40% requested the service when it was offered in the second trimester. Thus the Confidential Enquiry was able to elicit the clear preferences of a minority group who rarely articulate their needs at present. Of course, all women have a similar preference for early diagnosis and the results indicate an overall need to make early diagnosis available to all at-risk couples in every pregnancy.

The Enquiry findings were clear. Carrier screening as part of routine antenatal care cannot meet couples' need for early information and access to early prenatal diagnosis. Ideally genetic information and screening should be provided in primary care, either before pregnancy or as soon as a pregnancy is notified (6, 27) and antenatal screening should be an essential safety net rather than the approach of choice. Thus an audit based on the registers was able to evaluate the present service and show how future services should develop.

Following publication of the results of the Confidential Enquiry, which demonstrated the legitimacy and importance of enquiring confidentially into the circumstances surrounding new affected births, a question on informed parental choice was added to the new patient registration form for the UK Thalassaemia Register. This audit contributed to the decision to include linked programmes of antenatal and neonatal screening for haemoglobin disorders in, the UK National Plan for the Health Service (2000) (29).

Up to now, audits in most countries have been promoted by a dedicated individual, without allocated funding or as a research project. However, the results have shown that ongoing audit is an essential part of a thalassaemia control programme. The requirement for audit should be made explicit, and audit should be built into the system so that it is independent of specific individuals.

When a steering committee is involved in a thalassaemia control programme, an audit sub-group should be appointed and appropriate funding made available. The requirements are relatively modest. On the basis of experience in the UK, a large country requires the following: one doctor 1/5th time, one full-time curator (who can travel to outlying centres and collect data), computer support 1/5th time, an office with a computer and office and travel expenses. Results should be reported at an annual or biennial national meeting.

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CHAPTER 10

Piero Giordano

NEWBORN SCREENING FOR HAEMOGLOBINOPATHIES

10.1 INTRODUCTION

Newborn screening (NBS) is generally applied for the early recognition of babies affected by disorders that benefit from early treatment to avoid irreversible health problems. Examples include metabolic disorders and sickle cell disease. It also potentially supports the identification of at-risk couples who can then be offered genetic counseling, with the option of prenatal diagnosis in subsequent pregnancies - often referred to as "secondary prevention".

In the context of the haemoglobinopathies, newborn screening may be considered an additional strategy within a prevention programme. Only during the last decade have Public Health Authorities started to include haemoglobinopathies into newborn screening, a choice which is more suitable for early treatment of the affected newborn rather than for prospective primary prevention. It is of note that to date none of the endemic countries (with long prevention experience) have implemented newborn screening; instead there are national premarital or early pregnancy screening campaigns. However in non-endemic countries, sickle cell disease and some other haemoglobinopathies have been incorporated into ongoing newborn screening (NBS) programmes for rare metabolic diseases, creating a strategy of morbidity prevention rather than of primary prevention, potentially supporting state of the art management of these diseases (1-3).

10.2 THE HB FRACTIONS AS A BASIS FOR NEWBORN SCREENING

The first step in newborn screening is the direct separation and measurement of the Hb fractions present. To understand the significance of the Hb fractions present at birth in normal and in pathological conditions one must be aware of the genes involved in haemoglobin production before and after birth (see Figure 10.1). During adult life the Hb fractions present in the normal lysate are the major component HbA ($\alpha_2\beta_2 \approx 96\%$), the minor component HbA_2 ($\alpha_2\delta_2 \approx 2.5\text{-}3.5\%$), and traces of foetal HbF ($\alpha_2\gamma_2 <1\%$). The Hb fractions present at birth are mainly HbF (≈80%) with some HbA (≈20%). Measurement of HbA, HbF and any abnormal fractions (HbS, C, E, D, Bart's etc.) which may be present supports the correct interpretation of the results.

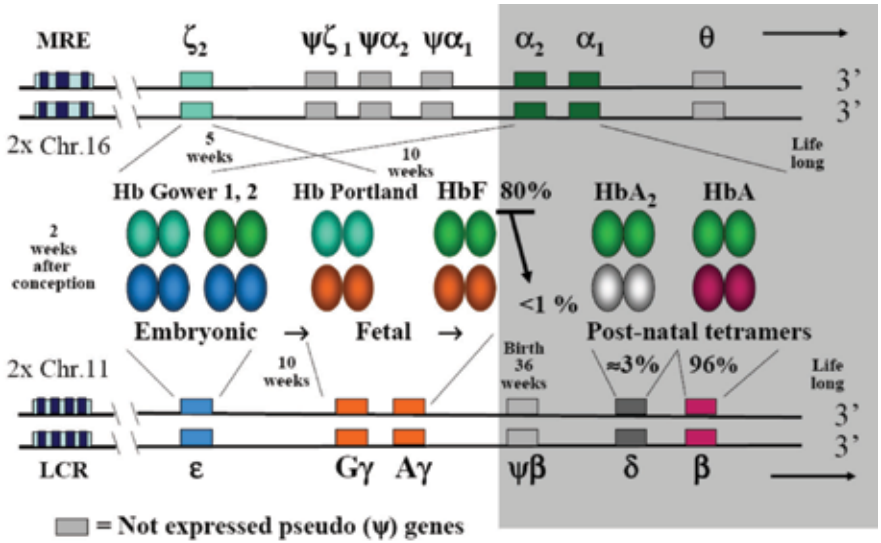


Figure 10.1 Genes coding for globin chains during embryonic, foetal and post-natal life.

The embryonic genes ζ_2 and ε are the first to be activated during early embryonic life, producing Hb Gower-1, Hb Gower-2 and Hb Portland. The two α-genes begin to be expressed later in embryonic life and maintain their expression during foetal life producing foetal haemoglobin (HbF) in combination with the two γ-genes. During adult life, the α-genes remain active producing HbA and HbA_2 in combination with the β- and δ-gene products, respectively. The two δ- and two β-globin genes become significantly expressed after birth, (as highlighted in the grey square), and together with the four α-genes remain fully expressed in post-natal life. The two δ-genes have a physiologically low expression and defects of the δ-genes solely are not associated with pathology.

10.3 THE TECHNOLOGIES FOR NEWBORN SCREENING

Little has changed over the last few decades regarding the elements of basic diagnostics for haemoglobinopathies. The two fundamental methodologies remain the complete blood count (CBC) and the separation and measurement of the haemoglobin fractions.

Separation and measurement of the haemoglobin fractions may be achieved by a variety of methods (see Volume 2). Those most commonly used in neonatal screening are high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and isoelectric focusing (IEF).

High-performance liquid chromatography. The first dedicated device for newborn screening, which has been thoroughly tested, is the Variant NBS (Bio-Rad Laboratories, Hercules, CA, USA)

(4). This is a robust system and it is currently used by many laboratories as single NBS method without significant problems.

Capillary electrophoresis. A capillary electrophoresis device, the Capillarys® Neonat (Sebia, Lisses, France) has also been tested for routine diagnostics (5) and for newborn screening using both fresh and dry samples (6). The validation studies showed good results for both applications, indicating likely good performance under constant high- throughput conditions.

The diagnostic potential of both systems is comparable, with sensitivity levels near 100% and high specificity. Identification of variants demonstrates a high level of specificity for the common variants, although it is always putative and requires definitive confirmation.

In some NBS programmes, quality regulations indicate the application of more than one technology, usually for samples identified with a positive result (7). For example in many of the UK newborn screening laboratories, automated HPLC is used as the first screening method, followed by IEF as the confirmatory technique.

10.4 IDENTIFICATION OF THE MAJOR HB FRACTIONS ON HPLC & CE

Identification of the major fraction HbF in the newborn is straightforward. Of note is that on CE the HbF fraction migrates more slowly than the HbA fraction (like on the traditional alkaline electrophoresis or IEF), while with HPLC, the HbF fraction has a lower retention time and thus will elute before HbA (Figure 10.2)

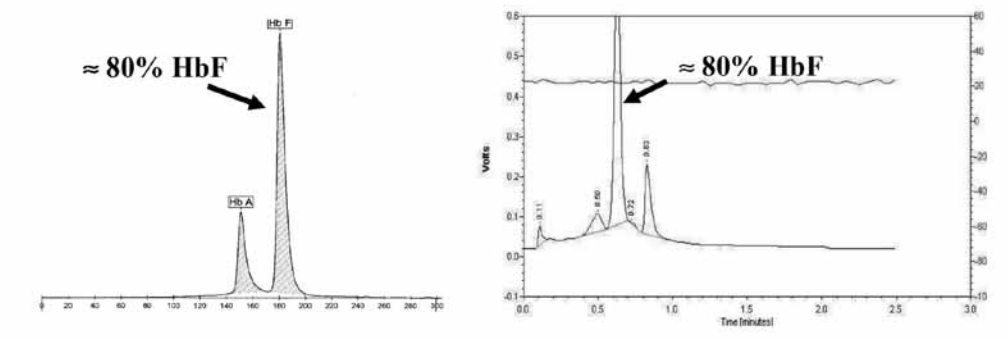


Figure 10.2 The pattern of newborn Hb fractions determined by CE and HPLC.

The left hand pattern in Figure 10.2 shows the newborn pattern of HbF relative to Hb A in fresh cord blood when separated by CE (Sebia). The right hand pattern depicts the newborn Hb pattern from a dried blood sample when separated by HPLC (Bio-Rad Variant). To the left to the HbF peak is the acetylated HbF fraction, and the sum of the both peaks is calculated for the amount of HbF. The very first window is Hb Bart's. The HbA peak elutes after HbF and in between the two is a very small peak of derivates (not to be confused with HbA).

The condition of the sample is important in newborn screening, and is usually either dry blood or fresh cord blood:

Fresh cord blood. Fresh cord blood, analyzed within 24 hours, gives the best separation patterns. The fresh cord blood samples can either be loaded directly from the tube or be haemolysed first. However, fresh cord blood is not always available. Transportation to remote laboratories may be required but the sample quality may be compromised by clotting, or contamination with maternal blood.

Dry blood. An alternative of choice, suitable for large scale regional or national screening programmes are dry blood samples collected from a heel-prick within one week after birth. A few drops are absorbed on filter paper (Guthrie card) and sent by mail to the reference laboratory serving the region.

Dry blood samples have some drawbacks. They degrade with time and chromatograms usually present with an elevated base line along with some degradation peaks, as shown in Figure 10.3. Samples up to 3 weeks after collection usually maintain the inter-fractions rate, allowing a reliable provisional diagnosis (4). For either dry blood or fresh cord blood samples, analysis takes only a few minutes. Manufacturers provide full technical support, including software for highlighting abnormal samples and storing results. Of note is that a reliable integration mode is important to calculate the areas of the peaks correctly.

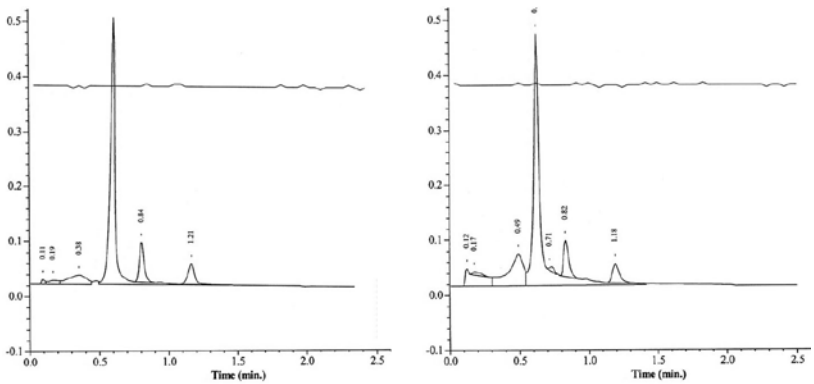


Figure 10.3 The difference between results obtained from fresh and dry cord blood using the Bio-Rad Variant NBS.

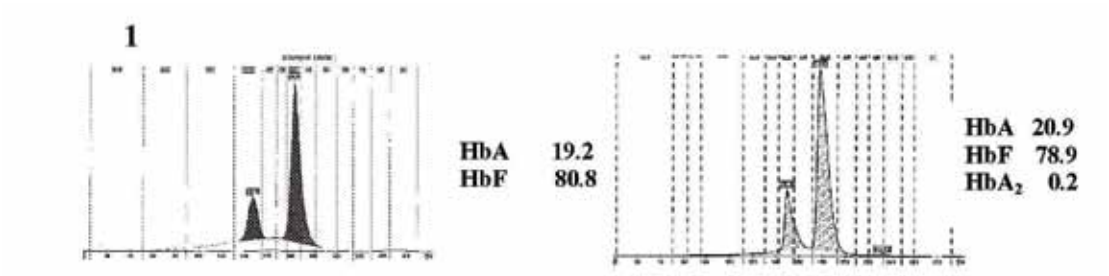
The left hand side of Figure 10.3 shows good HPLC separation (Bio-Rad NBS) of a fresh newborn sample of an HbAS carrier (sickle cell trait). The HbA (9.8%) and the HbS fractions (7.5%) are well represented at a ratio 56/44, as one can expect in a non premature SCT newborn without α-thalassemia in the presence of about 80% HbF (70.2 HbF + 9.7 acetyl F = 79.9%) and some 3% derivates. The base line is low as expected from a fresh sample. The right hand side of Figure 10.3 shows the same sample after it has been spotted on paper, dried for 24 hours and eluted. Due to degradation the base line is elevated and all fractions are slightly less represented. The HbA (6.3%) and HbS fraction (4.7%) have however maintained the same ratio (57/43).

10.5 INTERPRETATION OF THE RESULTS

Straightforward samples. As shown in Figures 10.4 and 10.5, severe conditions like sickle cell disease and thalassaemia major are recognized immediately at 100% sensitivity and at a high level of specificity when caused by common variants like HbS, C, E and D and virtually any β-thalassaemia mutation. Nevertheless, all these are putative results that must be confirmed at the DNA level to avoid spurious conclusions, since some rare Hb variants may mimic the common variants (8). In addition, hereditary persistence of foetal haemoglobin (HPFH), a mild condition, can be mistaken for thalassaemia major.

Complex samples. Over 1000 abnormal haemoglobin have been described, mostly recessive, and a number are clinically significant when combined with β-thalassaemia or HbS. In addition, the presence of Hb Bart's indicates α globin defects, which may be associated with intrauterine or perinatal pathology (hydrops foetalis and HbH disease) (9). Finally, alpha chains variants which will associate with γ- and β-globin chains may generate complex four-peak patterns. Patterns may become even more complex when β gene variants are co-inherited, generating 6 different Hb fractions. Analysis at the DNA level is for all these defects crucial.

Putative characterization of Hb fractions. As mentioned above, common Hb variants are detected at high specificity, simply because they are the most frequent. However, in fact we may expect to find several other common or uncommon variants in the so-called specific "windows" for the common Hb variants (8). Only the HbS variant can easily be confirmed, using a simple sickle test, although this is only possible if fresh blood is available (10).



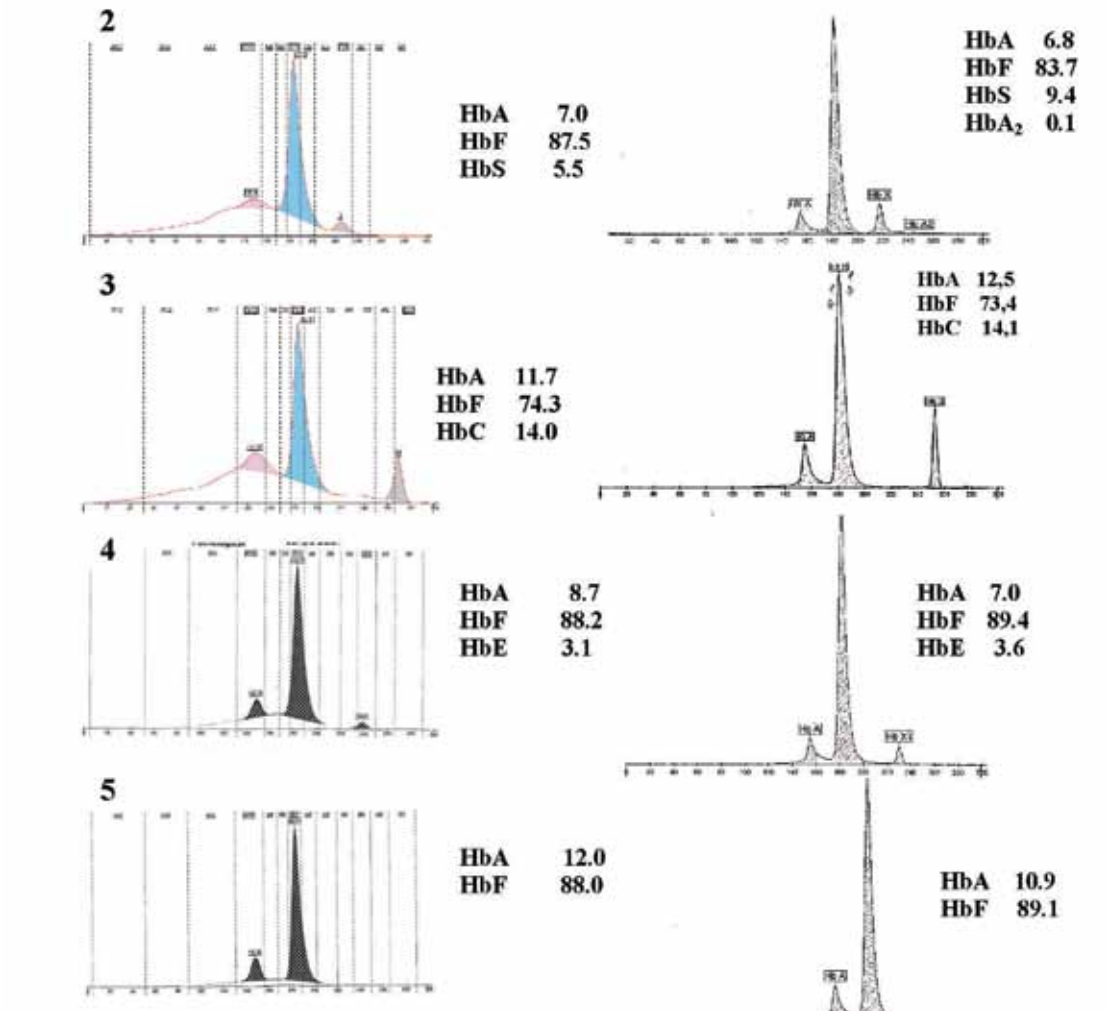


Figure 10.4 Some common examples of neonatal Hb patterns by CE.

Dry newborn samples were analysed on the Capillarys ® Neonat (left) and the same samples were analysed as fresh lysates on the Capillarys ® 2 (right). The different samples depicted above in Figure 10.5 are: 1; normal sample (left HbA, right HbF fraction), 2; HbS heterozygote (HbA preceding and HbS following HbF), 3; HbC heterozygote, 4; HbE heterozygote, and 5; a term ß thalassemia carrier. Adapted from (6)

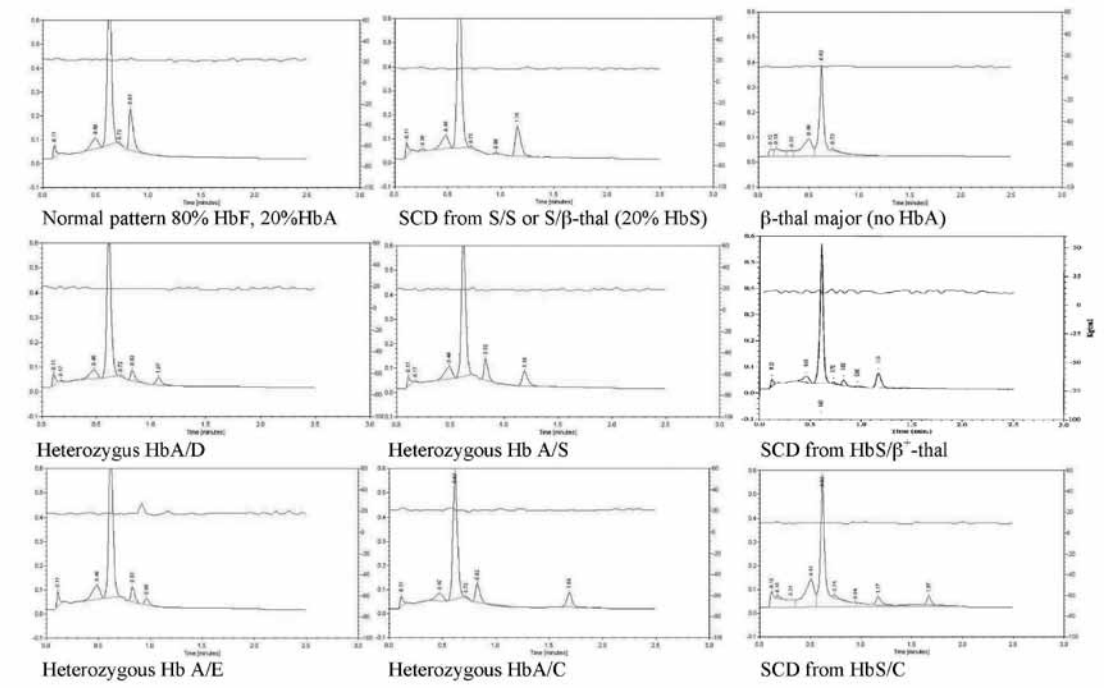


Figure 10.5 Some common examples of neonatal Hb patterns on HPLC.

Some common examples of neonatal Hb patterns on the Bio-Rad NBS are shown above in Figure 10.5. The normal Hb separation pattern of the newborn consists of two fractions with some derivates. After 36 weeks pregnancy the main fraction is HbF with an expression average of 80%. The second fraction is HbA with on average 20% expression. In case of sickle cell disease or ß-thalassaemia major no HbA will be present. In sickle cell disease, HbA is substituted by the HbS fraction only or by the common combinations HbS/HbC, HbS/HbE or HbS/HbD. Other combinations should be considered suspect and investigated further. Carriers of these common traits are also clearly detectable in the presence of HbA. Because of the short cycles, traces of normal fractions can be carried over to the next run. Therefore when the HbA level is below 5% and the neonate is not significantly premature, probable ß-thalassaemia major or intermedia must be considered and investigated further. At birth, the HbA2 is usually below detection levels. When HbA_2 is visible, HbF is lower and HbA is higher than expected, then a blood transfusion has probably been given to the baby, either intrauterine or shortly after birth. These cases should be investigated to exclude large deletion defects or other intrauterine haemoglobinopathies (8).

10.6 THE STEPS FOLLOWING PUTATIVE IDENTIFICATION ON NEWBORN SCREENING

Based on a likely pathological NBS result, provisional action includes referral to specialized care and/or laboratories. According to the confirmed genotype, subsequent steps include counseling the family on their options, including treatment of an affected child, testing the parents and information on their reproductive options. However, the steps followed and the information provided will differ for newborn affected babies than for carriers and misinformation and unnecessary anxiety should be avoided.

The affected child. For parents who receive test results indicating a presumed affected child health, practitioners should direct them and their child to have a confirmatory DNA test. If the disease is indeed confirmed the baby should be referred to a specialist Haemoglobinopathy centre for management. The parents should be fully informed about the expected prognosis and treatment options. In addition, in the context of retrospective primary prevention, the parents should be referred for extensive counseling about their reproductive options although this may have been done prior to or in early pregnancy.

The healthy carrier. For parents who receive a result that their child is a presumed healthy carrier, it should be emphasized by the health practitioners that a carrier is not a disease-state. However, both parents should be offered tests to investigate their carrier status and if at risk be offered genetic counseling. For the common beta thalassaemia traits this is possible without molecular analysis. However when both parents are beta thalassaemia carriers, they should be referred to a specialized lab for genotype confirmation and phenotype prediction. It is preferable that the DNA tests, evaluation of results, counseling and eventually prospective primary prevention (prenatal diagnosis) for the next child (if requested) be done in a single genetics centre and the same laboratory. The flowchart for referral of patients and carriers is summarized in Figure 10.6.

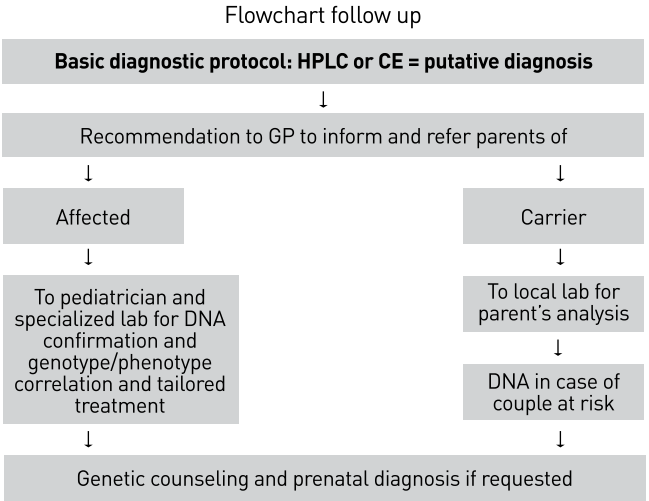


Figure 10.6: Flowchart for referral of patients and carriers following NBS

Why genotype confirmation?
Genotype analysis is essential for both parents and newborns for several reasons: 1) to exclude false positive cases like variants mimicking HbS, C, E or D (33) and false thalassaemia major pattern caused by Hereditary Persistence of Foetal Haemoglobin (HPFH); 2) to support the management of the disorder and thus the most appropriate therapy; 3) to predict the genetic risk for the next child and to provide genetic information to the at risk couple, so they can make an informed reproductive choices for future pregnancies.

10.7 DETECTING AND REPORTING CARRIERS FOUND DURING NEWBORN SCREENING.

Like with any other Hb separation method, healthy carriers are easily detected with NBS methods. Carriers of the common abnormal Hb's are easily recognized by the presence of both normal HbA and the abnormal HbS, C, E, D etc. Reporting carriers is also an advantage and especially relevant for carriers of HbS who may risk serious complication during anesthesia if not diagnosed (11, 12). Other less common Hb variants must also be reported for characterization to exclude or confirm a possible associated risk. As discussed above, it may also lead to the identification of at risk couples.

Alpha thalassaemia, patients and carriers. In contrast with the severe ß-globin gene mutations that are usually asymptomatic at birth, the α-globin gene mutations are already expressed during embryonic, foetal and postnatal life. Therefore, newborns with defective α-globin genes will be slightly or severely anemic already in utero, depending on the number of compromised α-globin genes.

In all newborns with α-thalassaemia the non functional abnormal fraction Hb Bart's will always be detected. To some extent, the amount of Hb Bart's correlates with the number of defective α-globin genes. A simple mild condition with a single gene defect (-α/αα) will present with very little Hb Bart's (1-4%), while in samples with two defective α-genes, either in cis (--/αα) or in trans (-α/-α), the Hb Bart's will rise to 5-15%, reaching 25% and higher in HbH disease with 3 defective α-genes (--/-α) (13-15).

Reporting HbH disease has the advantage of providing a definitive cause for anaemia management and treatment. Reporting α-thalassaemia carriers potentially provides early diagnosis to a large category of carriers, who otherwise may be at risk for drawn-out mistaken and expensive attempts at diagnosis and likely unnecessary treatment with iron. Moreover, at risk couples for having offspring with severe hydrops foetalis or severe HbH disease may be identified and informed of their reproductive, and thus the potential for retrospective or prospective primary prevention.

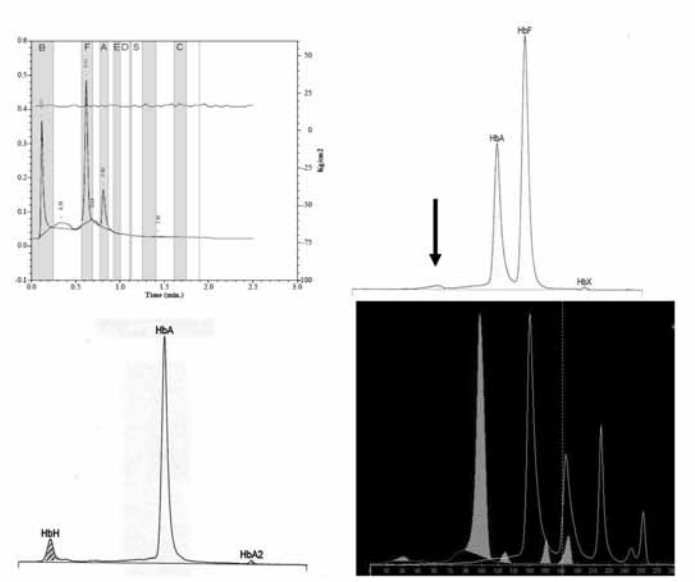


Figure 10.7 Newborn screening results for α-thalassaemia

Alpha thalassaemia screening results are shown above in Figure 10.7. Above left: a newborn with Hb Bart's in a percentage compatible with HbH disease (courtesy of M. Bouva et al. 2011). Above right: Hb Bart's in a very low amount (↓) on CE as found in a newborn carriers of the most common single gene deletion (-$\alpha^{3.7}$/αα). Below left: a postnatal pattern of an HbH disease (-α/--) on CE. Below right again on CE (Sebia), the pattern of an α^{o}-thalassaemia homozygote (--/--), the severe Hb Bart's hydrops foetalis, with from left to right: traces of HbH (β4); Hb Bart's (γ4); probably (ε4); probably Hb Gower 1 ($\zeta_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$).

The β-thalassamia carrier. Carriers of β-thalassaemia cannot be diagnosed by measuring their elevated HbA_2 expression at birth. This is because HbA_2 is not sufficiently expressed to be a diagnostic parameter until one year of age. Nevertheless, carriers can be putatively diagnosed at birth measuring the HbA expression (see Table 10.1).

Table 10.1 Details of 4 examples of newborn β-thalassaemia carriers putatively detected by their low % HbA at 40 week of gestation and confirmed by molecular analysis (16).

Weeks of pregnancy	% HbA	Mutation (HGVS)	Mutation	Mutation type
40	13.8	HBBc.*96T→C	+1570 t>c	$Beta^{+}$
41	8.2	HBBc.-50-30T→A	–30 (t→a)	$Beta^{+}$
40	8.6	HBBc.316-125A→G	IVS2-726a>g	$Beta^{o}$
40	12.1	HBBc.*96T→C	+1570 t>c	$Beta^{+}$

Newborn carriers of ß-thalassaemia do not express one of their ß-globin genes and are born with a reduced HbA level. Carriers can then be recognized with reasonable sensitivity by measuring the expression of HbA at birth according to the weeks of gestation (16). Once the suspected carrier is putatively recognized, the carrier state can be confirmed in the parents. This will also allow an informed reproductive choice in future pregnancies to any at risk couples identified, who would otherwise remain undetected until the birth of the first affected child. Moreover, extended family analysis will allow detection of carrier siblings and even other at risk couples. In addition, early detection of the newborn carrier will prevent unnecessary iron therapy and attempts at definitive diagnosis, as is common the case for carriers of α-thalassaemia.

10.8 WHAT CAN GO WRONG WITH NEWBORN SCREENING?

Some possible causes of technical and follow up errors are summarized below. Although technically the detection of haemoglobinopathies at birth is simple, mistakes can occur if the results are not correctly interpreted (17).

- **Missing ß-thalassaemia major.** As it is established that the level of normal or abnormal fractions at birth in non premature newborn must be ≈ 80% HbF and ≈ 20% HbA; the absence of HbA is pathomnemonic for ß-thalassaemia major. However, some HbA (2-3% or less) can also be present in $ß^+$-thalassaemia major (or $ß^+$-thalassaemia /HPFH). Samples with 3% or even up to 5% HbA should be considered as possible ß-thalassaemia intermedia or beta thalassaemia carriers and should be investigated further.

- **HPFH.** The absence of HbA can also be caused by homozygosity for deletional HPFH (very rare) or a ß0-thalassaemia/HPFH combination.

- **Homozygous HbS.** The absence of HbA and the presence of about 20% HbS indicates homozygous HbS or the compound heterozygous genotype of HbS/ß-thalassaemia. These cases, as well as all unusual fractions must be reported and investigated at the molecular level.

- **Homozygous HbE.** This mild condition shows HbF and HbE without HbA however the same pattern is compatible will severe HbE/ß0-thalassaemia. Likewise, homozygous HbC, HbD etc. can be confused with combined heterozygosis with ß0-thalassaemia.

- **Hb Bart's.** The presence of this fraction is the simplest way to identify α-thalassaemia at birth. However, by reporting only the cases with Hb Bart's compatible with HbH disease, a significant health gain of newborn screening will be wasted (18).

- **Unstable ß-gene variants.** These semi dominant traits will not be detected with newborn screening but will manifest after about 6 months with haemolysis of variable severity.

10.9 CONCLUSIONS

The health gain obtained with newborn screening of the haemoglobinopathies can be summarized in five categories:

1. morbidity prevention for the affected newborns;
2. retrospective primary prevention for the parents who are at risk and had an affected child;
3. prospective primary prevention for those couples who are at risk and had a carrier child;
4. health gain in the treatment of an anaemic carrier and accident prevention particularly during anaesthesia for HbS carriers
5. long term information for healthy carriers for future partner testing and reproductive choices.

As discussed in the introduction, newborn screening is not a method designed for primary prevention but for morbidity prevention. Even reporting all carriers and having full compliance and collaboration from parents and health professionals, prospective primary prevention is unlikely to reach 50% of the couples at risk of having an affected child. This is because at risk couples who have a healthy child are not detected and couples who have an affected child, have already missed prospective prevention. Nevertheless, where newborn screening is ongoing, it should continue both for morbidity and prevention while for primary prevention, public health authorities should focus on the detection of carriers at an earlier stage.

Ethical objections. Objections have been made, mainly in non-endemic countries. The concerns are that giving information to healthy carriers of recessive traits could be harmful rather then beneficial. Information could cause unnecessary worry and influence their reproductive choice, in contrast to the process of natural and non-informed reproduction (19). Many of the ethical objections concern also revealing the carrier status to minors. These concerns seem however misplaced when informing parents of newborns or school children. Obviously, it is not the newborn or the child that receive the information in these cases, but the parents who will then decide how to use the information for themselves and how and when to inform their children in the future. In two studies, one among informed carriers in the UK and the other among parents of screened secondary school children receiving information, no particular harmful effect were registered (20-21).

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ANNEXE 01

GLOBAL EPIDEMIOLOGY TABLES

Notes:
These tables are copied from the TIF database. The information provided is derived from published reports where possible but is supplemented or modified from information gathered by TIF delegations and contacts with local experts.

Published results of surveys are often inaccurate since they depend on small samples often not representing the whole population. Each publication is therefore judged and used according to its merits, including sample size, the population group studied, geographical location within a country, methodology used etc. For a minority of countries, micro-mapping has been possible.

For Europe and the Americas the population structure was studied with particular reference to the populations that have migrated from high prevalence parts of the world. For each population group the carrier rate was calculated and the total carriers were added to the carriers of the indigenous population so that an updated carrier rate could be calculated for each country. The main source of migrant numbers is the Eurostat database for Europe and other internet sources (such census reports) for the Americas.

Because of the difficulties in obtaining accurate information, this database is regularly reviewed and new information is considered.

Concerning patient numbers the information relies on the rare national registries and on information obtained from local associations and medical collaborators.

Because of these difficulties the tables presented must be regarded as the best current estimates. It is however a dynamic database which is useful for planning both for the interested NGOs and health authorities.

Annexe Table 1.1 East Mediterranean region.

Country	Population millions	% HbS carriers	Annual SCD Births expected	% ß-thal. Carriers	Expected homozygote births/year	Known ß-thal. homozygotes	Known SCD patients
Afghanistan	28.4	0	0	3	269	na	na
Algeria	35.7	1.25	100	2	61	3000	4711
Bahrain	1.2	11	61	3.5	4	300	600
Egypt	82.08	0.2	109	5.3	1423	9912	1166
Iran	77.9	1	371	6	1027	18616	na
Iraq	30.4	0.7	157	4.8	502	10808	3620
Jordan	6.5	1.5	40	3.5	38	1300	150
Kuwait	2.6	6	100	3	12	129	500
Lebanon	4.14	2	28	3.5	19	678	387
Libya	6.6	2	40	1.5	9	na	na
Morocco	34.8	1.76	164	1.67	51	375	na
Oman	3.02	5.7	105	3.1	16	591	4704
Pakistan	187.3	0.27	356	6	4600	50000	na
Palestine Gaza	1.7	1	14	4.2	26	162	na
Palestine West Bank	3.8	1.5	31	3.8	33	526	131
Qatar	0.84	5	18	3	3	146	280
Saudi Arabia	26.1	4.2	473	2.37	184	600	26000
Sudan	45.04	2.25	917	3.9	617	na	na
Syria	22.5	0.5	71	5	338	7700	100
Tunisia	10.3	1.9	49	2.21	20	742	1526
UAE	5.1	1.1	36	8	123	1000	na
Yemen	24.1	2.2	489	4.4	391	800	na

Abbreviations: SCD, sickle cell disease; thal, Thalassaemia; homoz, homozygote/s

Annexe Table 1.2 America

Country	Population millions	% HbS carriers	Expected SCD births/year	% ß-thal. Carriers	Expected ß-thal births/year	Known ß-thal patients	Known SCD patients
Argentina	41.8	0.7	44	1.35	33	na	na
Bahamas	0.316	10	15	1	<1	na	na
Barbados	0.29	7	5	0.5	<1	na	na
Brazil	203.4	4	2417	1.3	155	na	25-50000
Canada	34.3	0.3	3	0.5	4	300	na
Colombia	45.2	2.4	187	0.8	12	na	na
Cuba	11.05	3.08	39	0.75	2	na	4000
Dominican Republic	10.1	4.8	160	1	5	na	na
Haiti	9.8	15	1492	1	6	na	6000
Jamaica	2.89	10	177	1.5	3	na	na
Trinidad & Tobago	1.22	9.9	74	3.5	7	63	na
USA	312.8	0.75	115	0.4	24	716	70-100000
Venezuela	28.04	1.96	63	0.2	1	na	na

Abbreviations: SCD, sickle cell disease; thal, Thalassaemia; homoz, homozygote/s

Annexe Table 1.3 Europe

Country	Population millions	Immigrants (%) from at risk populations	% ß-thal. Carriers immigrants + indig	% HbS carriers immigrants + indig	Expected ß-thal Births/year if no prevention	Known ß-thal. homozygotes	Known SCD patients	Known ß-thal patients
Albania	3.2		5	1.4	18	12	530	500
Armenia	2.97		2	0	4	0	0	199
Austria	8.2	4.70%	0.24	0.06	<1	<1	na	na
Azerbaijan	9.5		8	0	263	0	0	642-2000
Belgium	10.4	3.80%	0.28	0.42	<1	1	358	62
Bosnia	4.61		1.2	0	1-2	0	0	na
Bulgaria	7.36	0.40%	2.5	0	11	0	0	270
Croatia	4.5		0.8	0	1	0	0	na
Cyprus	0.84	9.60%	14.4	0.27	52	2	40	681
Czech Rep	10.3	0.8	0.12	0.002	<1	0	0	na
Denmark	5.5	4.70%	0.26	0.11	<1	<1	na	26
France	64.05	4.80%	0.7	0.62	12	26	10000	378
Georgia	4.61		3	0	11	0	na	na
Germany	82.3	5.50%	0.28	0.06	1	1	1500	1600
Greece	10.7	5.80%	8.1	0.6	167	26	1080	3241
Ireland	4.2	1.30%	0.13	na	<1	<1	na	na
Israel	7.59		0.5	1	1	7	70	373
Italy	58.1	4.30%	4.3	2.1	220	267	829	6000
Kazakhstan	15.5		1	0	6	0	0	na
Kyrgyzstan	5.6		1.5	0	7	0	0	na
Luxenburg	0.49	22.80%	0.5		<1	<1	na	na
Malta	0.4		3		1			21
Netherlands	16.7	7.30%	0.4	0.18	1	1	616	140
Norway	4.66	4.80%	0.3		<1	<1	na	48
Portugal	10.7	3.44%	1.44	1.13	6	12	500	40
Romania	21.9	0.30%	1	0.002	5	0	0	200-300
Russian Fed.	147.2	2.30%	0.18		1	<1	na	na
Serbia	7.38		1.2		2	<1	na	na
Slovenia	20.5	5.90%	0.18		<1	<1	na	na
Spain	40.5	5.40%	1.64	0.3	27	11	350	27
Sweden	9.05	5.40%	0.32	0.09	<1	<1	100	50
Switzerland	7.6	13.80%	0.4		<1	<1	na	na
FYR Macedonia	2.06		2.6	0	4	<1	na	na
Turkey	79.7		2.2	0.44	173	76	1050	3264
Turkmenistan	5.05		1.2	0	4	0	na	na
United Kingdom	62.6	8%	0.44	2.5	4	142	12000	900
Uzbekistan	28.6		3	0	112	0	na	188

Abbreviations: SCD, sickle cell disease; thal, Thalassaemia; homoz, homozygote/s; indig. indigenous

Annexe Table 1.4 Asia

Country	Population millions	% HbE carriers	% ß-thal carriers	Expected ß-thal syndromes Births/year	Known patients
East Asia					
Bangladesh	161.1	6.1	4.1	5477	6880
Bhutan	0.7	4	na	na	na
India	1270.0	1	3.9	16200	100000
Maldives	0.4	0.9	18	54	574
Myanmar	54.6	22	2.2	2398	4079
Nepal	29.4	4.4	4	836	400
Sri Lanka	21.5	0.5	2.2	64	3410
Brunei Darussalam	0.4		2	1	179
Indonesia	245.6	6(1-25)	5(3-10)	9619	5000
Thailand	67.1	30	5	6983	35000
Timor Leste	1.2	6	5	65	na
Asia Pacific					
Cambodia	14.7	30	3	1762	na
Lao Peop.Dem.Rep.	6.6	18	6	1106	na
Malaysia	28.7	3.4	4.5	727	1500
Philippines	101.8	0.4	1.2	153	600
Singapore	4.7	0.64	3	13	154
Austarlia	21.6	0.4	0.4	3	332
Viet Nam	90.5	1	2.6	424	1000
Taiwan	22.9	0.027	2	21	400
Chinese territories					
Hong Kong	7.05	0.3	3.5	19	363
Guanxi	49.2	0.42	6.78	902	na
Guangdong	91.9	0.06	2.54	182	na
Yunnan	44.5	1.6	3.7	418	na
Guizhu	39.3	na	1.1	17	na
Sichuan	87.2	na	2.18	94	na
Hainan	82.8	na	2.09	132	na
Macau	0.55	na	3	2	na
Fujian	36	na	1.32	18	na

Abbreviations: thal, thalassaemia

ANNEXE 02

BETA THALASSAEMIA MUTATION DATA TABLES

Annexe Table 2.1 Beta thalassaemia point mutations

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
–190 (G→A)	c.-240G→A		β^{++}	Moroccan	442
–102 (C→A)	c.-152C→A		β^{++} (silent)		417
–101 (C→T)	c.-151C→T		β^{++} (silent)	Mediterranean	1
–101 (C→G)	c.-151C→G		β^{++} (silent)	Italian	362
–93 (C→G)	c.-143C→G		β^{+}	Surinam	GGS pc
–92 (C→T)	c.-142C→T		β^{+} (silent)	Mediterranean	2-5
–90 (C→T)	c.-140C→T		β^{+}	Portuguese	6
–88 (C→T)	c.-138C→T		β^{++}	African-American, Asian Indians	7, 8
–88 (C→A)	c.-138C→A		β^{+}	Kurds	9
–88 (C→G)	c.-138C→G		?	British	GGS pc
–87 (C→G)	c.-137C→G		β^{++}	Mediterranean	10, 11
–87 (C→T)	c.-137C→T		β^{++}	German, Italian	11, 12
–87 (C→A)	c.-137C→A		β^{++}	African-American	13
–86 (C→G)	c.-136C→G		β^{+}	Thai, Lebanese	2, 14
–86 (C→A)	c.-136C→A		β^{++}	Italian	11
–83 (G→A)	c.-133G→A		β^{+}	Gabon	487
–73 (A→T)	c.-123A→T		β^{++}	Chinese	397
–71 (C→T)	c.-121C→T		β^{+}	Omani	467
–56 (G→C)	c.-106G→C		?	Moroccan. Algerian	GGS pc, 442
–50 (G→A)	c.-100G→A		?	Chinese	440
–41 (A→C)	c.-91A→C		β^{+}	Iranian	478
–32 (C→A)	c.-82C→A		β^{+}	Taiwanese	15
–32 (C→T)	c.-82C→T		β^{+}	Hispanic	412
–31 (A→G)	c.-81A→G		β^{+}	Japanese	16
–31 (A→C)	c.-81A→C		β^{+}	Italian	17
–30 (T→A)	c.-80T→A		β^{+}	Mediterranean, Bulgarian	18
–30 (T→C)	c.-80A→C		β^{+}	Chinese	19
–30 (T→G)	c.-80A→G		β^{+}		GGS pc
–29 to –26 (–AA)	c.-79_78delAA		β^{+}	African-American	355
–29 (A→G)	c.-79A→G		β^{+}	African-American, Chinese	20, 21
–29 (A→C)	c.-79A→C		β^{+}	Jordanian	371
–29 (G→A)	c.-79G→A		β^{+}	Turkish	428

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
–28 (A→C)	c.-78A→C		β+	Kurds	22
–28 (A→G)	c.-78A→G		β+	African-American, Southeast Asians	20, 21, 23
–27 (A→T)	c.-77A→T		β+	Corsican	24
–27 (–AA)	c.-77_-76delAA		?		412
–26 (A→C)	c.-76A→C		β+		469
–25 (G→C)	c.-75G→C		β+		412
–23 to +23 (+45 bp duplication)	c.-73_-8del45		β+	Maori	360
CAP+1 (A→C)	c.-50A→C		β++ (silent)	Asian Indians	25
CAP+8 (C→ T)	c.-43C→T		β++ (silent)	Chinese	26, 406
CAP+10 (–T)	c.-41delT		β++ (silent)	Greeks	27
CAP+20 (C→T)	c.-31C→T		β++	Bulgarian	1
CAP+22 (G→A)	c.-29G→A		β++	Mediterranean, Bulgarian,Turkish	28, 29, 363
CAP+33 (C→G)	c.-18C→G		β++ (silent)	Greek Cypriot	30
CAP+39 (C→T)	c.-18C→T		β+	Chinese	453
CAP+40 to +43 (–AAAC)	c.-11_-8delAAAC		β+	Chinese	31
CAP+45 (G→C)	c.-3G→C		β++ (silent)	Italian	359
Initiation Cd (ATG→GTG)	c.1A→G		β0	Japanese	99
Initiation Cd (ATG→CTG)	c.1A→C		β0	British	357
Initiation Cd (ATG→ACG)	c.2T→C		β0	Yugoslavian	100
Initiation Cd (ATG→AGG)	c.2T→ G		β0	Chinese	101
Initiation Cd (ATG→AAG)	c.2T→ A		β0	N European	102
Initiation Cd (ATG→ATC)	c.3G→C		β0	Japanese	58
Initiation Cd (ATG→ATA)	c.3G→A		β0	Italian, Swedish	103, 104
Initiation Cd (ATG→ATT)	c.3G→T		β0	Iranian	45
Cd 1 (–G)	c.4delG		β0	Mediterranean	117
Cd 2-4 (–9, +31 bp)	c.7_15delinsCCT GAGGTGAAGT CTGCCTGAGG AGAAGTCT		β0	Algerian	118
Cd 3 (+T)	c. 9_10insT		β0	Turkish	379
Cd 3 (+T), CD 5 (–C) [Leu-Thr-Pro Ser-Asp-Ser]	c.[16delC; 9_10insT]	Hb Antalya	β+	Turkish	367
Cd 5 (–CT)	c.17_18delCT		β0	Mediterranean	119

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
Cd 26 (GAG→TAG)	c.79G→T		β⁰	Thai	107
Cd 26 (+T)	c.79_80insT		β⁰	Japanese	130
Cd 27 (GCC→TCC) [Ala→Ser]	c.82G→T	Hb Knossos	β⁺	Mediterranean	87-90
Cd 27/28 (+C)	c.84_85insC		β⁰	Chinese, Thai	131, 132
Cd 28 (–C)	c.85delC		β⁰	Egyptian	133
Cd 28 (CTG→CGG) [Leu→Arg]	c.86T→G	Hb Chesterfield	Dominant	English	192
Cd 28/29 (–G)	c.88delG		β⁰	Japanese, Egyptian	58, 133
Cd 29 (C→T) or IVSI (–3) (GGC→GGT)	c.90C→T		β⁺	Lebanese	65
Cd 30 (A→G) or IVSI (–2) (AGG→GGG)	c.91A→G		β⁰	Sephardic Jews	32
Cd 30 (A→C) or IVSI (–2) (AGG→CGG)	c.91A→C		β⁺		401
Cd 30 (G→A) or IVSI (–1) (AGG→AAG)	c.92G→A	Hb Monroe	β⁰	Bulgarian	420
Cd 30 (G→C) or IVSI (–1) (AGG→ACG) [Arg→Thr]	c.92G→C		β⁰	Mediterranean, African-American, N Africans, Kurds, UAE	9, 33-36
IVSI-1 (G→A)	c.92+1G→A		β⁰	Mediterranean	38
IVSI-1 (G→T)	c.92+1G→T		β⁰	Asian Indian, SE Asian, Chinese	39
IVSI-1 (G→C)	c.92+1G→C		β⁰	Japanese	330
IVSI-2 (T→G)	c.92+2T→G		β⁰	Tunisian	33
IVSI-2 (T→C)	c.92+2T→C		β⁰	African-American	40
IVSI-2 (T→A)	c.92+2T→A		β⁰	Algerian, Italian	41, 42
IVSI-5 (G→C)	c.92+5G→C		β⁰	Asian Indian, SE Asian, Melanesian	10, 60
IVSI-5 (G→T)	c.92+5G→T		β⁺	Mediterranean, N European	61, 62
IVSI-5 (G→A)	c.92+5G→A		β⁺	Mediterranean, Algerian	63
IVSI-5 (G→A) + Corfu del	c.92+5G→A		β⁺	Greeek	421,422
IVSI-6 (T→C)	c.92+6T→C		β⁺	Mediterranean	38, 64
IVSI-7 (A→T)	c.92+7A→T		β⁺	Italian	GGS pc
IVSI-109 (-T)	c.93-20delT		?	Iranian	478
IVSI-110 (G→A)	c.93-21G→A		β⁺	Mediterranean	73, 74
IVSI-116 (T→G)	c.93-15T→G		β⁰	Mediterranean	75
IVSI-128 (T→G)	c.93-3T→G		β⁺	Saudi Arabian	66
IVSI-129 (A→C)	c.93-2A→C		β⁰	Sri Lankan	49
IVSI-129 (A→G)	c.93-2A→G		β⁰	German, Chinese	67, 455
IVSI-130 (G→C)	c.93-1G→C		β⁰	Italian, Japanese, UAE	36, 50, 51
IVSI-130 (G→A)	c.93-1G→A		β⁰	Egyptian	52, 53
IVSI-130 (+1) or CD 30, (G→C); (AGG→AGC)	c.93G→C		β⁰	Middle East	36
IVSI [3' end] (–17 bp)	c.93-17_93-		β⁰	Kuwaiti	46

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
	1delTATTTTCC CACCCTTAG				
IVSI [3' end] (–25 bp)	c.93-21_96del		β⁰	Asian Indian, UAE	47
IVSI [3' end] (–44 bp)	c.76_92+27del		β⁰	Mediterranean	48
IVSI [3' end] (+22bp)			β⁰	Thai	429
Cd 30/31 (+CGG) [+Arg]	c.93_94insCGG		Dominant	Spanish	193
Cd 31 (–C)	c.94delC		β⁰	Chinese	135
Cd 32 (CTG→CAG) [Leu→Glu] in cis with Hb Köln: CD 98 (GTG→ATG) [Val→Met]	c.[295G→A; 98T→A]	Hb Medicine Lake	Dominant	US Caucasian	194
Cd 35 (–C)	c.108del		β⁰	Malay	81
Cd 33-34 (–GGT) [–Val]	c.100_102delGGT	Hb Korea	Dominant	Korean	195
Cd 33-34 (–G)	c.103delG		β⁰	Thai	438
Cd 33-35 (–6 bp) [Val-Val-Try→Asp]	c.101_106del TGGTCT	Hb Dresden	Dominant	German	196
Cd 35 (TAC →TGA)	c.107 A→G		β⁰		435
Cd 35 (TAC→TAA)	c.108C→A		β⁰	Thai	14, 108
Cd 36 (–C)	c.109delC		β⁰	Chinese	536
Cd 36/37 (–T)	c.112delT		β⁰	Kurd, Iranian	9, 45
Cd 36-39 (–8 bp)	c.111_118del TTGGACCC		β⁰	Asian Indian	364
Cd 37 (–G)	c.114delG		β⁰	Kurd	
Cd 37 (TGG→TGA)	c.114G→A		β⁰	Saudi Arabian	109
Cd 37 (TGG→TAG)	c.113G→A		β⁰	Afghanistani, Chinese	361, 374
Cd 37-39 (–7 bp (–GACCCAG)	c.114_120del GACCCAG		β⁰	Turkish	136
Cd 38/39 (–CC)	c.116¬_117delCC		β⁰	Belgian	138
Cd 38/39 (–C)	c.118delC		β⁰	Czechoslovakian	137
Cd 39 (CAG→TAG)	c.118C→T		β⁰	Mediterranean	110-112
Cd 40 (–G)	c.123delG		β⁰	Japanese	58
Cd 40 (+86 bp)	c.121_122ins86		β⁰	Portuguese	134
Cd 40/41 (+T)	c.123_124insT		β⁰	Chinese	135
Cd 41 (–C)	c.126delC		β⁰	Thai	139
Cd 41/42 (–TTCT)	c.124_127delTTCT		β⁰	Chinese, SE Asian, Indian	39, 140
Cd 42/43 (+T)	c.129_130insT		β⁰	Japanese	141
Cd 42/43 (+G)	c.129_130insG		β⁰	Japanese	58
Cd 43 (GAG→TAG)	c.130G→T		β⁰	Chinese, Thai	113
Cd 44 (–C)	c.135delC		β⁰	Kurdish	9, 142
Cd 45 (–T)	c.136delT		β⁰	Pakistani	36
Cd 45 (+T)	c.136_137insT		β⁰	Turkish	413
Cd 45/46 (+A)	c.38_139insA		β⁰		411
Cd 47 (+A)	c.143_144insA		β⁰	Surinmese	143
Cd 48 (+ATCT)	c.146_147insATCT		β⁰	Asian Indian	144, 145

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
Cd 48 (–T)	c.146delT		β⁰	Uruguayan	533
Cd 49 (–C)	c.149delC		β⁰	Jordanian	371
Cd 50 (–T)	c.153delT		β⁰		434
Cd 51 (–C)	c.154delC		β⁰	Hungarian	146
Cd 53 (–T)	c.161delT		Dominant	Chinese	398
Cd 53/54 (+G)	c.162_163insG		β⁰	Japanese	114
Cd 54 (–T)	c.165delT		β⁰	Swedish	147
Cd 54/55 (+A)	c.165_166insA		β⁰	Asian Indian	144
Cd 54-58 (–13 bp)	c.165_177delTA TGGGCAACCCT		β⁰	Chinese	436
Cd 55 (–A)	c.166delA		β⁰	Sri Lankan	49
Cd 56-60 (+14 bp duplication)	c.170_183ins14		β⁰	Iranian	148
Cd 57/58 (+C)	c.174_175insC		β⁰	Asian Indian	145
Cd 59 (–A)	c.178delA		β⁰	Italian	149
Cd 59 (AAG→TAG)	c.178A→T		β⁰		331
Cd 60 (GTG→GAG) [Val→Glu]	c.182T→A c.186_192delGG	Hb Cagliari	Dominant	Italian	197
Cd 61-63 (–7 bp) (–GGCTCAT)	CTAT c.184A→T		β⁰	Italian	98
Cd 61 (AAG→TAG)			β⁰	African-American	71
Cd 64 (–G)	c.193delG		β⁰	Swiss	150
Cd 66 (AAA→TAA)	c.199A→T		β⁰		373
Cd 66 (–A)	c.201delA		β⁰		476
Cd 67 (–TG)	c.202_203delGT		β⁰	Filipino	151
Cd 69 (–T)	c.210delT		β⁰	Indian	460
Cd 71/72 (+T)	c.216_217insT		β⁰	Chinese	152
Cd 71/72 (+A)	c.216_217insA		β⁰	Chinese	76
Cd 72-73 (–5, +1 bp) (–AGTGA, +T)	c.217_221delinsT		β⁰	British	153
Cd 72/73 (+T)	c.219_220insT		β⁰	British	GGS pc
Cd 74/75 (–C)	c.225delC		β⁰	Turkish	154, 155
Cd 76 (–GC)	c.229_230delGC		β⁰	North African	416
Cd 76 (–C)	c.230delC		β⁰	Italian	156, 157
Cd 77/78 (–C)	c.232delC		β⁰	Mexican	334
Cd 80/81 (–C)	c.243delC		β⁰	Iranian	372
Cd 81-87 (–22 bp)	c.244_265del22		β⁰	Asian Indian	366
Cd 82 (A→T)	c.247A > T)		?	Asian Indian	477
Cd 82/83 (–G)	c.250delG		β⁰	Czech, Azerbaijan	158, 159
Cd 83-86 (–8 bp) (–CACCTTTG)	c.252_259del		β⁰	Japanese	130
Cd 83 (+65 bp duplication)	c.251.252ins65		β⁰	Ghanaian	539
Cd 84/85 (+C)	c.255_256insC		β⁰	Japanese	58
Cd 84-86 (+T)	c.258_259insT		β⁰	Japanese	58

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
Cd 88 (+T)	c.266_267insT		β^0	Asian Indian	79
Cd 88 (–TG)	c.266_267del		β^0	Japanese	130
Cd 89/90 (–GT)	c.269_270delGT		β^0	Japanese	58
Cd 90 (GAG→TAG)	c.271G→T		β^0	Japanese	114
Cd 91 (–T) [→156aa]	c.275delT	Hb Morgan Town	Dominant	Irish	335
Cd 91 (+T)	c.275_276insT		β^0	Belgian	33
Cd 94 (+TG) [→156aa]	c.283_284insTG	Hb Agnana	β^0	S. Italian	198
Cd 95 (+A)	c.287_288insA		β^0	SE Asian	160, 161
Cd 98 (GTG→ATG) [Val→Met] in cis with Cd 32 (CTG→CAG) [Leu→Glu]	c.[295G→A; 98T→A]	Hb Medicine Lake	β^0	Caucasian	194
Cd 100 (–3,+8 bp) (–CTT, +TCTGAGAACTT) [→158aa]	c.301_303delins TCTGAGAACTT		Dominant	S. African	199
Cd 104 (–G) [→156aa]	c.314delG		β^0	German	GGS pc
IVSII-1 (G→A)	c.315+1G→A		β^0	Mediterranean, African-American	43, 44
IVSII-1 (G→C)	c.315+1G→C		β^0	Iranian	45
IVSII-1 (G→T)	c.315+1G→T		β^0	Surinam	GGS pc
IVSII-2 (T→C)	c.315+2T→C		β^+	Indian	GGS pc
IVSII-2 (T→A)	c.315+2T→A		β^+	Turkish	GGS pc
IVSII-2 (–T)	c.315+2del		β^0	Chinese	26
IVSII-2 (–13 bp)	c.315+2_315+13del TGAGTCTATGGG		?	British	GGS pc
IVSII-2,3 (+11, –2 bp)	c.315+2_315+delins ACGTTCTCTGA		Dominant	Iranian	217
IVSII-4/5 (–AG)	c.315+4_315delAG		β^0	Portuguese	6
IVSII-5 (G→C)	c.315+5G C		β^+	Chinese	68
IVSII-535 to CD108 (+23, –310, +28 bp)	c.[316-300_327 delinsCAGGTGC CATCTGTCACCC TTTTCTTTG;316 -316_316-315ins AATATATTTTTAA TATACTTTTT]	Hb Jambol	Dominant	Bulgarian	426
IVSII-613 (C→T)	c.316-238C→T		?	Indian	439
IVSII-654 (C→T)	c.316-197C→T		β^{0}/β^+	Chinese, SE Asian, Japanese	76, 77
IVSII-705 (T→G)	c.316-146T→G		β^+	Mediterranean	78
IVSII-726 (A→G)	c.316-125A→G		?	Moroccan	GGS pc
IVSII-745 (C→G)	c.316-106C→G		β^+	Mediterranean	38
IVSII-761 (A→G)	c.316-90A→G		?		GGS pc
IVSII-781 (C→G)	c.316-70C→G		?	African	GGS pc
IVSII-815 (C→T)	c.316-36C→T		?	Thai	438
IVSII-837 (T→G)	c.316-14T→G		?	Asian Indian	79

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
IVSII-843 (T→G)	c.316-8T→G		β^{+}	Algerian	69
IVSII-844 (C→A)	c.316-7C→A		β^{++} (silent)	Ghanain	427
IVSII-844 (C→G)	c.316-7C→G		β^{++} (silent)	Italian	42, 70
IVSII-848 (C→A)	c.316-3C→A		β^{+}	African-American, Egyptian, Iranian	66, 71
IVSII-848 (C→G)	c.316-3C→G		β^{+}	Japanese	72
IVSII-849 (A→G)	c.316-2A→G		β^{0}	African-American	20, 54
IVSII-849 (A→C)	c.316-2A→C		β^{0}	African-American	55
IVSII-850 (G→C)	c.316-1G→C		β^{0}	Yugoslavian	56
IVSII-850 (G→A)	c.316-1G→A		β^{0}	N European	57
IVSII-850 (G→T)	c.316-1G→T		β^{0}	Japanese	58
IVSII-850 (–G)	c.316-1delG		β^{0}	Italian	59
Cd 106 (CTG→GTG) [Leu→Val]	c.319C→G	Hb L'Aquila	β^{+}		410
Cd 106 (–12 bp)	c.319_330delCTG GGCAACGTG			African	GGS pc
Cd 106 (CTG→CGG) [Leu→Arg]	c.320T→G	Hb Terre Haute	Dominant	N European, French	376, 213
Cd 106/107 (+G)	c.321_322insG		β^{0}	African-American, Egyptian	162, 163
Cd 108-112 (–12 bp) [Asn-Val-Leu-Val-Cys→Ser]	c.326_337delACG TGCTGGTCT		Dominant	Swedish, German	200, 433
Cd 109 (–G) [→156aa]	c.328delG	Hb Manhattan	Dominant	Lithuanian, British	201, 357
Cd 110 CTG→CCG) [Leu→Pro]	c.332T→C	Hb Showa-Yakushiji	Dominant	Japanese	202
Cd 112 (TGT→TGA)	c.339T→A		β^{0}	Slovenian	115
Cd 113 (–G) [→156aa]	c.340delG		Dominant	Canadian	425
Cd 114 (CTG→CCG) [Leu→Pro]	c.344T→C	Hb Durham-N.C.	Dominant	US Irish, Italian	203, 204
Cd 114 (–CT, +G) [156aa]	c.343_344delinsG	Hb Geneva	Dominant	Swiss-French	205
Cd 114 (+TGTGCTG)	c.345_346insTGTG CTG	Hb Hradec Kravlove	β^{0}	Irish	GGS pc
Cd 115 (GCC→GAC) [Ala→Asp]	c.347C→A		Dominant	Czech	206
Cd 116 (+TGAT)	c.349_350insTGAT		β^{0}	Sri Lankan	332
Cd 117 (–C)	c.354delC		Dominant	British	GGS pc
Cd 118 (–T) [→156aa]	c.355delT	Hb Sainte Seve	Dominant	French	336
Cd 120 (-A)	c.361delA		Dominant		434
Cd 120/121 (+A)	c.363_364insA		Dominant	Filipino	164
Cd 121 (GAA→TAA)	c.364G→T		Dominant	Caucasian, N European	14, 207, 208, 51, 116
Cd 123 (–A) [→156aa]	c.370delA	Hb Makabe	Dominant	Japanese	209
Cd 123-125 (–ACCCC ACC) [→135aa]	c.370_378delACC CCACCA	Hb Khon Kaen	Dominant	Thai	210

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
Cd 124 (–A) [→156aa]	c.375delA		Dominant	Russian	203
Cd 124-125 (+A)	c.375_376insA		β^0	Irish	GGS pc
Cd 124-126 (+CCA) [+Pro]	c.378_379insCCA		Dominant	Armenian	203
Cd 125 (–A) [→156aa]	c.378delA		Dominant	Japanese	58
Cd 125 (–CCAGTG)	c.376_381delCCA GTG		Dominant	British	GGS pc
Cd 126 (–T) [→156aa]	c.380delT	Hb Vercelli	Dominant	Italian	42
Cd 126 (GTG→GGG) [Val→Gly]	c.380T→G	Hb Neapolis	β^+	Italian, German, Thai	369
Cd 126-131 (–17 bp) [→132aa]	C.380_396delTGC AGGCTGCTATCAG	Hb Westdale	Dominant	Trinidad, Pakistan	211, 212
Cd 127 (CAG→TAG)	c.382C→T		Dominant	English	214
Cd 127 (CAG→CCG) [Gln→Pro]	c.383A→C	Hb Houston	Dominant	US English	201
Cd 127 (CAG→CGG) [Gln→Arg]	c.383A→G	Hb Dieppe	Dominant	French	213
Cd 127/128 (–AGG) [Glu-Ala→Pro]	c.383_385delAGG	Hb Gunma	Dominant	Japanese	209
Cd 128/129 (–4, +5, –11 bp) [→153aa]	c.[385_388delinsC CACA;397_407del AAAGTGGTGGC]		Dominant	Irish	14
Cd 130 (TAT→TAA)	c.393T→A		?		GGS pc
Cd 131 (CAG→TAG)	c.394C→T		β^0	Korean	365
Cd 131 (+A)	c.394_395insA		β^0	British	GGS pc
Cd 131/132 (+GCCT)	c.396_397insGCCT		?	German	433
Cd 131-132 (–GA) [→138aa]	c.396_397delGA		Dominant	Swiss	215
Cd 131-132 (–11bp) [→134aa]	c.396_397del		Dominant	Spanish	424
Cd 132 (AAA→TAA)	c.397A T		β^0	African	407
Cd 134-137 (–12, +6 bp) [Val-Ala-Gly-Val→Gly -Arg]	c.404_413delins GCAG		Dominant	Portuguese	216
Cd 137-139 (–TGGCTA) [Val-Ala-Asn→Asp]	c.413_418delTGG CTA	Hb Stara Zagora	Dominant	Bulgarian	337
Cd 141 (–C) [→156aa]	c.424delC	Hb Florida	Dominant	Argentinian	338
Cd 142 (–C) [→156aa]	c.428delC	Hb Montreal II	Dominant		452
Cd 142 (–CC)	c.428_429delCC		β^0	French	413
Terminating Cd+6, (C→G) [CAP +1480]	c.*+6C→G		β^{++} (silent)	Greek	95, 96
Terminating Cd+32 (C→T)	c.*+32A→C		β^+	Japanese	466
Terminating Cd+39	c.*+39C→T		β^+	Chinese	453

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
(C→T)					
Terminating Cd+47 (C G)	c.*+47C G		β^{++}	Armenian	98
Terminating Cd+90 (–13 bp) [CAP +1565 to +1577]	c.*+91_*+103del GCATCTGGATTCT		β^{+}	Turkish	97
Poly A (A→C) [AATAAA→CATAAA]	c.*+108A→C		β^{++}	Chinese	368
Poly A (A→G) [AATAAA→GATAAA]	c.*+108A→G		β^{+}	Czechoslavakian	430
Poly A (T→C) [AATAAA→AACAAA]	c.*+110T→C		β^{++}	African-American	91
Poly A (T→A) [AATAAA→AAAAAA]	c.*+110A→C		β^{++}	Tunisian	378
Poly A (T→G) [AATAAA→AATGAA]	c.*+111A→G		β^{++}	Mediterranean	92
Poly A (A→G) [AATAAA→AATAGA]	c.*+112A→G		β^{++}	Malay	92
Poly A (A→T) [AATAAA→AATATA]	c.*+112A→T		β^{++}	African	GGS pc
Poly A (A→G) [AATAAA→AATAAG]	c.*+113A→G		β^{++}	Kurd	9
Poly A (–AT) [AATAAA→A--AAA]	c.[*+109_*+110del AT or *+110_*+ 111delTA]		β^{+}	French, African-American	4, 93
Poly A (–AA) [AATAAA→ AAT--A]	c.*+111_*+112del AA		?	British	GGS pc
Poly A (–AATAA) [AATAAA→ -----A]	c.*+108_*+112del AATAA		β^{+}	Kurd, UAE	36, 94
Poly A (–AATAA) [AATAAA→ ------]	c.*+108_*+113del AATAAA		β^{+}	Nigerian	431

Abbreviations: GGS pc: Globin gene server, personal communication; Cd: Codon; aa: amino acids; bp: base pairs.

Annexe Table 2.2 : Beta thalassaemia deletions

Mutation	Size	HGVS	Origin	References
25 bp deletion	25 bp	c.93-21_96del	Middle East	47
26 bp deletion	26 bp	c.20_45del26bp	Indian	465
44 bp deletion	44 bp	c.76_92+27del	Greek, Macedonian	165, 166
105 bp deletion	105 bp	c.-74_31del	Thai	167
125 bp deletion	125 bp			542
290 bp deletion	290 bp	c.-176_92+25del	Turkish, Bulgarian	168, 169
468 bp deletion	468 bp			380
Black	532 bp		African-American	170
Asian Indian	619 bp	NG_000007.3:g.71609_72227del619	Asian Indian	171-173
Afghan	909 bp		Afghan	479
	1.357 kb		Taiwanese	403
Black, British	1.393 kb	NG_000007.3:g.70060_71452del1393	African-American, British	165, 174-177
Croatian	1.605kb	NG_000007.3:g.69561_71164del1604	Croatian	178
2kb deletion	2kb+		French	538
Thai	3.485 kb		Thai	179, 180
Indian	4.056 kb		Indian	454
Czech	4.237 kb		Czech-Slovakian	181
Leiden	7.4 kb		Turkish	482
Turk	7.6 kb		Turkish	182
Cape Verdean	7.7 kb		Cape Verde Islands	399
7719bp deletion	7.719 kb			542
Asian Indian	10.329 kb		Indian	183
Australian (Anglo Saxon)	12.023 kb		Australian	184
Dutch	12.620 kb	NG_000007.3:g.68071_80682del12612	Dutch	185-187
	27 kb		SE Asian	188
27,825 bp deletion	27.825 kb +25 bp			542
Filipino	45 kb	NG_000007.3:g.66258_184734del118477	Filipino	189, 190
Italian	67 kb		Italian	191

ANNEXE 03

BETA THALASSAEMIA MUTATIONS: FREQUENCY AND DISTRIBUTION TABLE

Annexe Table 3.1: Northern European countries

Mutation	UK	Netherlands	Denmark	Germany	France	Czech	Hungary	Romania	Azerbaijan	Russia
–101 (C→T)	1.3	0.5								
–90 (C→T)	0.6	0.7								
–88 (C→A)	0.1	0.7							0.5	
–88 (C→T)	4.8	4.9								
–87 (C→T)	0.2	1.5		1.5						
–87 (C→A)	0.3									
–87 (C→G)	0.1	1.7		3.0				3.2		
–31 (A→G)	0.1									
–30 (T→A)		0.2							1.1	
–29 (A→G)	2.3	2.9								
–29 (A→ C)	0.1								0.5	
–28 (A→G)	0.7									
CAP+1 (A→C)	4.8									
CAP+22 (G→A)									0.8	
Initiation Cd (G→A)				0.5						
Initiation Cd (A→G)				0.5						
Initiation Cd (T→G)	0.1									
Initiation Cd (T→C)	0.1			0.5			13.8			1.6
Cd 5 (–CT)	3.2	1.9	5					2.4	0.3	
Cd 6 (–A)	0.4			1.5				4.0		
Cd 8 (–AA)	0.1	8.5	3					3.2	30.4	38.7
Cd 8/9 (+G)	9.8	6.8	12						6.4	
Cd 15 (–T)	0.1	0.2								
Cd 15 (–G)		0.2		1.0						
Cd 15 (TGG→ TAG)	0.3	0.2								
Cd 15 (TGG→TGA)	3.7		3						1.6	6.5
Cd 16 (–C)	0.2								0.5	
Cd 17 (AAG→TAG)	0.6	1.2	2							
Cd 24 (GGT→ GGA)	0.2									
Cd 26 (G→T)	0.1									
Cd 27/28 (+C)	0.1									
Cd 28 (T→ G)	0.1									
Cd 29 (C→T)	0.1								0.8	

Mutation	UK	Netherlands	Denmark	Germany	France	Czech	Hungary	Romania	Azerbaijan	Russia
Cd 30 (G→C)	2.4								0.8	
Cd 30 (A→G)	0.3									
IVSI-1 (G→A)	1.9	3.6	7	7.5	10.5	45.2	31.0	7.2	1.9	11.3
IVSI-1 (G→T)	1.6	1.0							1.3	
IVSI-2 (T→C)	0.1			1.5						6.5
IVSI-2 (T→A)	0.1									
IVSI-5 (G→C)	22.5	9.2	13	2.5					1.6	
IVSI-5 (G→T)		0.2							0.3	
IVSI-5 (G→A)	0.1									
IVSI-6 (T→C)	2.2	1.9	5	1.0	8.6	15.5	6.9	17.0	4.5	1.6
IVSI-110 (G→A)	5.5	11.2	11	22.0	25.7	5.4		34.7	11.9	6.5
IVSI-128 (T→G)									0.3	
IVSI-130 (G→C)	0.2			2.0						
IVSI-130 (G→A)	0.1									
Cd 35 (–C)		0.4								
Cd 36/37 (–T)	0.2	0.4	1						1.3	
Cd 37/38/39 (–7 bp)		1.0								
Cd 38/39 (–C)						1.5				
Cd 39 (C→T)	3.4	15.3	9	32.5	41.9	2.2	34.5	13.7	2.1	
Cd 41/42 (–TCTT)	9.0	7.5	4							
Cd 43 (GAG→TAG)	0.1									
Cd 44 (–C)	0.4								2.4	
Cd 45 (–T)	0.2									
Cd 47 (+A)	0.1									
Cd 51 (–C)							3.4	0.8		
Cd 71/72 (+T)	0.1									
Cd 76 (–C)		0.2								
Cd 77/78 (–C)	0.2									
Cd 82/83 (–G)	0.2			5.0		1.5				
Cd 90 (G→T)									3.7	
Cd 95 (+A)		0.2								
IVSII-1 (G→A)		9.4	11		1.0	14.0	6.9		15.9	4.8
IVSII-2,3 (+11, –2 bp)	0.3									
IVSII-654 (C→T)	1.6	2.7								1.6
IVSII-705 (T→G)				1.5						
IVSII-745 (C→G)	1.7	1.0		3.5		4.3	3.4	12.1	0.8	
IVSII-843 (T→G)	0.1									
IVSII-848 (C→A)		0.2								
IVSII-848 (C→G)					2.8					
IVSII-849 (A→C)		0.2								
IVSII-849 (A→G)	0.6									
IVSII-850 (G→A)	0.3									
IVSII-850 (G→T)	0.3									
Cd 114 (T→C)										9.7
Cd 121 (G→A)				0.5						

Mutation	UK	Netherlands	Denmark	Germany	France	Czech	Hungary	Romania	Azerbaijan	Russia
Cd 121 (G→T)	0.2	0.2				11.8				
Cd 124 (–A)										1.6
Cd 125 (+CCA)										1.6
Cd 126 (T→ G)	0.3									
Cd 127 (C→T)	0.3									
Cd 131/132 (G→A)	0.2									
+1480 (C→G)	0.2									
Poly A (AATAAA→AAT	0.4	0.4						0.8		
GAA)										
Poly A (AATAAA→AAT		0.2								
AGA)										
Poly A (AATAAA→AAC	0.4									
AAA)										
Poly A (–AT)	0.1									
25 bp deletion	0.4									
619 bp deletion	1.7									
1.4 kb deletion	0.1									
Unknown / Others	0.3	0.0	14	4.0	9.5				7.7	1.6
Total Chromosomes	1117	411		256	105	93	29	124	377	62
References	223, 489	490	491	492	222	116	146	493	159, 236-7	203, 239

Annexe Table 3.2 Mediterranean countries

Mutation	Portugal	Spain	Italy	Sardinia	Sicily	Malta	Greece	Cyprus	Turkey	Macedonia	Croatia	Serbia & Montenegro	Albania	Bulgaria
–101 (C→T)	1.2		0.4				0.5		0.4				1.4	0.5
–87 (C→T)			0.1					2.3						
–87 (C→A)					1.0					2.3				
–87 (C→G)			1.5				2.0		1.1	1.2		2.7		2.6
–30 (T→A)							0.1		3.1	1.2				
–28 (A→C)							0.1		0.3					
Initiation Cd (T→ C)				0.1										
CAP+33 (C→G)							0.1							
Cd 5 (–CT)							0.3		2.3	3.0				3.1
Cd 6 (–A)		1.0	1.2	2.1			2.8	0.1	0.4	4.2				6.2
Cd 8 (–AA)		0.2					0.5	0.2	5.4	1.2	11.4			3.6
Cd 8/9 (+G)		14.1					0.1		1.6					3.6
Cd 9/10 (+C)									0.1					
Cd 27 (G→T)							0.1		0.1					
Cd 29 (C→T)										1.2				
Cd 30 (G→C)			0.1				0.1							
IVSI-1 (G→T)							0.1							
IVSI-1 (G→A)	21.0	25.0	10.4	0.1	3.1		13.4	6.0	4.7	8.9	11.4	2.7	3.2	4.1

Mutation	Portugal	Spain	Italy	Sardinia	Sicily	Malta	Greece	Cyprus	Turkey	Macedonia	Croatia	Serbia & Montenegro	Albania	Bulgaria
IVSI-1 (G→C)							0.1							
IVSI-5 (G→C)			0.2						1.3				0.7	
IVSI-5 (G→T)									0.4					
IVSI-5 (G→A)			0.1						0.1					
IVSI-6 (T→C)	19.0	9.4	10.1	0.1	28.9	71.4	8.4	6.4	11.3	18.6	4.6	2.7	14.4	9.8
IVSI-110 (G→A)	11.5	10.2	23.5	0.5	26.8	12.5	42.5	78.4	39.7	47.3	27.3	17.0	41.8	27.3
IVSI-116 (T→G)									0.2					
IVSI-130 (G→C)	0.8		0.4						0.2					
Cd 37 (G→A)		0.2					0.1				2.3		0.7	
Cd 39 (C →T)	37.3	35.5	41.0	95.7	36.1	5.4	19.6	2.5	4.4	3.0	4.6	22.0	21.2	19.6
Cd 44 (–C)			0.7				0.1		0.1			2.7	2.7	
Cd 76 (–C)			0.4	0.7	1.0									
Cd 82/83 (–G)											2.3			
IVSII-1 (G→A)			3.9	0.1		10.7	2.0		6.3	0.6	9.1	0.7	2.1	2.1
IVSII-745 (C→G)				0.2	3.1		4.8	5.7	3.2	3.0	4.6	12.0	0.7	8.2
IVSII-848 (C→ G)		1.5	5.2				0.1			1.2	2.3			
IVSII-848 (C→ A)							0.1	0.1						
IVSII-850 (–G)			0.1				0.1				2.3			
Cd 127 (G→T)	0.4													
Poly A (A→G)							0.1			2.4			2.1	0.5
Terminating Cd+6 (C→G)							0.1							
25 bp deletion							1.0							
290 bp deletion			0.1				0.1							
Hb Lepore												33.0		
Unknown / Others	0.4	9.2	0.4				1.6	1.9	8.2	3.0	13.6		8.9	8.8
Total Chromosomes	252	411	893	3000	97	56	1142	1017	937	1017	167	44	73	194
References	6, 218,	220,	59	117	156	495	224	227,	53,154	228-233	228-	496	229,	37, 233
	219	221,					235	235,	155,		233		234	
		494						238	225,					
									226					

Annexe Table 3.3 North African countries.

Mutation	Egypt	Tunisia	Morocco	Algeria
–101 (C→T)			1.0	
–87 (C→A)	0.1			
–87 (C→G)	1.9	1.4		
–30 (T→A)	1.5	1.2		0.3
–28 (A→C)				3.0
–28 (A→G)			1.0	
–29 (A→G)			7.0	
CAP+20 (C→T)			1.0	
Cd 5 (–CT)	1.9	0.7		
Cd 6 (–A)	0.3	4.2	10.0	17.7
Cd 8 (–AA)	0.3	0.3	15.5	
Cd 15 (G→A)	0.1			
Cd 25-26 (+T)		0.7		
Cd 26 (+T)	0.2			
Cd 27 (G→T)	1.0			0.3
Cd 30 (G→C)		2.9		
Cd 30 (A→G)				0.7
IVSI-1 (G→A)	15.1	3.9	13.0	10.5
IVSI-1 (G→C)	0.1			
IVSI-2 (T→C)			3.0	3.3
IVSI-2 (T→G)		2.7		
IVSI-2 (T→A)				1.0
IVSI-5 (G→C)		0.9		0.3
IVSI-5 (G→A)		1.4		0.7
IVSI-6 (T→C)	19.7	1.7	14.8	3.3
IVSI-110 (G→A)	28.5	20.0	2.0	24.7
IVSI-130 (G→A)			1.0	
Cd 35 (C→A)				3.0
Cd 37 (G→A)	0.5		2.0	
Cd 39 (C→T)	2.1	45.4	15.5	27.6
Cd 44 (–C)		3.9		
IVSII-1 (G→A)	2.2	0.5	1.0	
IVSII-654 (C→T)	0.1			
IVSII-745 (C→G)	3.7	3.1	1.0	0.7
IVSII-843 (T→G)		0.3		0.3
IVSII-848 (C→A)	3.9	0.5		0.3
IVSII-849 (A→C)				0.3
Cd 106/107 (+G)	0.2			
Poly A (T→C)			2.0	
Unknown / Others	15.5	1.9	3.0	2.3
Total Chromosomes	942	473		305
References	163, 240	33, 129, 497		33,34, 498

Annexe Table 3.4 Middle Eastern countries

Mutation	Syria	Lebanon	Palestine	Israel	Jordan	Saudi Arabia	Yemen	Iran	Kuwait	U.A.E.	Oman
–101 (C→T)				1.4							
–90 (C→T)				0.2							
–88 (C→A)								0.9			
–88 (C→T)	2.0	0.6		0.2							
–87 (C→G)	2.0	0.6			2.2						
–30 (T→A)	7.0	0.6	2.1	0.7							
–28 (A→C)			2.1	6.0							
–31 (A→G)					3.3						
CAP+1 (A→C)						1.2					
Cd 5 (–CT)	9.0	3.1	2.1	1.7	3.3					4.7	0.5
Cd 6 (–A)			2.1	0.2		4.9					
Cd 8 (–AA)		5.0		2.5				3.7	6.2	3.4	
Cd 8/9 (+G)			2.1			1.2		4.6	2.7	8.5	
Cd 15 (G→A)	3.0			0.7						2.1	0.5
Cd 16 (–C)											
Cd 25-26 (+T)											
Cd 19 (A→G)								0.9			
Cd 27 (G →T)	0.7		2.1	0.2	3.3						
Cd 29 (C →T)		5.0			1.1						
Cd 30 (G→C)			2.1							0.9	
Cd 30 (A→G)		1.2									
Cd 30 (G→A)											0.5
IVSI-1 (G→C)				0.4				0.9		3.0	
IVSI-1 (G→A)	17.0	11.8	9.0	5.5				3.7	6.2		1.0
IVSI-5 (G→C)		3.1	2.1		5.5	14.8		7.4	15.9	56.6	61.6
IVSI 5 (G→A)				1.4							
IVSI-6 (T→C)	4.0	6.2	28.7	12.1	6.6			4.6	6.2	3.0	
IVSI-110 (G→A)	24.0	46.6	17.6	28.7	22.0	30.9	40.0	6.5	7.1	1.3	0.5
IVSI-116 (T→G)	1.0										
IVSI-130 (G→C)	0.7										
IVSI (– 25 bp)	0.7	0.6						3.7		4.6	
Cd 36/37 (–T)				1.1				1.9	10.6		1.0
Cd 37 (– G)										0.9	0.5
Cd 37 (G→A)	1.0		10.4	5.5	8.8						
Cd 38/39 (–C)											
Cd 39 (C→T)	6.0	1.2	4.6	11.6	2.2	14.8		5.5	16.8		1.0
Cd 41/42 (–TCTT)										4.3	
Cd 44 (–C)		1.2		9.4				3.7	0.9	0.9	9.6
IVSII-1 (G→A)	4.0	6.8	2.1	7.7	20.0	14.8	26.7	13.8	27.4	3.8	
IVSII-654 C→T	1.0										
IVSII-745 (C→G)	1.0	3.7	2.1	2.5	12.1			3.7			
IVSII-848 (C→A)			2.1		2.2						
Cd 106/107 (+G)			6.8	0.2							
Poly A (A→G)				6.0							

Mutation	Syria	Lebanon	Palestine	Israel	Jordan	Saudi Arabia	Yemen	Iran	Kuwait	U.A.E.	Oman
25 bp deletion										2.5	5.5
290 bp deletion		1.9									
619 bp deletion											4.0
Unknown / Others	12.0	0.6		4.9	4.4	17.3	33.3	36.1			3.0
Total Chromosomes	164	161	296	446	91	81	15	108	113	235	198
References	49	65, 243	500, 501	244	245	246	246	45	36	247, 248	502

Annexe Table 3.5 Asian Indian countries

Mutation	Pakistan	India	Sri Lanka	Bangladesh	Mauritius	Maldives
–88 (C→T)	0.2	0.3				
–28 (A→G)					3.0	
CAP+1 (A→C)	1.2	1.0				
Cd 5 (–CT)	2.1					
Cd 8/9 (+G)	24.1	12.0	2.0	10.0	4.0	
Cd 15 (G→A)	3.3	0.7	1.0	10.0	4.0	
Cd 15 (–T)			1.0			
Cd 16 (–C)	1.8	0.5	1.0			
Cd 30 (G→C)	2.3	1.0	1.0		4.0	15.4
Cd 30 (A→G)	0.5					
IVSI-1 (G→T)	10.4	23.0				
IVSI-1 (G→A)	0.5		27.0			9.0
IVSI-5 (G→C)	32.4	30.0	56.0	60.0	83.0	74.3
IVSI-129 (A→C)			0.2			
IVSI-130 (G→C)			1.0			
IVSI-130 (G→A)			0.7			
Cd 39 (C→T)	0.1					
Cd 41/42 (–TCTT)	6.3	7.0	2.0	20.0	3.0	1.3
Cd 47/48 (+4 bp)	0.4	0.7				
Cd 55 (–A)			0.2			
IVSII-1 (G→A)	0.6					
Poly A (AAT→AAC)			0.8			
619 bp deletion	13.1	20.0	0.2			
Unknown / Others	0.7	4.0	7.0			
Total Chromosomes	2490	674	560	10	55	58
References	212, 251	8, 249, 250, 503	49	249	49	504

Annexe Table 3.6 Southeast Asian countries

Mutation	Burma	Thailand	Malaysia		Singapore		Indonesia	China	Japan	Korea	South Vietnam
			Malays	Chinese	Malays	Chinese					
–86 (C→G)		0.2									
–31 (A→G)	0.3								17.2		
–29 (A→G)				1.4				2.3			
–28 (A→G)	2.3	5.8	3.1	11.3				12.4	0.4		7.3
–28 (A→C)			2.0	1.4							
CAP+1 (A→C)					6.5	8.8					
Initiation Cd (T→C)									0.8		
Initiation Cd (T→G)						1.0			21.8	28.9	
Initiation Cd(A→G)									4.2		
Initiation Cd (G→A)									1.9		
Initiation Cd (G→C)									0.8		
Cd 5 (–CT)	0.3										
Cd 8/9 (+G)	0.3	0.2			3.2				0.8		
Cd 14/15 (+G)		0.2						0.1			
Cd 15 (G→A)	0.3						6.8		2.3		
Cd 15 (–T)	0.3										
Cd 16 (–C)	0.3				3.2						
Cd 17 (A→T)	8.5	21.2	7.1	2.8		11.8	1.7	16.6	0.4	18.4	25.0
Cd 19 (A→G)		5.4	10.2	4.2	3.2						
Cd 24 (T→A)									0.8		
Cd 27/28 (+C)					3.2						
Cd 28/29 (–G)									0.4		
Cd 30 (G→C)	0.3						1.7				
IVSI-1 (G→T)	30.6	3.8	9.4	1.4			10.2	1.3	1.2		6.0
IVSI-1 (G→A)							1.7				
IVSI-2 (T→C)			1.0					1.0			
IVSI-5 (G→C)	19.4		45.9	1.4	35.5	1.0	54.2	1.8			
IVSI-5 (G→T)					3.3						
IVSI-128 (T→G)		0.3									
IVSI-130 (G→A)										5.6	
IVSI-130 (G→C)									0.4	5.6	
Cd 33/34 (–GTG)										5.6	
Cd 35 (C→A)	2.3	0.8							0.4		
Cd 35 (–C)			4.1				1.7				
Cd 39 (C→T)									0.4		
Cd 40 (–G)									0.4		
Cd 41 (–C)	0.3	0.2									
Cd 41/42 (–TCTT)	22.0	40.2	7.1	53.5	5.0	45	1.7	42.3	7.3	5.6	35.3
Cd 42/43 (+T)									0.4		
Cd 42/43 (+G)									0.4		
Cd 43 (G→T)						1.0					
Cd 44 (–C)	0.3										
Cd 53/54 (+G)									0.8		

Mutation	Burma	Thailand	Malaysia		Singapore		Indonesia	China	Japan	Korea	South Vietnam
			Malays	Chinese	Malays	Chinese					
Cd 71/72 (+A)	1.2	2.4		2.8				5.8			7.3
Cd 84/85 (+C)									1.2		
Cd 84-86 (+T)									2.3		
Cd 89/90 (–GT)										5.6	
Cd 90 (G→T)									16.8		
IVSII-1 (G→A)									11.1	105	
IVSII-654 (C→T)	2.3	6.0	4.1	19.7	3.2	32.4	11.9	12.1	14.6		7.3
IVSII-848 (C→ G)									0.8		
IVSII-850 (G →A)									0.8		
IVSII-850 (G→T)									0.4		
Cd 110 (T→ C)									1.2		
Cd 121 (G →T)										5.6	
Cd 123 (–A)									0.4		
Cd 123-125 (–8 bp)		0.2					0.2				
Cd125 (–A)									0.4		
Cd 127/128 (–3 bp)									4.2		
Poly A (AAA AGA)					6.5			1.0			
619 bp deletion	0.7										
Unknown / Others	6.2	3.8	5.1	8.0	12.9		8.5				
Total Chromosomes	304	533	98	71	31	102	59	710	261	65	60
References	252, 505	14, 253-5	256	256	257	257	258	269-264	58, 83	58, 506	507

Annexe Table 3.7 Sub-Saharan African countries

Mutation	African American	Jamaica	Guadeloupe
–90 (C→T)		0.7	
–88 (C→T)	24.3	15.1	4.1
–29 (A→G)	68.4	40.9	53.1
Cd 24 (T→ A)	2.7	1.4	6.2
Cd 30 (G→C)	0.9		
IVSI-5 (G→T)	1.8	0.7	
IVSI-5 (G→A)		0.7	18.3
IVSI-5 (G→C)		6.8	14.2
IVSI-6 (T→C)		0.7	
IVSII-848 (C→A)	0.9	0.7	
Poly A (AAT→ AAC)	0.9	0.7	4.1
Unknown / Others	0.9	0.7	
Total Chromosomes	111	132	49
References	508	509	510

ANNEXE 04

ALPHA THALASSAEMIA MUTATION DATA TABLES

Annexe Table 4.1: Northern European countries

Gene & Mutation	HGVS	Hb variant name	Phenotype	Origin	References
α1 CAP+22 (T→C)	HBA1:c.-16T>C		α+	Italian	GGS pc
α1 CAP+29 (G→C)	HBA1: c.-9 G>C		α+	Iranian	483
-α3.7 Initiation Cd–3/2 (ACCATG→--CATG)	HBA1: c.-3_-2del2bp		α+-α0	N African, Mediterranean	301
α1 Initiation Cd (ATG→GTG)	HBA1:c.1A>G		α+	Mediterranean	299
α2 Initiation Cd (ATG→ACG)	HBA2:c.2T>C		α+	Mediterranean	297
α2 Initiation Cd (ATG→A-G)	HBA2:c.2delT		α+	Vietnamese	298
α2 Initiation Cd (ATG→-TG)	HBA2:c.1delA		α+	Hmong	377
-α3.7 Initiation Cd (ATG→GTG)	HBA2:c.1A>G		α0	African-American	300
α2 Cd 8 (-C) [→48aa]	HBA2:c.56delC		α+	Chinese	463
α1 Cd 14 (TGG→CGG) [Trp→Arg]	HBA1:c. 43T>C	Hb Evanston	α+	Indian	310,451
α2 Cd 14 (TGG→CGG) [Trp→Arg]	HBA1:c. 43T>C	Hb Evanston	α+		
-α3.7 Cd 14 (TGG→CGG) [Trp→Arg]	HBA1:c. 43T>C	Hb Evanston	α0	African-American	311,451
-α4.2 Cd 14 (TGG→CGG) [Trp→Arg]	HBA1:c. 43T>C	Hb Evanston	α0	Asian Indian	451
α1 Cd 14 (TGG→TAG)	HBA1:c. 44A>G		α+	Iranian	343
α2 Cd 18 (–G)	HBA2:c.56delG		α+	Iranian	343
α1 Cd 20 (+T)	HBA1:c.62_63insT		α+	French	GGS pc
α2 Cd 21 (GCT→TCT) [Ala→Ser]	HBA2:c.64G>T	Hb Zoetermeer	α+	Dutch	409
α2 Cd 21/22 (+T)	HBA2:c.66_67insT		α+		GGS pc
α2 Cd 22 (GGC→GGT)	HBA2:c.69C>T		α+	Surinamese	350
α2 Cd 22 (–C)	HBA2:c.69delC		α+	Portuguese	356, 400
α2 Cd 23 (GAG→TAG)	HBA2:c.70G>T		α+	Tunisian	340
α2 Cd 24 (TAT→GAT) [Tyr→Asp]	HBA2:c.73T>G	Hb Creve Coeur	α+	North European	GGS pc
α2 Cd 24 (TAT→TAG)	HBA2:c.75T>G		α+	Dutch	475
α2 Cd 26 (GCG→ACG) [Ala→Thr]	HBA2:c.79G>A	Hb Caserta	α+	Italian	312
α2 Cd 29 (CTG→CCG) [Leu→Pro]	HBA2:c.89T>C	Hb Agrinio	α+	Greek	313
α2 Cd 30 (–GAG) [–Glu]	HBA2:c.91_93delGAG		α+	Southeast Asian	314
α2 Cd 31 (A→C)	HBA2:c.94A>C		α+		457
α2 Cd 31 (AGG→AAG)	HBA2:c.95G>A		α+		GGS pc
α1 IVSI-1 (AGGT→AGTT)	HBA1:c.95+1G>T		α+		481
α1 IVSI-1 (AGGT→AGAT)	HBA1:c.95+1G>A		α+	Thai	292, 481
α2 IVSI-1 (–5 bp) (GAGGTGA GG→GAGG-----)	HBA2:c.95+2_95+6del TGAGG		α+	Mediterranean, Middle East	289
α1 IVSI-4 (A→G)	HBA1: c.95+4 A>G		α+	Iranian	483
α2 IVSI-5 (G→A)	HBA2:c.95+5 G>A		α+	Ashkanazi Jews	GGS pc
α1 IVSI-5 (G→A)	HBA1:c.95+5 G>A		α+	African	GGS pc
α1 IVSI-45 (G→C)	HBA1:c.95+45G>C		α+		GGS pc
α2 IVSI-55 (G→A)	HBA2:c.95+55 G>A		α+	Surinam	GGS pc
α2 IVSI-116 (GCAGGA→GCGGGA)	HBA2:c.96-2A>G		α+	North European	290
α1 IVSI-117 (GCAGGA→GCAAGA)	HBA1:c.96-1G>A		α+	Indian	291

Gene & Mutation	HGVS	Hb variant name	Phenotype	Origin	References
-α$^{3.7}$ Cd 30/31 (GAGAGG→GAG--G)	HBA1:c.92_93delAG		α^{+}-α^{o}	Black	307
α2 Cd 31 (G→A)			α^{+}	Chinese	446
α2 Cd 32 (ATG→AGG) [Met→Arg]	HBA2:c.98T>G	Hb Rotterdam	α^{+}	Dutch	475
α2 Cd 32 (ATG→AGA) [Met→Ile]	HBA2:c.99G>A	Hb Amsterdam	α^{+}	Surinam	447
α2 Cd 33 (TTC→TCT) [Phe→Ser]	HBA2:c.101T>C	Hb Chartres	α^{+}	French	448
α2 Cd 35 (TCC→CCC) [Ser→Pro]	HBA1/2:c.106T>C	Hb Evora	α^{+}	Filipino	370
α1 Cd 37 (–CCC) [–Pro]	HBA1:c.112_114delCCC	Hb Heraklion	α^{+}	Mediterranean	344
α2 Cd 37 (-C)	HBA2:c.112delC		α^{+}		GGS pc
α1 Cd 38/39 (–ACC) [–Thr]	HBA1:c.118_120delACC	Hb Taybe	α^{+}	Arabian	326
α2 Cd 39-41(–9 bp, + 8 bp duplication)	HBA1:c.108_146del9;ins8		α^{+}	Yemenite-Jewish	306
α2 Cd 42 (TAC→CAC) [Tyr→His]	HBA2:c.127T>C	Hb Barika	α^{+}		415
α2 Cd 43 (–T) (TTC→-TC)	HBA2:c.130delT		α^{+}		534
α2 Cd 43/44 (–C)	HBA2:c.132delC		α^{+}	Chinese	532
α2 Cd 47 (–A)	HBA2:c.143delA		α^{+}		GGS pc
α2 Cd 49 (–GC)	HBA2:c.149_150delGC		α^{+}		408
α1 Cd 51-55 (–13 bp deletion)	HBA1:c.155_167delGCTCT GCCCAGGT		α^{+}	Spanish	308
α2 Cd 51/52 (+G)	HBA2:c.156_157insG		α^{+}		GGS pc
α2 Cd 54 (CAG→TAG)	HBA2:c.163C>T		α^{+}		402
α2 Cd 59 (GGC→GAC) [Gly→Asp]	HBA2:c.179G>A	Hb Adana	α^{+}	Southeast Asian	314
α1 Cd 59 (GGC→GAC) [Gly→Asp]	HBA1:c.179G>A	Hb Adana	α^{+}	Mediterranean	315
α2 Cd 59 (GGC→CGC) [Gly→Arg]	HBA2:c.178G>C	Hb Zurich Albisrieden	α^{+}		385
α1 Cd 60/61 (–AAG) [–Lys]	HBA1:c.184_186delAAG	Hb Clinic	α^{+}	Spanish	327
α1 Cd 62 (–GTG) [–Val]	HBA1:c.187_189delGTG	Hb Aghia Sophia	α^{+}	Greek	310
α1 Cd 62 (GTG→-TG)	HBA1:c.187delG		α^{+}	African American	400
α2 Cd 63-76 (-42 bp)	HBA2:c.190_231del		α^{+}		GGS pc
α1 Cd 64-74 (–33 bp)	HBA1:c.183_215del33		α^{+}		381
α2 Cd 66 (CTG→CCG) [Leu→Pro]	HBA2:c.200T>C	Hb Dartmouth	α^{+}	Cambodian	316
α2 Cd 74 or 75 (–GAC) [– Asp]	HBA1:c.226_228delGAC	Hb Watts	α^{+}	Mexican	328
α1 Cd 78 (–C)			α^{+}	African American	377
α2 Cd 81 (–T)	HBA1:c.244delT		α^{+}		461
α2 Cd 82/83/84 del 5bp ins	HBA1:c.247_255delGCCC TGAGC		α^{+} α^{+}	Portugese	GGS pc
α2 Cd 90 (AAG→TAG)	HBA2:c.271A>T		α^{+}	Middle East	341
α2 Cd 93 (GTG→GGG) [Val→Gly]	HBA2:c.281T>G	Hb Bronte	α^{+}	Italian	312
α1 Cd 93-99 (+21 bp)	HBA1:c.280_300ins21		α^{+}		381
α2 IVSII-2 (GT→ GA)	HBA2:c.300+2T>A		α^{+}	North European	342
α2 IVSII-149 (AG→AA)	HBA2:c.301-1G>A		α^{+}	Argentinian	351
α1 IVSII-148 (AG→GG)	HBA1:c.301-2A>G		α^{+}	Iranian	343
α1 IVSII-147 (C→ G)	HBA1:c.301-3C>G		α^{+}		456
α2 Cd 101 (CTA>CCA)	HBA2:c.305T>C		α^{+}		GGS pc
α2 Cd 103 (CAC→CTC) [His→Leu]	HBA2:c.311A>T	Hb Bronovo	α^{+}	Turkish	449
α1 Cd 104 (TGC→AGC) [Cys→Ser]	HBA1:c.313T>A	Hb Oegstgeest	α^{+}	Surinamese	347
α2 Cd 104 (TGC→TAC) [Cys→Tyr]	HBA2:c.314G>A	Hb Sallanches	α^{+}	Mediterranean	317
α1 Cd 104 (TGC→TGG) [Cys→Trp]	HBA1:c.315C>G		α^{+}		GGS pc
α1 Cd 106 (CTG→CCG) [Leu→Pro]	HBA1:c.320T>C	Hb Charlieu	α^{+}	French	487
α2 Cd 107 (–T) [→132aa]	HBA2:c.323delT	Hb Lynwood	α^{+}		468
α2 Cd 108 (ACC→AAC) [Thr→Asn]	HBA2:c.327C>A	Hb Bleuland	α^{+}	Surinamese	346
α2 Cd 108 (–C)	HBA2:c.327delC		α^{+} -α^{o}	Jewish	445
α2 Cd 109 (CTG→CGG) [Leu→Arg]	HBA2:c.329T>G	Hb Suan Dok	α^{+}	Southeast Asian	318
-α$^{3.7}$ Cd 109 (CTG→CGG) [Leu→Arg]	HBA2:c.329T>G	Hb Suan Dok	α^{o}		405
α? Cd 110 (GCC→GAC) [Ala→Asp]	HBA1 or HBA2:c.332C>A	Hb Petah Tikva	α^{+}	Iraqi-Jewish	319
α2 Cd 112/113 (–C)	HBA2:c.339delC		α^{+}		414

Gene & Mutation	HGVS	Hb variant name	Phenotype	Origin	References
α2 Cd 112 (C→G) [His→Gln] & Cd 113-116 (–12 bp) [Leu-Pro-Ala-Glu - →0]	HBA2:c.[339C>G;340_351 delCTCCCCGCCGAG]	Hb Lleida	α+	Spanish	329
α2 Cd 113/114 (–C)	HBA2:c.342delC		α+	African American	377
α2 Cd 115 (–GAGTTCACCCC) [→166 aa]	HBA2:c.349_359 delGAGT TCACCCC		α+		535
α2 Cd 116 (GAG→TAG)	HBA2:c.349G>T		α+	African-American	309
α2 Cd 117 (TTC→TCC) [Phe→Ser]	HBA2:c.353T>C	Hb Foggia	α+	Italian	464
α1 Cd 117/118 (+ATC) [+Ile]	HBA1:c.354-355insATC	Hb Phnom Penh	α+	Chinese	459
α1 Cd 119 (CCT→TCT) [Pro→Ser]	HBA1:c.358C>T	Hb Groene Hart	α+	Moroccan	349, 414
α1 Cd 124-128 (–13 bp)	HBA1:c.333_345delCGCC CACCTCCCC		α+		GGS pc
α1 Cd 123 (GCC→CCC) [Ala→Pro]	HBA1:c.370G>C	Hb Voreppe	α+	French	404
α2 Cd 125 (CTG→CCG) [Leu→Pro]	HBA2:c.377T>C	Hb Quong Sze	α+	Southeast Asian	320
α2 Cd 125 (CTG→CGG) [Leu→Arg]	HBA2:c.377T>G	Hb Plasencia	α+	Spanish	352
-α$^{3.7}$ Cd 125 (CTG→CAG) [Leu→Gln]	HBA2:c.377T>A	Hb Weste-Einde	α+	Kurdish-Jewish	306
α1 Cd 129 (CTG→CCG) [Leu→Pro]	HBA1:c.391T>C	Hb Tunis- Bizerte	α+	North African	322
α2 Cd 129 (CTG→CCG) [Leu→Pro]	HBA2:c.389T>C	Hb Utrecht	α+	Unknown	321
α2 Cd 130 (GCT→CCT) [Ala→Pro]	HBA2:c.391G>C	Hb Sun Prairie		Indian-Pakistani	323
α2 Cd 131 (TCT→CCT) [Ser→Pro]	HBA2:c.394T>C	Hb Questembert	α+	Yugoslavian	324
α1 or 2 Cd 132 (GTG→GGG) [Ser→Pro]	HBA1 or HBA2:c.398T>G	Hb Caen	α+	French	450
α1 Cd 132 (+T) [→175aa]	HBA1:c.396_397insT	Hb Pak Num Po	α+	Thai	354
α2 Cd 136 (CTG→CCG) [Leu→Pro]	HBA2:c.410T>C	Hb Bibba	α+	Caucasian	325
α2 Cd 142 (TAA→CAA) [→172aa]	HBA2:c.427T>C	Hb Constant Spring	α+	Southeast Asian	302
α2 Cd 142 (TAA→AAA) [→172aa]	HBA2:c.427T>A	Hb Icaria	α+	Mediterranean	303
α2 Cd 142 (TAA→TCA) [→172aa]	HBA2:c.428A>C	Hb Koya Dora	α+	Indian	304
α2 Cd 142 (TAA→GAA) [→172aa]	HBA2:c.427T>G	Hb Seal Rock	α+	African-American	305
α2 Cd 142 (TAA→TAT) [→172aa]	HBA2:c.429A>T	Hb Paksé	α+	Laotian	123
α2 3'UTR+46 (C→A)	HBA2:c.*+46C>A		α+	Iranian	537
α2 3'UTR (–16bp) (giving rise to CATAAA)	HBA2:c.*+74_*+89delCCT TCCTGGTCTTTGA		α+	Arabian	296,424
α2 Poly A (AATAAA→AATAAG)	HBA2:c.*+94A>G		α+ -α°	Middle East, Mediterranean	293
α2 Poly A (AATAAA→AATGAA)	HBA2:c.*+92A>G		α+ -α°	Mediterranean	294
α2 Poly A (AATAAA→AATA--)	HBA2:c.*+93_*+94delAA		α+ -α°	Indian	295
α2 Poly A (AATAAA→AATAAC)	HBA2:c.*+94A>C		α+ -α°	Surinam	444
α1 Poly A (AATAAAG→AATAAAA)	HBA1:c.*+96G>A		α+		GGS pc
α2 +832 (G→A)	HBA2:c.*+107A>G		α+	Italian	540

Annexe Table 4.2 Alpha thalassaemia deletions

Mutation	Size kb	HGVS	Phenotype	Origin	References
α⁺-thal					
-α$^{3.7}$	3.7 (type I)	NG_000006.1:g.34164_37967del3804	α⁺	Worldwide	265
-α$^{3.7}$ II	3.7 (type II)		α⁺	Southeast Asian	541
-α$^{3.7}$III	3.7 (type III)		α⁺	Melanesian, Polynesian	541
-α$^{4.2}$	4.2		α⁺	Worldwide	265
-α$^{2.4}$	2.4		α⁺	Chinese	384
-α$^{2.7}$	3.7	NG_000006.1:g.36664_39364del2701	α⁺	Chinese	266
-α$^{3.5}$	3.3	NG_000006.1:g.37464_40964del3501	α⁺	Indian	267
-α$^{5.3}$	5.3	NG_000006.1:g.28684_33930del5246	α⁺	Italian	418
-α$^{7.9}$	7.9		α⁺	Indian	353
-α^{18}	18		α⁺	Polish	268
-α$^{27.6}$	27.6	NG_000006.1:g.9079_36718del27640	α⁺	Chinese	473
α⁰-thal					
--SEA	20	NG_000006.1:g.26264_45564del19301	α⁰	Southeast Asian	269
--THAI	34-38	NG_000006.1:g.10664_44164del33501	α⁰	Thai	270
--FIL	30-34	NG_000006.1:g.11684_43534	α⁰	Philippinos	270
--MEDI	17.5	NG_000006.1:g.24664_41064del16401	α⁰	Mediterranean	269
--MED II	26.5	NG_000006.1:g.10864_40864del30001	α⁰	Mediterranean	271
--(α)$^{20.5}$	20.5	NG_000006.1:g.15164_37864del227	α⁰	Mediterranean	272
--SA	22.8-23.7	NG_000006.1:g.19464_43064del23601	α⁰	Indian	273
--(α)$^{5.2}$	5.2		α⁰	Greek, Italian	269
--GEO	8.5	NG_000006.1:g.32864_42264del9401	α⁰	African-American	274
--SPAN	10.5-12.0	NG_000006.1:g.30864_41414del10551	α⁰	Spanish	275
--MA	22	NG_000006.1:g.18964_39864del20901	α⁰	Spanish	48
--BRIT	26	NG_000006.1:g.16364_45664del29301	α⁰	British	276
--CL	40+	NG_000006.1:g.13314_124664del111351	α⁰		419
--CI			α⁰	Spanish	277
--CAL	32	NG_000006.1:g.8464_40664del32201	α⁰	Spanish, Italian	278
--115	115		α⁰	African-American	279
--RT	36.5-40	NG_000006.1:g.12264_49164del36901	α⁰	British	280
--YEM			α⁰	Yemenite	281
--MC	46+	NG_000006.1:g.164_43364del43201	α⁰	British	282
--CANT	14-15.4	NG_000006.1:g.30864_41414del10551	α⁰	Spanish	283
--KOL	33.3		α⁰	Indian	345
--11.1	11.1		α⁰	Chinese	383
--OH			α⁰	Dutch	443
--AW	8.2		α⁰	Dutch	472
--BGS	131.6		α⁰	Greek	461
--CAMPANIA			α⁰	Italian	480
--27.9	27.9		α⁰	Filipino	484
--ED	80		α⁰	Spanish	485
--GP	145		α⁰	Spanish	485

ANNEXE 05

ALPHA THALASSAEMIA MUTATIONS: FREQUENCY AND DISTRIBUTION TABLES

Notes: The data in these tables have been adapted from Disorders of Hemoglobin, chapter 32, (courtesy of Professor L.F.Bernini).

Annexe Table 5.1 Mediterranean Countries (Southwest and Central Europe).

α-thal alleles	Sardinia	Sicily	Calabria	Spain	Portugal	Greece	Cyprus	Turkey
Gene frequency								
α^{o}-thal	0.002-0.003	≤0.001		0.001	<0.005	0.007	0.01	>0.003
α^{+}-thal (deletion)	0.13+0.02(north) 0.18+0.06(south)	0.05+0.013		0.011+0.007	0.05+0.03	0.037+0.017	0.09	0.022
α^{+}-thal (point mutation)	0.02	0.004		<0.001	<0.001	0.015	0.05	0.01
α^{+} (relative frequency %)								
$-\alpha^{3.7}$	83	84	76	97	70	70	61	52
$-\alpha^{4.2}$ →	1.2	6			30			11
α2 Initiation Cd (T→C)	11	6		<1		2		
α1 Initiation Cd (A G)	1.3			1				
α2 IVSI (–5 bp)	3.2	4		2	rare	3.75	22	15
α1 Cd 59 (G A)								4
(Hb Adana) →						3.75		
α2 Cd 142 (T A)						16	10	
(Hb Icaria) →							7	18
α2 Poly A: (AATAAA→AATAAG)			24			4.5		
α2 Poly A; (AATAAA AATGAA)								
(α2/α1)T (other)								
α^{o} (relative frequency %)								
- - MED I	100	71	present			73	71	35
- $(\alpha)^{20.5}$			present			27	24	52
- - CAL		29	present					
- - MED II							4	13
Total chromosomes	868	1276	600	800	200	454	1000	276
reference	526	526	526	526	526	526	526	526

Annexe Table 5.2 Mediterranean Countries (North Africa and the Middle East)

α-thal alleles	Israel Middle East	Israel (Yemen)	Israel (Arab)	Egypt	Tunisia	Algeria	Iran
Gene frequency							
α^{o}-thal				0.08±0.05			
α^{+}-thal (deletion)							
α^{+}-thal (point mutation)					0.05		
α^{+} (relative frequency %)							
$-\alpha^{3.7}$	50	90	20	96	72	75	79.1
$-\alpha^{4.2}$				4	7		1.7
$-\alpha^{3.7}$ (Hb G-Philadelphia)						5	
$-\alpha^{3.7}$ II						10	
α2 IVSI (–5 bp)					7		4.3
α2 Cd 18 (-G)							
α2 Poly A: (AATAAA→AATAAG)	36		60		14		12.2
α2 Cd 39-41 deletion/ins		10					
α? Cd 110 (C→A)	15		20				
(Hb Petah Tikva)						10	2.7
(α2/α1)T (other)							
α^{o} (relative frequency %)							
- - MED I	100		75				
- $(\alpha)^{20.5}$			25			66	
- - Yem		100					
- - ?						33	
Total chromosomes				124	608		114
reference	526	526	526	526	527	526	528

Annexe Table 5.3 Arabian Peninsula

α-thal alleles	Saudi Arabia	Oman	Kuwait	U.A.E.
Gene frequency				
α^{o}-thal				<0.005
α^{+}-thal (deletion)	0.275±0.02	0.67±0.06	0.27	0.29+0.03
α^{+}-thal (point mutation)			0.135	0.014
α^{+} (relative frequency %)				
$-\alpha^{3.7}$	95	95	67	92
$-\alpha^{4.2}$	5	5		2.3
α2 IVSI (–5 bp)			25	2.3
α2 Cd 142 (T→C) (Hb Constant Spring)				<1
α2 Poly A: (AATAAA→AATAAG)			8	1
α2 Poly A; (AATAAA→AATGAA)				<1
(α2/α1)T (other)				
α^{o} (relative frequency %)				
- - MED I				100
Total chromosomes	1688	254	120	836
reference	526	526	526	526

Annexe Table 5.4 India (non-tribal populations)

α-thal alleles	South Nepal	Punjab	Madhya Pradesh	Andhra Pradesh
Gene frequency				
α^{o}-thal				
α^{+}-thal (deletion)	0.03-0.09	0.1	0.074	0.12±0.05
α^{+}-thal (point mutation)				
α^{+} (relative frequency %)				
$-\alpha^{3.7\ I}$	100	100		75
$-\alpha^{43.7\ II}$			50	8
$-\alpha^{4.2}$			50	17
α2 Cd 142 (A→C) (Hb Koya Dora)				
(α2/α1)T (other)				
Total chromosomes	34	126	54	170
reference	526	526	526	526

Annexe Table 5.5 South East Asia

α-thal alleles	Thailand	Singapore	Indonesia	Laos	Vietnam	Kampuchea	Malaysia
Gene frequency							
α^{o}-thal	0.036-0.025	0.02-0.03?	<0.01?			0.009	0.045
α^{+}-thal (deletion)	0.08±0.02	0.01-0.03?	0.03±0.02	0.11	0.035	0.155	0.16
α^{+}-thal (point mutation)		0.03±0.01					
α^{+} (relative frequency %)							
$-\alpha^{3.7}$	87	68	50	90	100	100	28
$-\alpha^{4.2}$	4	14	50	10			7
α2 Cd 125 (T→C) (Hb Quong Sze)		3					
α2 Cd 142 (T→C) (Hb Constant Spring)	9	8					58
(α2/α1)T (other)		2					7
α^{o} (relative frequency %)							
- - SEA	100	98					
- - THAI		1.6					
- - FIL		0.3					
Total chromosomes	812	792	206	88	88	116	
reference	526	526	526	526	526	526	526

Annexe Table 5.6 China

α-thal alleles	South China (Guangxi)	Hong Kong	Taiwan (Chinese)	Taiwan (Ami)	Taiwan (Bunun)	Taiwan (Atayal)	Taiwan (Paiwan)
Gene frequency							
α^{o}-thal	0.15	0.023	0.02	0.042±0.012	0.002	0.002	0.009
α^{+}-thal (deletion)		0.003	0.01	0.041	0.004		
α^{+}-thal (point mutation)							
α^{+} (relative frequency %)							
$-\alpha^{3.7}$	23	66		98	100		90
$-\alpha^{4.2}$	27	33		2			10
$-\alpha^{2.7}$	3						
α2 Cd 125 (T→C) (Hb Quong Sze)	5						
α2 Cd 142 (T→C) (Hb Constant Spring)	42						
(α2/α1)T (other)							
α^{o} (relative frequency %)							
- - SEA	97	100	97	21	100		57
- - THAI	3			77			43
- - FIL			3	2		100	

Annexe Table 5.7 Oceania

α-thal alleles	Australia	Papua NG (Highlands)	Papua NG (Lowlands)	Vanuatu	Micronesia (overall)	Polynesia	Tahiti
Gene frequency							
α^{o}-thal	0.034	0.053	0.38	0.26	0.026	0.06	0.114±0.015
α^{+}-thal (deletion)				0.05			
α^{+}-thal (point mutation)							
α^{+} (relative frequency %)							
$-\alpha^{3.7\ I}$	90	19	14	9	6		1
$-\alpha^{4.2\ II}$							
$-\alpha^{2.7\ III}$	10		3	68	67	96	97
$-\alpha^{4.2}$			83	23	27	4	2
		81					
Total chromosomes	292	226	753	1079	844	458	1732
reference	526	526	526	526	526	526	526

Annexe Table 5.8 Northern Europe

α-thal alleles	UK	NL	Denmark
Gene frequency			
α^{o}-thal			
α^{+}-thal (deletion)			
α^{+}-thal (point mutation)			
α^{+} (relative frequency %)			
$-\alpha^{3.7}$	81	84	95
$-\alpha^{4.2}$	6	5.2	5
α1 Cd 14 (T>C) (Hb Evanston)	0.4	1	Not specified
α2 Cd 18 (–G)	0.4		Not specified
α2 IVS1 (–5 bp)	4.3	3.1	Not specified
α2 Cd 125 (T→C) (Hb Quong Sze)	1.3		Not specified
α2 Cd 130 (G→C) (Hb Sun Prairie)	0.9		Not specified
α2 Cd 142 (T→C) (Hb Constant Spring)	1.6	2	Not specified

α-thal alleles	UK	NL	Denmark
α2 Poly A: (AATAAA→AATAAG)	1.8		Not specified
α2 Poly A; (AATAAA→AATGAA)	0.6		Not specified
α2 Poly A: (AATAAA→AATA--)	0.8	1	Not specified
(α2/α1)T (other)	0.9	3.7	Not specified
α^0 (relative frequency %)			
- - SEA	74	77	73
- - THAI	0.2		2
- - FIL	9.5		1
- - SA	1.5		
- - BRIT	7		
- - Dutch I		3	
- - Dutch II		5.7	
- - MED I	6.6		20
- (α)20.5	1	8.6	
- - MED II	0.2	5.7	
Total chromosomes	1617	139	511
reference	529	530	531

Annexe Table 5.9 Sub-Saharan Africa

α-thal alleles	Nigeria	Gambia	Senegal	Zambia	Zaire	Togo	CAR	Ivory Coast	Kenya	Benin and Upper Volta	South Africa
Gene frequency											
α^0-thal											
α^+-thal (deletion)	0.24-0.26	0.08±0.05	0.1±0.077	0.27±0.06	0.2	0.26±0.05	0.22-0.25	0.22±0.04	0.19	0.16-0.28	0.06
α^+-thal (point mutation)											
α^+ (relative frequency)											
$-\alpha^{3.7}$	100	90	100	100	100	100	100	100	100	100	100
$-\alpha^{4.2}$		10									
(α2/α1)T (other)											
α^0 (relative frequency)											
	none	none	none	none	none	none	none	none	none	none	none
Total chromosomes	1126	106	118	200		340	98	468		204	508
reference	526	526	526	526	526	526	526	526	526	1	1

ANNEXE 06

DELTA THALASSAEMIA MUTATION TABLE

Mutation	HGVS nomenclature	Hb variant name	Phenotype	Origin	References
-80 (G→A)	c.-130G>A		δ^+	Portuguese	511
-77 (T→C)	c.-127T>C		δ^0 or δ^+?	Japanese	512
-76 (A→T)	c.-126A>T		δ^0	Italian	513
-68 (C→T)	c.-118C>T		δ^+	Dutch	525
-65 (A→G)	c.-115A>G		δ^+	Greek, Cypriots	514
-55 (T→C)	c.-105T>C		δ^+	Greek, Cypriots	514
-36 (C→A)	c.-86C>A		δ^+	Greek	514
-31 (A→G)	c.-81A>G		δ^+	Italian	515
-30 (T→C)	c.-80T>C		δ^+	Greek	GGS pc
CAP+53 (G→A)	c.-1G>A		δ^0	Chinese	GGS pc
Initiation Cd (ATG→ATA)	c.3G>A		δ^0	Chinese	474
Cd 4 (ACT→ATT)	c.14C>T		δ^+	Greek Cypriot	516
Cd 27 (GCC→TCC)	c.82G>T	Hb A_2-Yialousa	δ^+	Greek, Sardinian	517,518
Cd 30 (AGG→ACG)	c.92G>C		δ^0	Italian	519
IVSI-2 (T→C)	c.92+2T>C		δ^0	Italian	517
IVSI-5 (G→T)	c.92+5G>T		?	Iranian	470
IVSI 3′ (AG→AC)	c.93-1G>C		δ^0	Greek	GGS pc
Cd 37 (TGG→TAG)	c.113G>A		δ^0	Sardinian	520
Cd 59 (–A)	c.178delA		δ^0	Egyptian	521
Cd 83 (A→T)	c.247A>T		?	Indonesian	GGS pc
Cd 91 (+T)	c.286_287insT		δ^0	Belgian	522
Cd 98 (GTG→ATG)	c.295G>A	Hb A_2-Wrens	δ^+	African	523
IVSII-1 (G→A)	c.315+1G>A		δ^0	African	474
IVSII-6 (T→A)	c.315+6T>A		unclear		513
IVSII-894, (C→T)	c.316-5C>T		δ^+	Greek Cypriot	GGS pc
IVSII-897 A→G	c.316-2A>C		δ^0	Greek Cypriot	518
Cd 107 (G→A)	c.323G>A		δ^+	Indian	458
Cd 116 (CGC→TGC)	c.349C>T	Hb Troodos	δ^+	Greek Cypriot	518
Cd 141 (CTG→CCG)	c.425T>C		unclear	Greek Cypriot	518
Poly A+69 (G→A)	c.*+199G>A		unclear	Sardinian	524

ANNEXE 07

DELTA BETA THALASSAEMIA AND HPFH MUTATION DATA TABLE

Mutation	Size kb	HGVS nomenclature	Phenotype	%Hb F in heterozygotes	Ethnic Origin	References
Fusion genes						
Hb Lepore -Hollandia	7.398	NG_000007.3:g.63290_70702d	δβ fusion		Bangladesh, Papua New Guinea,	569
Hb Lepore -Baltimore	7.398	NG_000007.3:g.63564_70978del	δβ fusion		Yugoslavia, Spain	570
Hb Lepore – Boston/ Washington	7.398	NG_000007.3:g.63632_71046del	δβ fusion		Rumania,Yugoslavia, Turkey, Cyprus, Jamaica, Cuba, Greece, England, Australia, Mexico	571
Hb Lepore – Leiden	7.398		δβ fusion		Dutch	572
Corfu (δβ)^0	7.201	NG_000007.3:g.57237_64443del7207	(δ)^0		Mediterranean	573
Hb Kenya	22.675	NG_000007.3:g.48194_70985del	γβ fusion		Kenyan, Ugandan	574
Hb Gγ-β Ulsan	27,707	NG_000007.3:g.42947_70653del	γβ fusion		Korean	623
HPFH						
HPFH-1; Black	84.918	NG_000007.3:g.59478_144395del84918	HPFH	20-30	Africa	575, 576
HPFH-2; Ghanaian	83.679	NG_000007.3:g.54867_139178del84312	HPFH	20-30	Africa	575, 576
HPFH-3; Indian	47.733	NG_000007.3:g.50509_83170del32662	HPFH	22-23	India	576, 577
HPFH-4; Italian 1	~40		HPFH	14-30	Italian	578
HPFH-5; Italian 2	12.910		HPFH	16-20	Sicilian	579
HPFH-6; SE Asian	79.278	NG_000007.3:g.45595_124872del79278	HPFH	18-27	Thai	580
HPFH -7;Vietnamese	~30		HPFH	14-27	Vietnam, Cambodia, China	581
δβ-thalassaemia						
Indian (δβ)^0	32.624	NG_000007.3:g.50509_83170del32662	$^G\gamma^A\gamma$ (δβ)^0	5-15	India	582
Sicilian (δβ)^0	13.377	NG_000007.3:g.64336_77738del13403	$^G\gamma^A\gamma$ (δβ)^0	4-19	Mediterranean	583
Japanese (δβ)^0	113.629	NG_000007.3:g.51483_165148del113666	$^G\gamma^A\gamma$ (δβ)^0	5-7	Japan	584
Spanish (δβ)^0	~115	NG_000007.3:g.60375_153285del92911	$^G\gamma^A\gamma$ (δβ)^0	5-15	Sp[ain	585
Black (δβ)^0	11.767	NG_000007.3:g.(60530_60730)_(72351_72551)del11822	$^G\gamma^A\gamma$ (δβ)^0	25	African	586
East European (δβ)^0	9.124		$^G\gamma^A\gamma$ (δβ)^0	13-18	Eastern European	587
Laotian (δβ)^0	12.584		$^G\gamma^A\gamma$ (δβ)^0	11	Laotian, Vietnamese	588
Thai (δβ)^0	~30		$^G\gamma^A\gamma$ (δβ)^0	10	Thai	589
Macedonian(δβ)^0/ Turkish inv-del (δβ)^0	11.465 + 1.593del 7.6 inv		$^G\gamma^A\gamma$ (δβ)^0	7-4	Macedonian, Turkish	590, 591
Turkish (δβ)^0	30		$^G\gamma^A\gamma$ (δβ)^0		Turkish	592
Leiden 7.4 kb (δβ)^0	7.4		$^G\gamma^A\gamma$ (δβ)^0	18.5	Turkish	618
Thai 11.3 (δβ)^0	11.3		$^G\gamma^A\gamma$ (δβ)^0	23.2	Thai	621
Japanese 2 (δβ)^0	27		$^G\gamma^A\gamma$ (δβ)^0		Japan	622
Indian (Aγδβ)^0 (inv)	0.834 + 7.460del 15.5 inv		$^G\gamma^A\gamma$ (δβ)^0	10-18	India, Bangladesh, Kuwait, Iran	593
German (Aγδβ)^0	~52	NG_000007.3:g.(45922_46319)_(98640_99640)del	$^G\gamma^A\gamma$ (δβ)^0	10-13	German	594

Mutation	Size kb	HGVS nomenclature	Phenotype	%Hb F in heterozygotes	Ethnic Origin	References
Cantonese (Aγδβ)^0			$^G\gamma^A\gamma$ (δβ)^0	19-20	Cantonese	595
Malaysian-1 (Aγδβ)^0			$^G\gamma^A\gamma$ (δβ)^0	25	Malay	596
Malaysian-2 (Aγδβ)^0	~42	NG_000007.3:g.(47376_47553)_(89149_90149)del	$^G\gamma^A\gamma$ (δβ)^0	unknown	Malay	597
Turkish (Aγδβ)^0	36.211	NG_000007.3:g.45410_81665del36256	$^G\gamma^A\gamma$ (δβ)^0	10-14	Turkish	575, 583
Belgian (Aγδβ)^0	~50		$^G\gamma^A\gamma$ (δβ)^0	14-15	Belgian	598
Black (Aγδβ)^0	35.811	NG_000007.3:g.49040_84889del35850	$^G\gamma^A\gamma$ (δβ)^0	6-21	African	599
Chinese (Aγδβ)^0	78.847	NG_000007.3:g.48795_127698del78	$^G\gamma^A\gamma$ (δβ)^0	9-20	Chinese	600
Yunnanese (Aγδβ)^0	~88		$^G\gamma^A\gamma$ (δβ)^0	9-17	Chinese	601
Thai (Aγδβ)^0 [HPFH6]	79.278	NG_000007.3:g.45595_124872del79278	$^G\gamma^A\gamma$ (δβ)^0	17-23	Thai	580
Italian	~52		$^G\gamma^A\gamma$ (δβ)^0	12-17	Italian	602
Canadian	55.1		$^G\gamma^A\gamma$ (δβ)^0	9.7-17.3	Canadian	617
Leiden 69.5	69.5		$^G\gamma^A\gamma$ (δβ)^0	10.6	Italian	618
Asian	49.3		$^G\gamma^A\gamma$ (δβ)^0		Asian	620
εγδβ–thalassaemia						
Anglo-Saxon (εγδβ)^0	95.501	NC_000011.8:g.5204501_5300223del	(ε$^G\gamma^A\gamma$δβ)^0	3.1	British	603
Irish (εγδβ)^0	~205	NC_000011.8:g.5110112_5312961del	(ε$^G\gamma^A\gamma$δβ)^0	1.1	Irish	604
Canadian (εγδβ)^0	>185		(ε$^G\gamma^A\gamma$δβ)^0		Canadian, Dutch	605
Scottish - Irish	~205		(ε$^G\gamma^A\gamma$δβ)^0		British	606
English I (εγδβ)^0	~100		(ε$^G\gamma^A\gamma$δβ)^0	0.8	English	607
Mexican (εγδβ)^0	>105		(ε$^G\gamma^A\gamma$δβ)^0		Mexican-American	608
Hispanic (εγδβ)^0	30	NC_000011.8:g.5257379_5297161del	(ε$^G\gamma^A\gamma$δβ)^0	0.2	Hispanic	609
Croatian (εγδβ)^0	>148		(ε$^G\gamma^A\gamma$δβ)^0	2.0	Croatian	605
Dutch I (εγδβ)^0	99.611	NC_000011.8:g.5207238_5307069del	(ε$^G\gamma^A\gamma$δβ)^0		Dutch	610
English II	98		(ε$^G\gamma^A\gamma$δβ)^0		British	611
English III	114		(ε$^G\gamma^A\gamma$δβ)^0		British	611
English IV	439		(ε$^G\gamma^A\gamma$δβ)^0		British	611
Chilean	150		(ε$^G\gamma^A\gamma$δβ)^0		Chilean	612
Dutch II	>400		(ε$^G\gamma^A\gamma$δβ)^0		Dutch	613
Dutch III	112		(ε$^G\gamma^A\gamma$δβ)^0		Dutch	614
Dutch IV	~240		(ε$^G\gamma^A\gamma$δβ)^0		Dutch	615
Dutch V	~160		(ε$^G\gamma^A\gamma$δβ)^0		Dutch	615
Dutch VI	~175		(ε$^G\gamma^A\gamma$δβ)^0		Dutch	615
Japanese	~1400		(ε$^G\gamma^A\gamma$δβ)^0		Japanese	616
French	100		(ε$^G\gamma^A\gamma$δβ)^0		French	619

ANNEXE 08

NON-DELETIONAL HPFH MUTATION DATA TABLE

Mutation	HGVS nomenclature	Hb variant name	Phenotype (%Hb F in heterozygotes)	Origin	References
$^{G}\gamma$–gene					
-567 (T→G)	HBG2:c.-610T>G		5.9-10.2	Iranian	564
-202 (C→G)	HBG2:c.-255C>G		14-21	African	543
-200 (+C)	HBG2:c.-253_254insC	Tunisian	18-28	Tunisian	566
-196 (C→T)	HBG2:c.-249C>T		8.6	Greek	565
-175 (T→C)	HBG2:c.-228T>C		17-30	African	544
-114 (C→G)	HBG2:c.-167C>G	Australian	8.6	Australian	545
-114 (C→A)	HBG2:c.-167C>A	Algerian	0.6-3.5	Algerian	546
-114 (C→T)	HBG2:c.-167C>T	Japanese	11-14	Japanese	547
-110 (A→C)	HBG2:c.-163A>C	Czech	3.1	Czech	548
-109 (G→T)	HBG2:c.-162A>C	Greek	4.1	Greek	563
-37 (A→T)	HBG2:c.-90A>T		2.3	Dutch	568
$^{A}\gamma$–gene					
-211 (C→T)	HBG1:c.-264C>T	Venezuelan	2.7-6.3	African	549
-202 (C→T)	HBG1:c.-255C>T		1.6-3.4	African	550
-201 (C→T)	HBG1:c.-254C>T		10.2	Greek	565
-198 (T→C)	HBG1:c.-251T>C	British	3.5-12	British	551
-196 (C→T)	HBG1:c.-249C>T	Italian	14-21	Italian, Chinese	552, 562
-195 (C→G)	HBG1:c.-248C>G	Brazilian	4.5-7.0	Brazilian	553
-175 (T→C)	HBG1:c.-228T>C	Black	36-41	African	554,555
-158 (C→T)	HBG1:c.-211C>T	Cretan	2.9-5.1	Greek	556
-117 (G→A)	HBG1:c.-170G>A	Greek-Italian,	7.1-19	Greek, Italian, African	557,558, 559, 560
-114 to -102 (13bp)	HBG1:c.-167_-155del CAATAGCCTTGAC	13bp deletion Georgia	30-32	African	567
-114 (C→T)	HBG1:c.-167C>T		3-6.5	African	561

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