MUTATION ANALYSIS AND CONFIRMATION OF BETA THALASSEMIA IN NEPALESE POPULATION

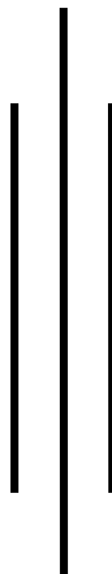
M.Sc. Thesis

2014

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University
Kirtipur, Kathmandu, Nepal



By

Raju Lama
Roll No. BT 076/068
TU Registration No. : 5-3-28-22-2011

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Supervisors

Dr. Tilak R. Shrestha (PhD)
Assoc. Professor
Central Department of Biotechnology, TU
Kirtipur, Nepal

Dr. Zilfalil Alwi (MD, PhD)
Professor
Universiti Sains Malaysia,
Kota Bharu, Kelantan,
Malaysia

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// Abstract

Mutation Analysis and confirmation of beta thalassemia in Nepalese population.

Beta (β) thalassemia is genetic disorder which passes from parents to their offspring in an autosomal recessive inheritance pattern. This disease is directly related to the haemoglobin chemical anatomy and functioning. This genetic disease leads to defective beta globin haemoglobin chain which means partial or complete loss of beta globin chain synthesis. This chemistry in RBCs make them vulnerable to lysis of cells themselves prematurely resulting in anaemia. Beta-thalassemia hence requires continuous blood transfusion along with other care to maintain the normal homeostasis of RBCs and other systems of body. This management procedure is costly, sensitive and tedious and became serious health problem in developing nation like Nepal. 107 subjects were selected of which 61 were already clinically distinguished cases and remaining were one of their immediate family members as carrier unaffected according to clinical data. These two groups were carefully recruited and evaluated by means of multiplex ARMS PCR. The multiplex ARMS PCR results were validated by direct sequencing. The group of 21 major mutations were investigated using allele-specific primers categorised in 6 different panels in this study but only 9 mutations were revealed during study. Unaffected family member were analysed to find the link among them. The most common mutations were found as IVS 1-5(G-C) and Cd 26(G-A) with 23% followed by 619 deletion (20%), Cd 8/9(+G) 12%, Cd 16 (-C) 8%, Cd 41/42(-TTCT) 6%, IVS 1-1 (G-T) 4%, Cd 19 (A-G) 3% and Cd 17(A-T) 1% respectively. Heterozygous and homozygous mutation types were analysed using internal controls. The result of this study reveals that the mutational profile of Nepal resembles with two neighbouring countries China, India and other South Asian countries. Previously it was assumed that thalassemia is common in Terai region only but our study reflects its distribution all over Nepal in most of the ethnic groups. This technique has proved its value in β thalassemia studies and has been used widely for the analysis of β thalassemia worldwide. It is found reliable, simple, quicker and affordable. It is recommended to participate in the thalassemia screening programmes before marriage in endemic areas. And for the married couples to take prenatal diagnosis of foetus in high risk populations to predict and prevent or stop the frequency of the new patients in early future.

Keywords: (β) thalassemia, autosomal recessive, Multiplex ARMS PCR, Sequencing, high risk population, prenatal diagnosis, Nepalese population.

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Acronyms

ARMS	Amplification refractory mutation system
ACE	Angiotensin converting enzyme
BTM	**Beta thalassaemia major**
BTI	**Beta thalassaemia intermediate**
CVS	**Chorionic villus sampling**
ECG	Electrocardiogram
FBS	**Foetal blood sampling**
HS	Hypersensitive
HPFH	High persistence of fetal haemoglobin
IVF	In-vitro fertilisation
LCR	Locus control region
NMD	Nonsense mediated decay
PGD	Pre-implantation genetic diagnosis
PASA	PCR amplification of specific alleles
RFLP	Restriction fragment ligation polymorphism
UTR	Untranslated regions

Chapter 1
INTRODUCTION

1.1Background

The term “Thalassemia” was coined only in 1933 by G.Whipple and W.Bradford; it derives from the Greek “thalassa”, which means the sea and “-haima”, which is the blood; so, its meaning is “sea in the blood”. But for Greeks the “thalassa” was the Mediterranean Sea, so “Thalassemia” also conveys the idea of Mediterranean in the blood or, better, the idea of anaemia coming from the Mediterranean Sea. In the past, it was largely distributed, mostly in people coming from the Mediterranean region. This fact led to the traditional conviction of the Mediterranean origin of β-thalassemia, the reason why it is also called “Mediterranean anaemia” (Wikipedia).

In 1925, Cooley and Lee (Cooley, Lee, 1925), working in Detroit, described a severe, hypochromic, microcytic, hemolytic anemia in children of Italian, Greek and Syrian ancestry. Somewhat earlier, a similar but milder syndrome had been delineated in Italy by a number of investigators working in that area. With the discovery of the asymptomatic carrier state (Dameshek, W. (1940) Smith, C. H. (1943): about 1940, the essential unity of these various disorders as well as the hereditary nature of the anemia was soon appreciated, although a thorough understanding of the genetic background has not yet been achieved (Banton, 1951). The prevalence of this disease among peoples of the northern and eastern shores of the Mediterranean Sea was obvious almost from the start and is reflected in such designations as Mediterranean anemia and Mediterranean syndromes as well as in the more popular name, thalassemia (Kunkel, Cappellini, 1957).

The origin of thalassemia is still controversial, but its distribution shows its probable origin and its spread from Greek population especially as far as Italy is concerned. It also shows interlinking relation with the malarial infection (www.demarchi.org/thalassemia.htm). The connection between thalassemia and malaria is well known: since the malarial Plasmodium cannot reproduce into the abnormal RBCs of thalassemia, this disease represents a protection against malarial infection. That’s the reason why the thalassemia gene could thrive in areas full of malaria. If we look at the map of distribution of β-thalassemia (fig 1), we can see that the areas affected are the same affected by malaria (Iandola, Ortner 2003, Wikipedia).

The history of malaria is very ancient: it began 60 Mya with the apparition of quartana’s Plasmodium. But, probably, it is only during Palaeolithic that the vector, the Anopheles mosquito appeared. Anyway, it seems that during all the Paleolithic it was a relative rare disturb. John Lawrence Angel (1927-1977) maintained that malaria started its diffusion

during Neolithic, when a series of climatic and social changes (e.g. glacier melting, birth and diffusion of agriculture, sedentary life in fertile plains, growth o population) led to a rapid and extensive spread of lots of infection (Alciati *et al*. 1987, Ortner 2003).

According to the hypothesis, the spread of malaria was strictly connected to the diffusion of agriculture from East to West. Before reaching the Western Europe, agriculture spread in Greece during 4th millennium BC; maybe, during that period Greece was one of the most full of malaria region of Mediterranean (Iandola, 2002).β-thalassemia must have originated just in such conditions, in Greece during Neolithic(John Lawrence Angel).Then, it would have spread with move of population, contacts and trades. As proof of his theory, Angel used the great percentage of thalassemia's marks (the porotic hyperostosis) that he found in skeletal specimens coming from Nea Nicomedia, a marshy site on Macedonian coast: 60% of those specimens showed evidence of porotic hyperostosis (while in Near East he founded lower percentage: 50% in Catal Hüyük and only 9% in Cyprus; Iandola, DeMarchi).

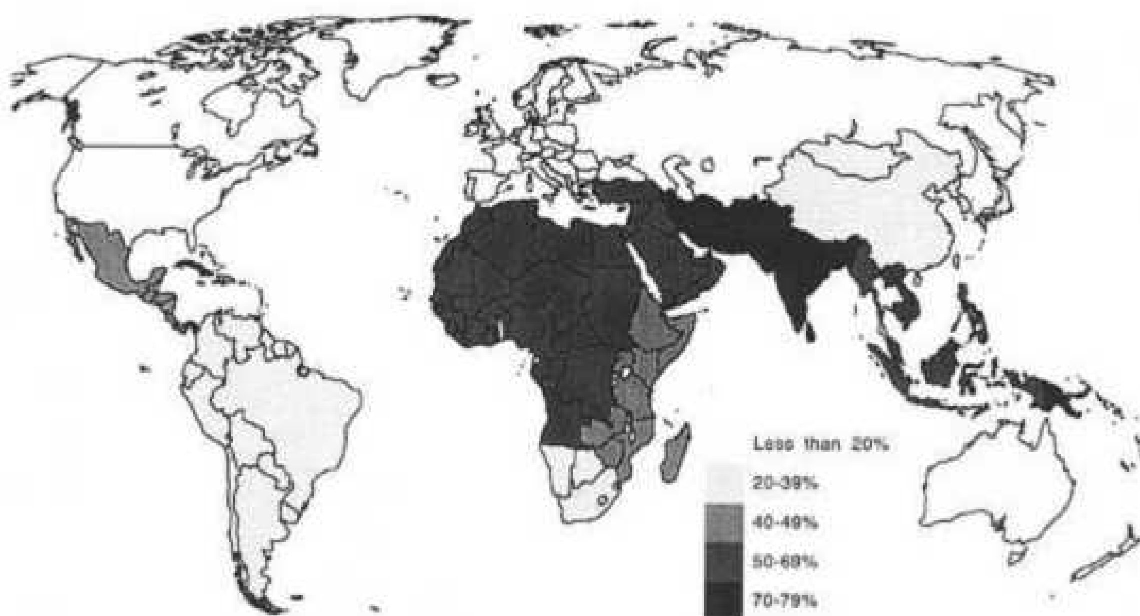


Fig.1, The distribution of thalassemia gene (Source Hillard 1996).

It is interesting to speculate on how thalassemia achieved so widespread a geographic distribution and in what way its incidence has been maintained at such high levels. Unfortunately, a definitive answer to each of these questions is lacking. Perhaps the most attractive hypothesis relative to the distribution of thalassemia is that mass migrations and commerce served to carry the genetic defect eastward to China from a single focus in the northern Mediterranean basin (Amoz, 1959). At least one individual, (Brumpt, 1955) contends, however, that thalassemia may have arisen in Indochina and moved westward while another, (Sheba, Personal communication) suggests an Armenian origin with spread

both to the east and west. It is possible to find prehistoric migrations as well as later movements of peoples to support each of these hypotheses. Although spontaneous mutations arising in a number of areas must always remain a possibility, this suggestion provides a less plausible explanation for the observed distribution of thalassemia (Amoz, 1959).

The rates of spontaneous mutation needed to offset the normal loss of the thalassemia gene through the mortality of the homozygote are far greater than have been ob-served in man. There is no evidence for the proposal that thalassemia hetero-zygotes have a higher index of fertility than other individuals (Montalenti, Siniscalco, 1950). There is, on the other hand, some evidence suggesting that a state of balanced polymorph-ism may be operating to maintain the thalassemia gene at its present level in a manner analogous to that which is presumed to operate in the case of sickle cell disease (Jacob, Raper, 1958). Although the data are far from conclusive, it has been suggested that partial resistance to malarial infection is the reward for harbouring this abnormal genetic constitution. Intensive studies are under way to test this proposal and their results will be awaited with great eagerness (Amoz, 1959).

1.2 Scenario at present

These diseases are mostly common in geographic areas extending from the Mediterranean region through tropical countries including Sub-Saharans Africa, the Middle East, India, Southeast Asia and Indonesia, where malaria was or still is endemic (Weatherall & Clegg, 2001). In many of these areas the estimated frequencies of these disorders range from 3 to 10 percent, even though in some specific areas the carrier frequencies may be higher, reaching 80-90% in some tribal populations in India (Harteveld & Higgs, 2010).

Because of their high frequencies, different hemoglobin defects may be co-inherited, giving rise to an extremely complex series of genotypes and clinical phenotypes. In fact, in many regions thalassemic defects coexist with structural Hb variants. It is also quite common for individuals from areas at high frequency of thalassemic defects to inherit genes for more than one type of thalassemia. Furthermore, some Hb variants are synthesized at reduced rate or are highly instable, leading to both functional and structural deficiency of the affected globin chain, thus resulting in a thalassemic condition, generally showing dominant inheritance. These complex interactions contribute to generate a wide range of clinical disorders that, taken together, constitute the thalassemic syndromes (Weatherall, 2001). The complex and heterogeneous spectrum of molecular defects underlying these inherited conditions is regionally specific and in most cases the geographic and ethnic distributions have been determined, providing support for prevention programs based on screening, genetic counselling and prenatal diagnosis in couples at risk. On the other hand, as the result of mass migration of populations from areas at high risk, hemoglobinopathies are

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

Approximately 1.5% of the global population are heterozygotes or carriers of the β-thalassemias. While the overall frequencies of carriers of these disorders are known in most countries, there have been few attempts at micromapping and wherever this has been done, significant variations are seen even within small geographic regions. Thus, the figures for the estimated numbers of births each year of homozygous β-thalassemia and the severe compound states involving other haemoglobin disorders may be an underestimate. Screening strategies have varied from premarital to antenatal in different countries depending on socio–cultural and religious customs in different populations. Prenatal diagnosis programs are ongoing in many countries and the knowledge of the distribution of mutations has facilitated the establishment of successful control programs. This will eventually help to bring down the burden of the birth of affected children with β-thalassemia and other haemoglobin variants disorder. (Roshan Colah, Ajit Gorakshakar & Anita Nadkarni ; 2010).

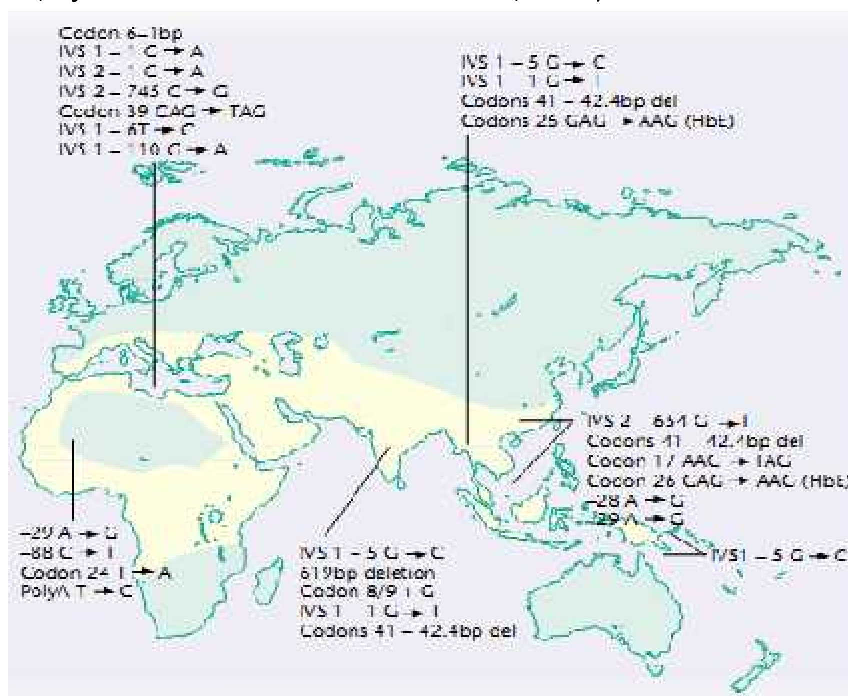


Fig.2; Worldwide distribution of β thalassemia.(Source- ENCYCLOPEDIA OF LIFE SCIENCES / & 2001 Nature Publishing Group / www.els.net)

In Europe, the highest concentrations of the disease are found in Greece, coastal regions in Turkey (particularly the Aegean Region such as Izmir, Balikesir, Aydin, Mugla, and Mediterranean Region such as Antalya, Adana, Mersin), in parts of Italy, particularly Southern Italy and the lower Po valley. The major Mediterranean islands (except the Balearics) such as Sicily, Sardinia, Malta, Corsica, Cyprus, and Crete are heavily affected in particular. Other Mediterranean people, as well as those in the vicinity of the Mediterranean, also have high rates of thalassemia, including people from West Asia and North Africa. Far from the Mediterranean, South Asians are also affected, with the world's highest concentration of carriers (16% of the population) being in the Maldives.

Nowadays, it is found in populations living in Africa, the Americas and Australia. Thalassemias are particularly associated with people of Mediterranean origin, Arabs (especially Palestinians and people of Palestinian descent), and Asians. The Maldives has the highest incidence of Thalassemia in the world with a carrier rate of 18% of the population. The estimated prevalence is 16% in people from Cyprus, 1% in Thailand, and 3–8% in populations from Bangladesh, China, India, Malaysia and Pakistan. Thalassemias also occur in descendants of people from Latin America and Mediterranean countries (e.g. Greece, Italy, Portugal, Spain, and others).

In Tharu people in the Terai region of Nepal and India, it is believed to account for much lower malaria sicknesses and deaths, accounting for the historic ability of Tharus to survive in areas with heavy malaria infestation, where others could not (Terrenato, Shrestha, 1988) .But the study reveals that many other Nepalese castes like Tamang, Rai, Limbu, Sherpa, Bhutia, Newar, Bhramins, Chhetri, Yadavs and Muslims also show the prevalence of thalassemia equally.(Mukherjee and Roy et al.,2012).

1.3 Hypothesis

There have been detected numerous mutations around the world. And the mutations were detected by various methods but Multiplex ARMS is found to be convenient, cost efficient and reliable. The main purpose of this study is to find out the extent of similarities of mutations in Nepal with respect to those found in India, China and South Asian Countries.

1.4 Broad objective

- Screening of beta thalassemia using Multiplex ARMS PCR of beta thalassemic patients from Nepal Thalassemia Society.
- Generation of DNA sequence database of beta thalassemia mutation of beta thalassemic patients in Nepal.
- Mutation analysis of beta thalassemia of various ethnic groups clinically reported at the Nepal Thalassemia Society.

- Introducing the multiplex ARMS PCR as a rapid, cost effective and reliable molecular method for diagnosis of Thalassemia disease.

1.5 Specific objective

- To collect the blood samples of the patients clinically diagnosed as beta thalassemia cases from the Nepalese Thalassemia Society.
- To isolate the Genomic DNA from the collected blood samples using Phenol-chloroform extraction method and quantify the DNA.
- To amplify the beta globin gene using Multiplex ARMS PCR for the screening and confirmation of the mutations.

1.6 Research plan

The work planned to accomplish the research objectives as shown in following chart.

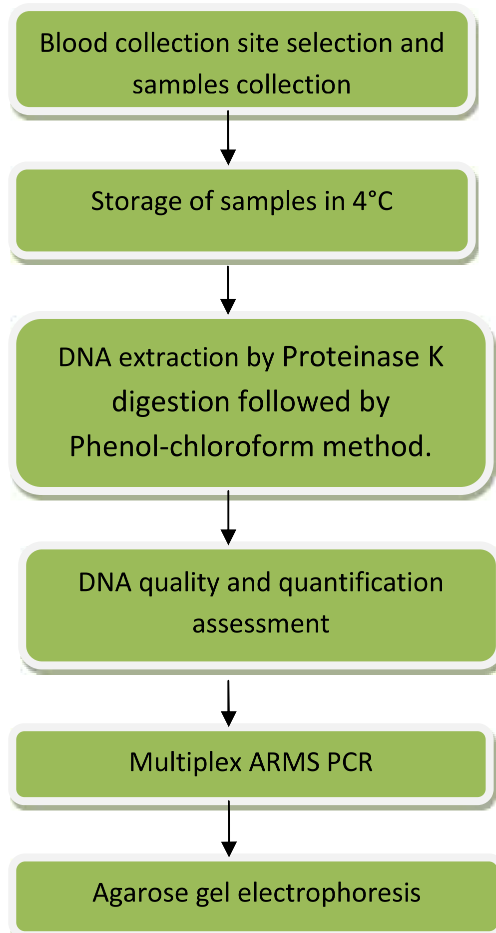


Fig.3; Work plan of research

1.7 Rationale

Nepal is the poorest country among the world and there is lack of health services all over its geography. In addition to that, thalassemia creates a serious health problem to Nepalese society. Many of the physicians are not aware of thalassemic cases and they got mis-diagnosed by the clinician themselves.

Due to the geographical make up, Nepal has very difficult places to counter such diseases. Many of the patients are not serious about themselves as they took it as curse from god or some bad result of previous life. Since, we have no good health services; it makes the situation worst ever.

Nepal Government has no such policy to address the disease like thalassemia. From the private sectors, there are some initiations to manage these cases under the fund from foreigner. But this is not enough to care and manage the thalassemic cases. It is the most ridiculous to know that many of clinicians don't know to diagnose thalassemic cases and Government officials have no idea and record of thalassemic incidence cases all over the nation. There is not a single such medical institute for the diagnosis of thalassemia in Nepal. The patients have to go other countries for diagnosis. This adds up the burden to patients' party and of course tedious job to handle.

Our study is dedicated to all the diagnosed and undiagnosed thalassemic patients who are facing such problems from the Government itself and society. As this disease directly link to the socio-economic status of society. If unmanaged, this disease can lead to early death of patients which is huge loss for nation. We wish our study may help to open the eyes of authorised sectors of Government as well as some other aristocrats to deliver their attention towards the scenario of such cases and address the problems faced by those patients to minimize the thalassemic cases, care and management of the big problem.

1.8 Scope

There have been many molecular tools used for the diagnosis and screening the mutations of β thalassemia. Among them, multiplex ARMS PCR is the most widely applied molecular tool for the genetic disease diagnosis like Thalassemia. MARMS PCR can differentiate the allele specific mutation showing the homozygosity and heterozygosity of the mutation. Moreover, MARMS can detect the number of mutations on single reaction on PCR. This quality makes the MARMS most convenient, reliable, and cost effective.

Nepal has large number of thalassemic patients. They were still unknown or undiagnosed due to lack of health education. There is no solid data of these patients but many

newspaper report as this disease is more than expected and still increasing day by day. The management and treatment cost of thalassemia is beyond the limit of Nepalese citizens. So my study in thalassemia can be useful in context of Nepal.

My study in thalassemia can be a milestone in thalassemia diagnosis and mutation analysis for the proper prevention, management, care and the treatment of the patients. My study shows, thalassemia is spreading rapidly all over the Nepal affecting many ethnic groups. This is a terrible situation. Now we have handful of conditions on which we can work on.

Prevention is better than cure. This statement plays worthy role on prevention of thalassemia. The high risk couple can undergo the pre-marital diagnosis of the thalassemia so that they can choose the better option. Similarly foetus can be diagnosed of thalassemia. This mean we can select the better option for not being born a new thalassemic patient.

This molecular technique can reduce the incidence of the thalassemia, hence decreasing thalassemic patients. This means we can save a lot of money, efforts and produce healthy citizens for our Mother Nation, Nepal.

Chapter 2
Literature Review

2.1 Thalassemia and inheritance

In β-thalassemia, in particular, there is a decreased or totally lack of β-globin synthesis, which results in an excess of free α chains, that accumulate and precipitate damaging both erythroid precursors in the bone marrow and circulating RBCs (Galonello, Renzo 2010) . In fact, red blood cells are made in the red bone marrow; here, stem cells multiply and differentiate into the different blood components, such as "erythrocytes", "leukocytes" (which fight against infections) and "platelets" (which are responsible for healing wounds). The destruction of erythroid precursors caused by the excedent α chains results in an ineffective erythropoesis; RBCs are smaller than normal (microcytosis) and have few levels of haemoglobin (hypocromatosis), so they are not able to carry enough oxygen through the body (Childrencentralcal 2006, eMedicine).

In 1925, Thomas Cooley and Pearl Lee described a form of severe anemia, occurring in children of Italian origin and associated with splenomegaly and characteristic bone changes. Over the next decade, a milder form was described independently by several Italian investigators. Because all early cases were reported in children of Mediterranean origin, the disease was later termed thalassemia, from the Greek word for sea, thalassa. Over the next 20 years, it became apparent that Cooley and Lee had described the homozygous or compound heterozygous state for a recessive Mendelian disorder not confined to the Mediterranean, but occurring widely throughout tropical countries. In the past 20 years, the two important forms of this disorder, α- and β-thalassemia, resulting from the defective synthesis of the α- and β-globin chains of hemoglobin, respectively, have become recognized as the most common monogenic diseases in humans. This article focuses on the β-thalassemias, the severe forms of which are by far the most important of all the thalassemias. The molecular and clinical aspects of the severe α-thalassemia syndromes have been reviewed elsewhere.

The purpose of this report is to review the geographic distribution of the thalassemia gene and to summarize briefly, from a historical standpoint, the evolution of our current concepts of the thalassemia syndromes. A discussion of the clinical, hematologic and pathologic aspects of the disease is beyond the scope of this paper. An attempt has been made to record some of the significant literature relative to the occurrence of thalassemia in areas out-side of Italy and Greece. A critical evaluation of all these reports, however, has not been carried out since many appear in journals not readily available. It is likely, therefore, that some of the publications quoted, particularly those appearing before 1940, deal with diseases other than thalassemia as it is currently defined. Nevertheless, it is believed that

the majority of the cases fulfil the major criteria of thalassemia and merit inclusion in this review.

The diagnosis of thalassemia has, in the past, been based almost entirely on morphologic grounds. Such diagnostic criteria as anisocytosis and poikilocytosis, hypochromia, microcytosis, stippling, target cells, the associated changes in osmotic fragility of the red cells, and evidence of a haemolytic anemia need not be dealt with at length. To these morphologic features have been added an appreciation of the refractoriness of thalassemia to iron therapy and more recently, the presence of normal or high serum iron levels. As time has gone by, the marked variability of the thalassemia states, both from a clinical and hematologic point of view, has become apparent. This realization has helped identify a number of mild, bizarre, hypochromic anaemias, particularly in families of patients with Cooley's anemia, as members of the thalassemia complex. Finally, the demonstration of increased amounts of fetal hemoglobin in most cases of thalassemia major as well as in some of the less severe examples of this syndrome has provided us with an additional diagnostic criterion. During this period of development, which we may refer to as Phase I, the genetic transmission of thalassemia was under investigation particularly by Angelini, Carninopetros, Valentine and Neel, and Silvestroni and Bianco. The concept developed that Cooley's anemia represented the homozygous state for the defect of thalassemia, while the less severe forms of the disease were manifestations of heterozygosity for the same genetic abnormality.

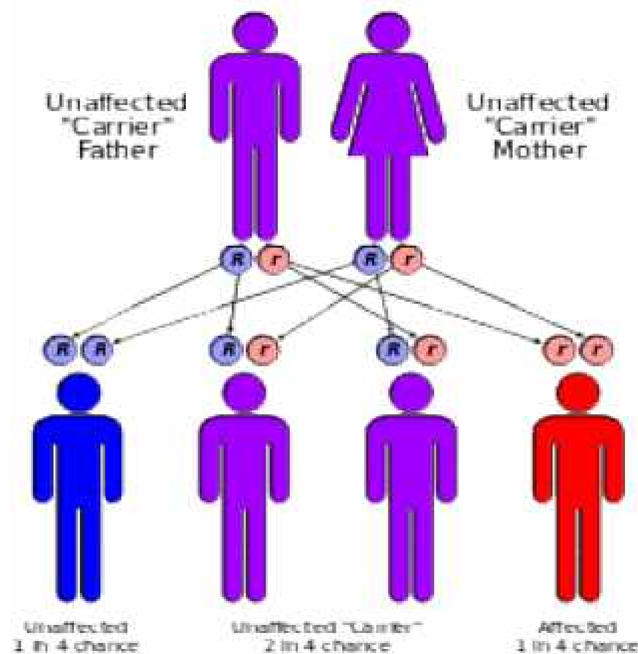


Fig.4, Autosomal recessive inheritance pattern (Source Wikipedia)

Thalassemia is a genetic disorder that involves the decreased and defective production of hemoglobin. Hemoglobin is the molecule found in red blood cells (RBCs) necessary to transport of gases, and to give red pigment to RBCs (eMedicine 2007). Normal hemoglobin

is a tetramer made up of two pairs of globin chains (which can be α. β, γ or δ chains) attached to a heme, containing a core of Iron (Peritz, Rossmann, 1960)Figs 5-6). Different combinations of different globin chains produce different types of haemoglobin. For example, haemoglobin of a new born child is only type F (fetal), made of 2 α and 2 γ chains, whereas the normal adult haemoglobin is 95% of type A (made of 2 α and 2 β chains), 3% of type A2 (2 α and 2 δ chains), 1% or less of type F (Badens et al. 2000, Hillard et al. 1996).

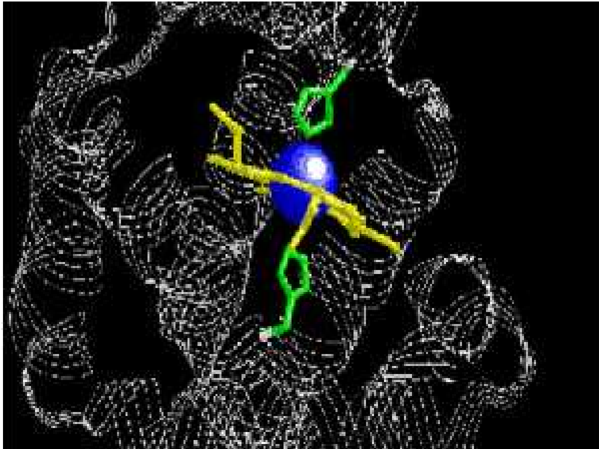


Fig.5, haemoglobin chain

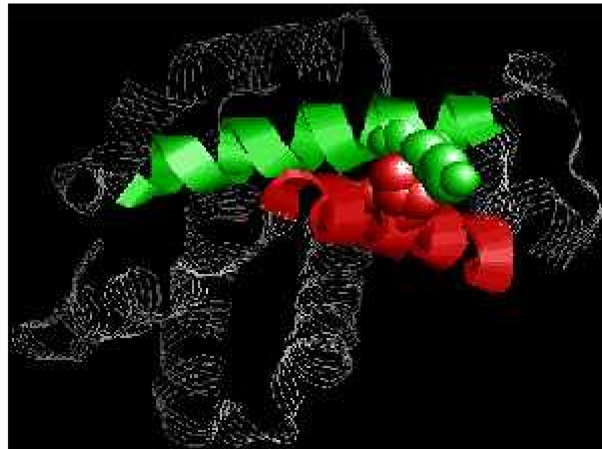


Fig.6, Globin chains tied together

Thalassemia disease originates from a defect in the gene or in the sequence close to the gene that settles the synthesis of the different globin chains (Hillard and Barkou, 1996) that leads to defective or totally absent production of the correspondent globin chain. This results in an imbalance between the different chains that form the haemoglobin, causing the clinical features of the disease: in fact, for normal functioning of RBCs it is necessary an equal production of α and non-α (β, γ or δ) globin. In adults, the most common imbalance is between α and β chains; that's because the greater part of adult haemoglobin is type A. Really, it can be possible to have also γ or δ thalassemia, but the amount of haemoglobin made up of such chains in adults is too little to develop any symptom (Badens et al. 2000).

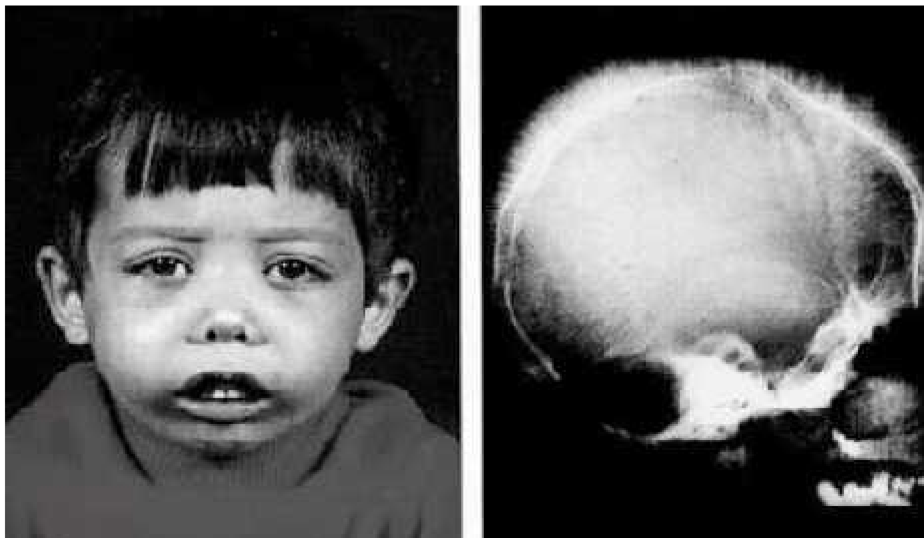


Fig 7, β-thalassemia patients with bone deformities.

2.2 Relation between genotype and phenotype

Several genetic factors may ameliorate the severity of β-thalassemia (Weatherall and Clegg, 1999). First, the underlying mutations vary widely in their effect on the synthesis of b-globin chains (Huisman, Carver, 1997). Co-inheritance of a-thalassemia may reduce the severity of the globin-chain imbalance. Many different interactions with structural hemoglobin variants may also result in a complex series of clinical phenotypes (Weatherall and Clegg, 1999). The interactions of β-thalassemia with two of these variants, hemoglobin S and hemoglobin E, are of global importance.

The clinical consequences of the interaction with hemoglobin S depend mainly on the b-thalassemia allele. If inherited with β°-thalassemia or severe β^+-thalassemia, the resulting clinical disorder may be indistinguishable from sickle cell anemia. By contrast, interactions with mild β^+-thalassemia alleles produce a milder sickling disorder.

Although hemoglobin E b-thalassemia is probably the most common serious hemoglobinopathy world-wide (Weatherall 1998), its natural history remains poorly under-stood (Weatherall and Clegg, 1999). The mutation that produces hemoglobin E activates a cryptic splice site in exon 1 in the b-globin gene; hence, hemoglobin E is associated with mild b-thalassemia. For reasons that are not well understood (Weatherall and Clegg, 1999), the interaction of hemoglobin E and b-thalassemia results in a wide spectrum of clinical disorders: some are indistinguishable from thalassemia major, and some are much milder and not transfusion-dependent. Finally, a number of acquired and environmental factors, including progressive splenomegaly, exposure to infections, socioeconomic factors, and the availability of medical care, may also modify the severity of the disease.

Phenotype	Genotype	Clinical severity
Silent carrier	Silent β/ β	-Asymptomatic -No haematological abnormalit- ies
Trait/Minor	β°/ β , β^+/ β or mild β^+/ β	-Borderline asymptomatic anae- mia -Microcytosis and hypochromia
Intermedia	-β°/mild β^+, β^+/ mild β^+ or mild β^+/ mild β^+ -β°/ Silent β, β^+/Silent β, mild β^+/Silent β, or Silent β/ Silent β -β°/ β°, β^+/ β^+, or β°/ β^+ and deletion or non deletion α-thalassemia -β°/ β°, β^+/ β^+, or β°/ β^+ and increased capacity for	-Late presentation -Mild to moderate anaemia -Transfusion dependent -Clinical severity is variable and ranges from minor to major.

	γ-chain synthesis -Deletion forms of δβ-thalassemia and HPFH - β°/ β or β^+/ β and ααα and αααα duplications -Dominant β-thalassemia (inclusion body)	
Major	β°/ β°, β^+/ β^+, or β°/ β^+	-early presentation -Severe anaemia -Transfusion dependent

Table 1; Genotype and phenotype association in beta thalassemia (Source THALASSAEMIA INTERNATIONAL FEDERATION*)*

2.3Molecular basis

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

These mutations may occur in exon or intron sequences, as well as in the promoter or the 5' and 3' flanking UTR sequences (Fig. 8). As a consequence of the type and the position in which these defects fall, they have been reported to affect expression of the β-globin gene at the following stages:

- Transcription efficiency, for mutations occurring in the promoter region, i.e., recognition sequences for proteins involved in transcriptional or post-transcriptional mechanisms such as the conserved TATA, CCAAT and CACCC boxes. Generally, such mutations are of β^+ or β^{++} types, thus resulting in mild forms of β-thalassemia;

- Maturation of pre-mRNA, if they fall into splicing or polyadenylation sites. RNA-splicing mutations are fairly common and represent a large portion of all β-thalassemic mutations. These mutations affect the splicing process at variable degree, depending on the position in which the mutation occurs. Mutations that affect either of the invariant dinucleotide at the intron-exon junction (the GT motif at the 5' or donor site and the AG motif at the 3' or acceptor site) completely abolish normal splicing or result in β^0-thalassemia. Mutations occurring in the splicing consensus sequences are instead of β+ type, resulting in variable degrees of defective splicing and causing milder types of β-thalassemia. Other mutations occurring in exon or intron sequences may activate a cryptic splicing site, thus leading to abnormal mRNA processing. Even in these cases defective splicing occurs at variable degrees, resulting in phenotypes that range from mild to severe;

- RNA stability, if they occur in the 5' UTR, Cap site, 3' UTR or the polyadenylation site. These mutations are generally associated with mild β-thalassemia phenotypes. In

particular, mutations occurring in the 5' UTR are so mild that they act as silent β-thalassemic alleles which generally show normal haematological phenotypes in heterozygotes.

- mRNA translation, if they generate premature nonsense codons. Premature termination of globin chain synthesis generally leads to the production of short, nonviable β-chains or to nonsense mediated decay (NMD) of abnormal mRNA. In all these cases mutations are of β^0-type and result in severe thalassemia;

- Protein instability, if they give rise to truncated or elongated globin chains which tend to form insoluble tetrameters. (Michela, Raffaele, Stella, Maria and Poala, 2012)

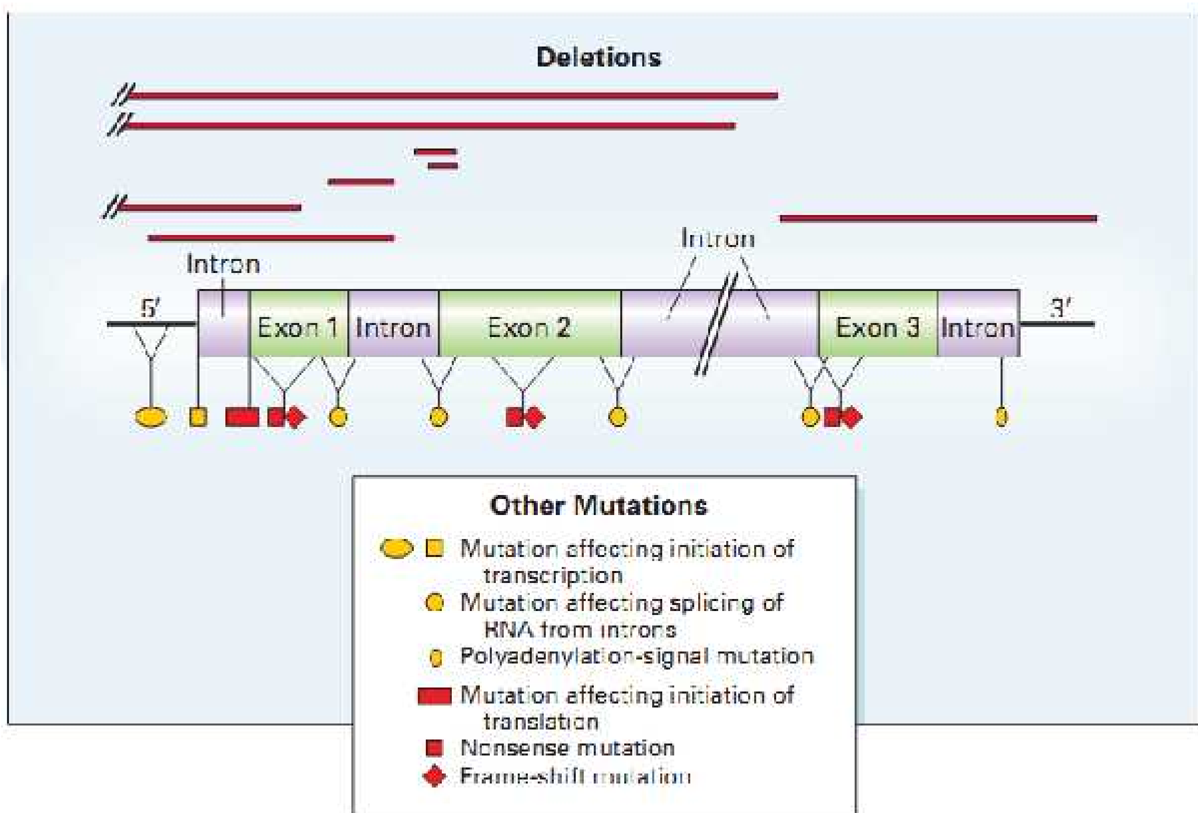


Fig.8, the Normal Structure of the b-Globin Gene and the Locations and Types of Mutations Resulting in β-thalassemia. (Source- Nancy ,1999; Massachusetts Medical Society).

Thalassemias is characterized by a reduced rate of synthesis of one or more globin chains of haemoglobin; conditions of high persistence of fetal haemoglobin in adulthood (HPFH) (Weatherall & Clegg, 2001). It is thought that the high prevalence of these defects could be due to selective advantage of the carrier state to malaria infection. However, in spite of epidemiological evidences supporting this hypothesis as well as of extensive haematological studies, the mechanisms underlying this protection still remain unknown.

Haemoglobin (Hb) is a tetramer of two α-like and two β-like globin chains. Each of these globin chains is covalently linked to a prosthetic oxygen-binding heme group. In healthy adults, approximately 95% of the Hb is Hb A (α2 β2) with <3.2% being Hb A2 (α2δ2) and <1% being foetal Hb (Hb F, α2γ2). The a globin chain is encoded by two a globin genes (α2 and α1), located on the tip of chromosome 16p, while the γ, δ and β chains are encoded by their respective genes ($^{G}\gamma$ and $^{A}\gamma$, δ and β) located in a cluster on chromosome 11p.Throughout human development, exquisitely co-ordinated expression of these α-like and β-like globin chains is required to generate a balanced and high concentration of haemoglobin within the red blood cells.

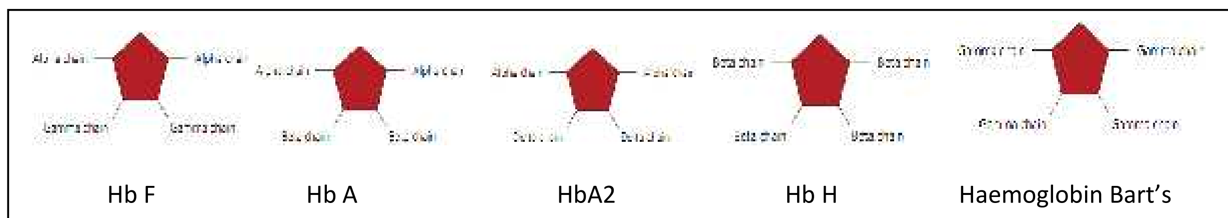


Figure 9; Hemoglobin varients.

2.3.1Organization and structure of human globin genes

Hemoglobin tetramer is composed by two α-like and two β-like globin chains which are encoded by genes localized in two clusters where they are arranged in a sequential mode in the 5'→3' direction, according to their order of activation and expression during ontogenesis (Weatherall & Clegg, 2001). The α-like gene cluster is located in a region of about 30 kb in the telomeric region on the short arm of chromosome 16(Fig.10).It includes in the 5'→3' order an embryonal gene (ζ2), three pseudogenes, (ψζ1, ψα2, ψα1), the α2 and α1 genes and the pseudogene θ. The β-like gene cluster is located in a region of DNA of about 60 kb on the short arm of chromosome 11(Fig.11).It includes in the 5'→3' order the genes ε, $^{G}\gamma$, $^{A}\gamma$ the pseudogene ψβ followed by the δ and β genes (Weatherall & Clegg, 2001). All globin genes share a similar structure which includes three coding exons separated by two introns. Conserved sequences critical for gene expression are found in the proximal promoter regions, at the exon-intron boundaries and in the 5’ and 3’ untranslated (UTR) regions. The fetal globin chains are encoded by two genes, $^{G}\gamma$ and $^{A}\gamma$ which share the same sequence, except in the proximal promoter region and at codon 136, where a glycine residue ($^{G}\gamma$) is replaced by alanine ($^{A}\gamma$) (Amanda, Cooper, 2009). Besides typical promoter and enhancer elements, each globin gene cluster has an upstream regulatory region which plays a crucial role to promote erythroid-specific gene expression and to coordinate the developmental regulation of each gene. In the β-gene cluster this region is known as Locus Control Region (LCR), a relatively large element, encompassing ~20 Kb. It is located approximately 25 Kb

upstream of the most proximal ε-globin gene and contains five DNase I hypersensitive (HS) erythroid specific sites (HS-1 HS-2 HS-3 HS-4 HS-5). These sites define sub-regions of open chromatin that are bound by multi-protein complexes. Similarly a regulatory region, known as HS-40, is located in the α-gene cluster, upstream of the embryonal α-like globin gene (Fig.10)(Cao & Moi, 2002; Ho & Thein, 2000; Weatherall & Clegg, 2001).

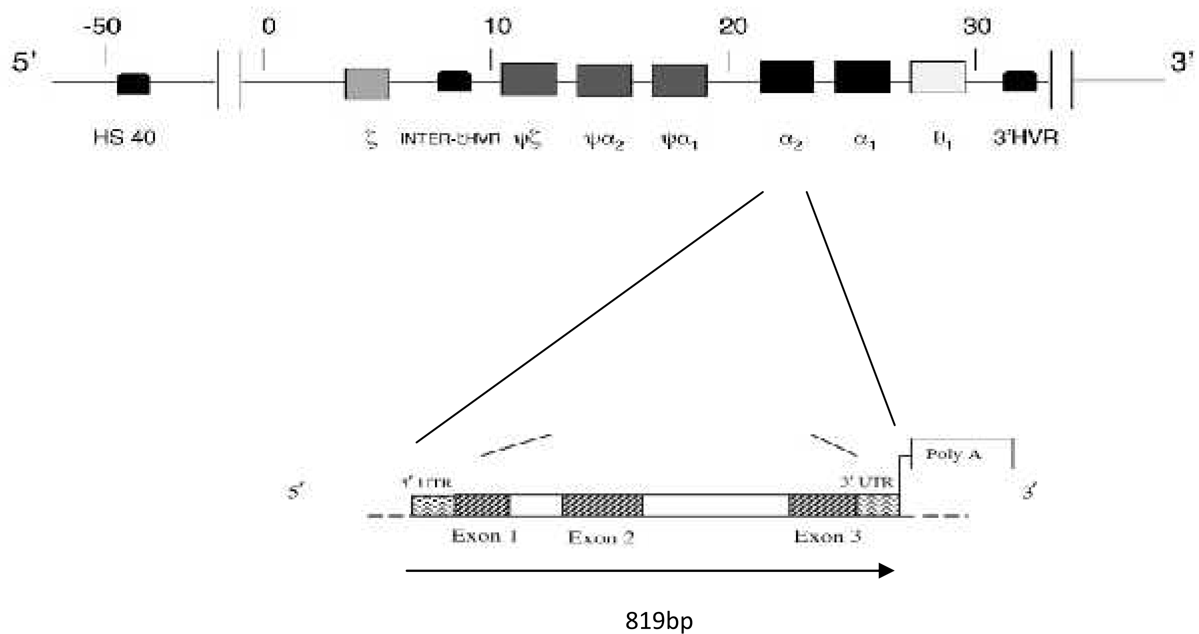
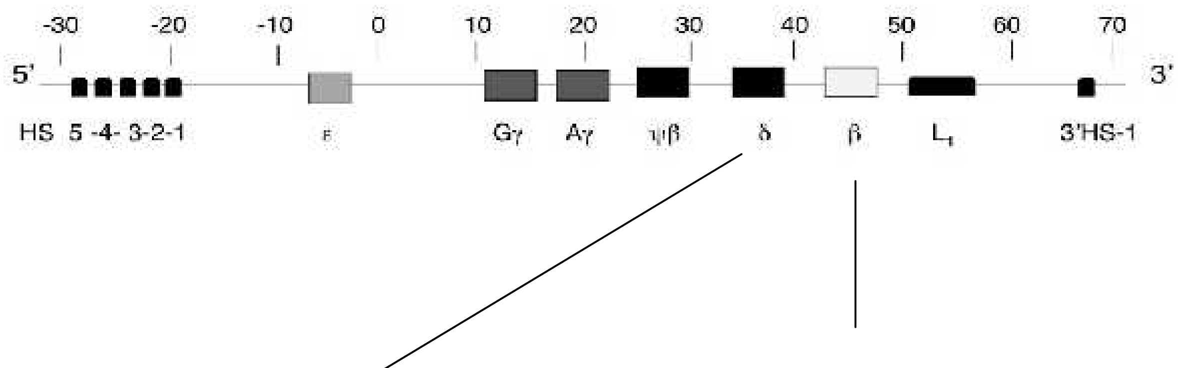


Fig. 10. Structure of the α-gene cluster on chromosome 16. The genes are arranged spatially in the order of their expression during ontogeny.



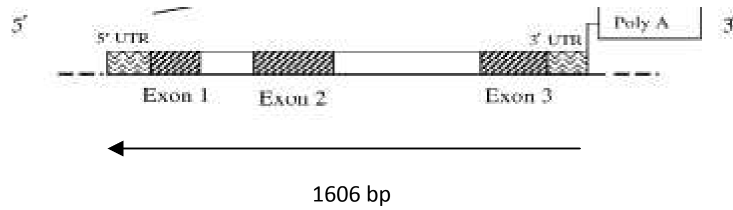


Fig. 11. Structure of the β-gene cluster on chromosome 11. The genes are arranged spatially in the order of their expression during ontogeny.

Beta globin gene sequence in chromosome 11.

5'UTR

ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACC**ATGGTGCACCTGACTCCTGA** } Exon-1
GGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGC
AGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAG
ACTCTTGGGTTTCTGATAGGCACTGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAG**GCTGCTGG**
TGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGG
CAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGAC } Exon 2
AACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACT
TCAGGGTGAGTCTATGGGACCCTTGATGTTTTCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTCATAG
GAAGGGGAGAAGTAACAGGGTACAGTTTAGAATGGGAAACAGACGAATGATTGCATCAGTGTGGAAGTCT
CAGGATCGTTTTAGTTTCTTTTATTTGCTGTTCATAACAATTGTTTTCTTTTGTTTAATTCTTGCTTTCT
TTTTTTTTCTTCTCCGCAATTTTTACTATTATACTTAATGCCTTAACATTGTGTATAACAAAAGGAAATA
TCTCTGAGATACATTAAGTAACTTAAAAAAAAACTTTACACAGTCTGCCTAGTACATTACTATTTGGAAT
ATATGTGTGCTTATTTGCATATTCATAATCTCCCTACTTTATTTTCTTTTATTTTTAATTGATACATAAT
CATTATACATATTTATGGGTTAAAGTGTAATGTTTTAATATGTGTACACATATTGACCAAATCAGGGTAA
TTTTGCATTTGTAATTTTAAAAAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTAATA
CTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTCTTTGCACCATTCTAAAG
AATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATTTCTGCATATAAATATTTCTGCATATAAAT
TGTAACTGATGTAAGAGGTTTCATATTGCTAATAGCAGCTACAATCCAGCTACCATTCTGCTTTTATTTT
ATGGTTGGGATAAGGCTGGATTATTCTGAGTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTT
ATCTTCCTCCCACAG**CTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCA**
CCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCA } Exon 3
CTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACT
GGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAATAAAAAACATTTATTTTCATTGC

3'

Exon 1[50 bp (5'UTR) + 92bp]

ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCC
GTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG

with ATG as start codon

Intron 1(130 bp)

GTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTG
ATAGGCACTGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAG

Exon 2(223bp)

GCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACC
CTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTT
GCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGG

Intron 2(850bp)

TCAGGGTGAGTCTATGGGACCCTTGATGTTTTCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTCATAG
GAAGGGGAGAAGTAACAGGGTACAGTTTAGAATGGGAAACAGACGAATGATTGCATCAGTGTGGAAGTCT
CAGGATCGTTTTAGTTTCTTTTATTTGCTGTTCATAACAATTGTTTTCTTTTGTTTAATTCTTGCTTTCT
TTTTTTTTCTTCTCCGCAATTTTTACTATTATACTTAATGCCTTAACATTGTGTATAACAAAAGGAAATA
TCTCTGAGATACATTAAGTAACTTAAAAAAAAACTTTACACAGTCTGCCTAGTACATTACTATTTGGAAT
ATATGTGTGCTTATTTGCATATTCATAATCTCCCTACTTTATTTTCTTTTATTTTTAATTGATACATAAT
CATTATACATATTTATGGGTTAAAGTGTAATGTTTTAATATGTGTACACATATTGACCAAATCAGGGTAA
TTTTGCATTTGTAATTTTAAAAAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTAATA
CTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTCTTTGCACCATTCTAAAG
AATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATTTCTGCATATAAATATTTCTGCATATAAAT
TGTAACTGATGTAAGAGGTTTCATATTGCTAATAGCAGCTACAATCCAGCTACCATTCTGCTTTTATTTT
ATGGTTGGGATAAGGCTGGATTATTCTGAGTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTT
ATCTTCCTCCCACAG

Exon 3(261bp)

CTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATC
AGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATT
AAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTA
ATAAAAAACATTTATTTTCATTGC

2.3.2 Regulation of globin gene expression

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

Transcriptional control of each globin gene expression requires distant upstream regulatory regions as well as proximal promoter regions. All proximal regulatory elements are located within the first 500 base pairs (bp), 5' to the transcriptional start (Cap) site. The promoters of all the globin genes share high homology but they also show unique sequences that may be responsible for their developmental stage-specific regulation. Three major regulatory elements with minor sequence variations are common to all globin promoter regions: the

TATA, CCAAT and CACCC boxes. In the β-globin gene promoter the TATA box is located at positions -28 to -31, the CCAAT box at positions -72 to -76 and the duplicated CACCC sequences at positions -86 to -90 (proximal element) and at position -101 to -105 (distal element), respectively. It is noteworthy that, with respect to the β-globin gene promoter, the γ-globin gene shows a single CACCC element and a duplication of the CAAT box, which may have implications in the different developmental regulation of these genes. All promoter regions also contain binding sites for specific erythroid transacting factors (Cao & Moi, 2002; Ho & Thein, 2000).

All these elements, through direct interactions with the LCR and transcriptional factors, act as positive regulators and are required for optimal transcription. In fact, mutations in these sequences lead to impaired globin gene expression levels. Several other positive regulatory elements known as enhancers have been identified within gene sequences or in intergenic regions which increase transcriptional activity of certain promoters. In the β-globin gene, enhancers are found in intron 2 and 3' to the gene, 600 to 900 bp downstream of the polyadenylation site. Silencer elements which repress gene expression play a role in the developmental control of globin gene expression, in the switch from embryonal to fetal to adult hemoglobin production. Indeed, these elements are found in the distal promoter region of the ε-globin gene and in the γ-globin genes (Oneal et al., 2006).

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

2.3.3 Switching of globin gene expression

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

Human hemoglobin synthesis requires two switches: from embryonic to fetal hemoglobin at 6 week of gestation and from fetal to adult production at birth (Fig. 12). The first genes to be expressed are those of the ζ-chain (α-like) and ε-chain (β-like), synthesized in the embryonic yolk sac until 4-5 weeks of gestation, which lead to the formation of Hb Gowers I (ζ2ε2). Then, with the change of the liver as the main erythropoietic compartment, synthesis of α and γ chains is activated. At this stage the embryonic Hb Gowers II (α2ε2) and Hb Portland (ζ2γ2) are progressively and completely substituted by the fetal hemoglobin Hb F

($\alpha 2\gamma 2$). Around birth, when the bone marrow becomes the main erythropoietic site, β-globin gene expression is activated to synthesize the adult Hb A ($\alpha 2\beta 2$), which at birth is about 20% of total hemoglobin. The switch from fetal to adult hemoglobin is completed within the first two years of life and leads to the pattern in which adult globin expression Hb A ($\alpha 2\beta 2$) comprises about 97%, HbA2 ($\alpha 2\delta 2$) 2-3% and HbF ($\alpha 2\gamma 2$) less than 1% of total hemoglobin, respectively (Stamatoyannopoulos G. & Gronsveld F., 2001).

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

The switching of the expression of β-globin genes is not only a fascinating and complex model used for studying regulation mechanisms of gene expression in space and time, but its full understanding could also have important therapeutic implications in the treatment of sickle cell anaemia and β-thalassemia. Indeed, the clinical picture of these conditions can improve in the presence of sufficiently high levels of HbF: in β-thalassemia syndromes, in fact, hereditary persistence or drug-mediated reactivation of γ-globin chain output may result in a reduction of the α/non α globin chain imbalance which represents the main pathogenetic factor influencing the severity of these conditions, whereas in sickle cell anemia an increase in HbF contributes to ameliorate the severity of disease by inhibiting the polymerization of sickle hemoglobin and its related pathophysiological effects (Fathallah & Atweh, 2006).

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

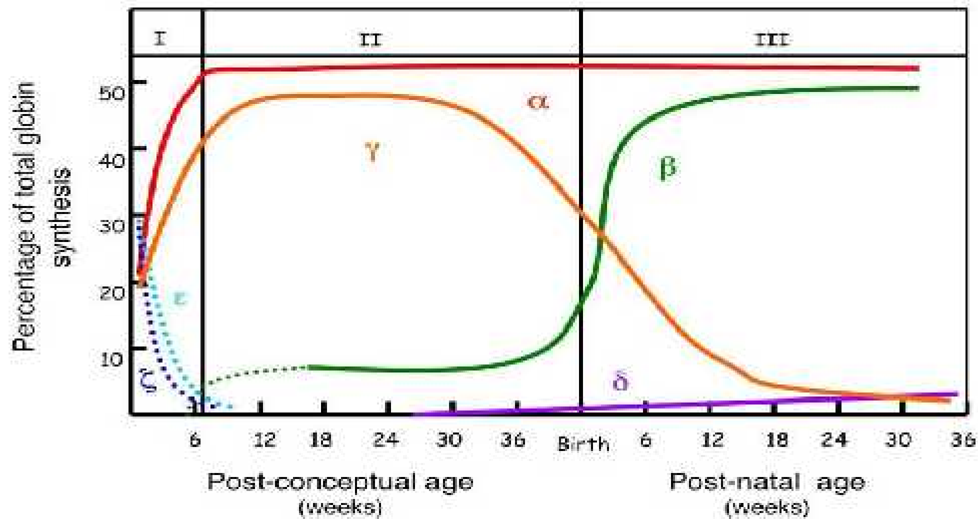
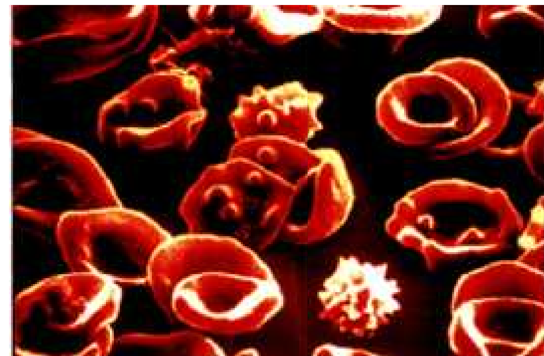


Fig.12. **Changes in globin gene expression profile during ontogeny**. The x-axis represents the age of the fetus in weeks. The y-axis corresponds to the expression of each globin gene as a percentage of total globin gene expression. Time of birth is denoted with a vertical line. The embryonic genes are expressed during the first six weeks of gestation. The first switch from ε- to γ-globin occurs within 6 weeks after conception, and the second switch from γ- to β-globin occurs shortly after birth (Nancy, 1999; Source- Massachusetts Medical Society).

2.4 Pathophysiological basis

Fig.13 Normal RBCs



Source: haematology medical journal

Fig.14, Thalassemic RBCs

The hemoglobinopathies refer to a diverse group of disorders caused by a disruption of this normal pattern of globin gene expression (Weatherall & Clegg, 2001). The disorders are characterized by either a reduced synthesis of one or more of the globin chains (thalassemias) or the synthesis of a structurally abnormal Hb variant. These characteristics, however, are not mutually exclusive (Weed, Robert, Reed, 1963). Some structural Hb variants are also ineffectively synthesized or the globin chain variants are so unstable that they are not able to form tetramers, resulting in a functional deficiency of the globin chain and a thalassemic phenotype. The former is sometimes referred to as thalassemic hemoglobinopathies, and includes the δβ fusion variants (Hb Lepore) and Hb E, β26 (Glu - Lys), in which the substitution at β-codon 26 (GAG AAG) also causes alternative splicing of the β globin mRNA leading to a reduction of the normally spliced β message encoding the Hb E variant. The hyper unstable globin chains act in a dominant negative fashion causing a

disease phenotype even when present in a single copy (Thein, 1999; Thein,2001); for example Hb Showa Yakushiji caused by a single base substitution in β codon 110 (CTG CCG) Leu Pro (Kobayashi et al., 1987). Another group of hemoglobinopathies, referred to as hereditary persistence of foetal haemoglobin (HPFH), is characterized by variable increases of Hb F in otherwise normal adults (Wood, 2001). Because of their concomitant increase in Hb F levels, the δβ- and γδβ-thalassaemias are often considered within the syndrome of raised Hb Fs, forming a continued spectrum within the HPFHs. For practical and clinical reasons however, the distinction between δβ-thalassemias and HPFH should be retained. Homozygotes or compound heterozygotes of δβ-thalassemias with β-thalassemia have a clinical picture of thalassemia intermediate or major. In contrast, HPFH homozygotes or compound heterozygotes with β-thalassemia tend to be clinically very mild (Rochette, Craig & Thein, 1994; Weatherall & Clegg, 2001).

As a group, the hemoglobinopathies are the commonest single gene disorder in the world and are found at high frequencies in tropical and sub-tropical regions where malaria is endemic (Flint *et al*., 1998). A full spectrum of >1000 mutations has been documented, from rearrangements and deletions of gene(s) to single-base substitutions altering amino acid sequence. The globin gene server is a comprehensive database of these mutations and is accessible at http://globin.cse.psu.edu/ (Hardison *et al*., 2002).In many regions, α- and β-thalassemia coexist with a variety of different structural Hb variants; these complex interactions give rise to an extremely wide spectrum of clinical phenotypes. There are four main categories of haemoglobin disorders that are associated with severe disease states and clinical significance.

• Sickle cell disease (major genotypes Hb S/S, Hb S/C and Hb S/b-thalassemia, and less common genotypes Hb S/D^{Punjab} , Hb S/O^{Arab} and Hb S/Lepore).

• β-thalassemia syndrome; including δβ-thalassemia and Hb E/b-thalassemias.

• α-thalassemia syndrome.

• Hb variants resulting in haemolytic anaemias, polycythaemias and, more rarely, cyanosis

Despite discoveries concerning the molecular abnormalities that led to the thalassemic syndromes, it still is not known how accumulation of excess unmatched α-globin in β thalassemia and β-globin in α -thalassemia leads to red blood cell haemolysis in the peripheral blood, and in the β thalassemias particularly, premature destruction of erythroid precursors in marrow (ineffective erythropoesis). Oxidant injury may cause haemolysis, but there is no evidence that it causes ineffective erythropoesis. Hemoglobin E/β thalassemia is now a worldwide clinical problem. The reasons underlying the heterogeneity and occasional severity of the syndrome remain obscure. Ineffective erythropoesis now appears to be caused by accelerated apoptosis, in turn caused primarily by deposition of α-globin chains in erythroid precursors. However, it is not clear how α-globin deposition causes apoptosis. (Curr Opin Hematol 2002, 9:123–126 ©2002 Lippincott Williams & Wilkins, Inc.)

2.4.1 Role of oxidant Injury

Evaluating the possible role of oxidant injury caused by the presence of reactive oxygen species (ROS) is potentially important because anti-oxidant therapy could be clinically useful while improvements from genetic-based therapies are awaited. ROSs include superoxide anion radical, hydrogen peroxide, singlet molecular oxygen, and hydroxyl radical; recently, the role of nitric oxide radicals as been appreciated (Kattamis and Kattamis, 2001). These ROSs are generated in increased amounts in thalassemic red blood cells (RBCs) because the deposition of excess unmatched globin chains (α in β thalassemia and β in α thalassemia) contain free iron, non-heme iron, and hemichromes.These compounds can generate ROS by several mechanisms, including action as a Fenton reagent. The failure of therapeutic trials with agents like vitamin E is not surprising given newer information on the highly specific actions of the different free radicals, which in turn produce equally specific alterations in membrane lipids, intracellular hemoglobin, and membrane proteins like band 3 (Kattamis and Celedon, Rodriguez, Espana *et al*,2001). The evidence supporting the role of oxidant injury in thalassemic RBCs can be reviewed briefly. Previous studies showed that in β thalassemia intermedia, band 4.1 had undergone partial oxidation and, accordingly, was only 50% effective in generating the important spectrin-actin-band 4.1 membrane skeleton ternary complex (Shinar, Rachmilewitz and Lux, 1989; Advani Sorenson and Shinar *et al*, 1992). Furthermore, α globin-associated hemichromes induced oxidant injury that led to clustering of band 3, which in turn produced a neoantigen that bound IgG and complement (Yuan, Kannan and Shinar *et al*, 1992). Membrane-associated IgG and complement provided signals for macrophages to remove such affected RBCs. The excess globin chains bound to the membrane skeleton had lost thiols, presumably because of oxidative attack (Advani, Sorenson and Shinar et al, 1992). More recent data showed that in comparison with children with iron deficiency anemia, children with β thalassemia had elevated plasma levels of conjugated dienes and thiobarbituric acid-reactive substances. These are markers of lipid oxidation. The levels of RBC protective antioxidant enzymes, superoxide dismutase, and glutathione peroxidase were increased (Meral, Tuncel and Surmen-Gur *et al*, 2000) implying that ongoing intra-erythrocytic oxidant injury led to induction of antioxidant mechanisms. Iron compounds that could generate reactive oxygen species have been identified on β thalassemia intermedia RBC membranes. Thus, membrane-bound free iron, nonheme iron, and heme compounds (mainly hemichromes and methaemoglobin) are very much increased, particularly in the RBCs of splenectomised subjects (Tavazzi, Duca and Grazidei *et al*, 2001). This finding suggests that the spleen normally removes the most heavily iron-loaded and thus severely damaged RBCs. The level of the RBC antioxidant, reduced glutathione, is reduced by almost 70% (Tavazzi, Duca and Grazidei *et al*, 2001 and Chakraboryt, Bhattacharya, 2001). To summarize: there is evidence of oxidant injury to RBC hemoglobin, membrane proteins, and lipids; the possible sources of generation of ROS in thalassemic RBC have been identified; antioxidant RBC enzyme activity is increased; and the RBC level of the antioxidant glutathione is much reduced.

Investigators are beginning to consider the role of nitric oxide in the hemoglobinopathies. Nitric oxide from the blood or endothelial cells diffuses into RBCs but cannot diffuse out to perform its vasodilatory function. Nitric oxide entering RBCs binds first to the heme of hemoglobin and then is transferred to the β chain cysteine 93, forming hemoglobin-derived S-nitrosothiol. S-nitrosothiol is associated with the RBC membrane, binding to the cytosol

face of band 3 (also known as anion ex-changer AE1), forming AE1–S-nitrosothiol. Upon deoxygenation, normally nitric oxide is released from this membrane site, entering the circulation and performing its vasodilatory function. In thalassemia, hemoglobin may be oxidatively altered at the β 93 cysteine site (Advani Sorenson and Shinar *et al*, 1992), and it is known that band 3 has undergone oxidative clustering (Yuan, Kannan and Shinar *et al*, 1992). These alterations could impair nitric oxide release, and the lack of vasodilator activity could contribute to the pulmonary hypertension now being reported in β thalassemia intermedia (Aessopos, Farmakis, Karagiorga et al, 2001).

2.4.2 Ineffective erythropoesis and apoptosis

Particularly in the β thalassemias, erythrokinetic and morphologic studies showed that the major site of cell death and, thus, the major cause of the anemia was death of erythroid precursors in the marrow and presumably other sites of extramedullary erythropoesis. The cause of this ineffective erythropoesis was unknown until it was shown in 1993 that β thalassemia major erythroid precursors, but not myeloid precursors, underwent accelerated apoptosis (Yuan, Angelucci and Lucarelli *et al*, 1993) as detected by an increase in DNA laddering, a sign of enhanced nucleosomal DNA cleavage. Quantitative studies subsequently showed that erythroid apoptosis in β thalassemia major was increased approximately fourfold above normal (Centis, Tabellini and Lucarelli *et al*, 2000). Of interest was the observation that approximately 2 to 3% of normal erythroid precursors, mostly late orthochromic erythroblasts, were undergoing apoptosis (Centis, Tabellini and Lucarelli *et al*, 2000 and DeMaria, Testa and Luchetti *et al*, 1993). In combined erythrokinetic and marrow analyses of patients with moderate to severe forms of α and β thalassemia, it was shown that there was a fairly tight correlation between ineffective erythropoiesis and marrow erythroid apoptosis (Pootrakul, Sirankapracha and Hemsorach *et al*, 2000). This finding led to the proposal that the mechanism of ineffective erythropoiesis in the thalassemias was enhanced apoptosis. The degree of apoptosis and ineffective erythropoiesis appeared to be controlled by the accumulation of α-globin chains in early erythroid precursors either α-A in severe β thalassemia or α-Constant Spring (CS) in patients with either Hb H/CS or Hb CS/CS. However, accumulation of β-globin chains in erythroid precursors in the more severe forms of α thalassemia could produce similar but lesser effects because there was a small but significant increase in both apoptosis and ineffective erythropoiesis in Hb H disease (Pootrakul, Sirankapracha and Hemsorach *et al*, 2000). There was considerable variation in measurements of apoptosis in each specific form of thalassemia; the variation was at least partially dependent on the extent of erythroid expansion in a given patient. One interpretation of this finding is that under extreme erythropoietic drive leading to greater erythroid expansion, the erythroid precursors are more likely to make the errors that turn on apoptotic programs (Centis, Tabellini and Lucarelli *et al*, 2000). Morphologic analysis of marrow in severe forms of β thalassemia showed a decrease in late orthochromic normoblasts (Yuan, Angelucci and Lucarelli *et al*, 1993 and Pootrakul, Sirankapracha and Hemsorach *et al*, 2000), and in vitro studies have led to the suggestion that apoptosis primarily occurs at the polychromatophilic normoblast stage (Mathias, Fisher and Zeng et al,2000).

The key question became how accumulation and deposition of excess (primarily α) globin in erythroid precursors led to activation of apoptotic programs and cell death. Preliminary

experiments pursuing this question were based on the following two lines of emerging information on apoptosis and erythropoiesis (Stanley, 2002).

Apoptosis

Apoptosis is a fundamental cellular mechanism for getting rid of unneeded or potentially dangerous cells. Apoptotic programs require the action of a family of cysteine-dependent and aspartate-specific proteases called caspases. It is proposed that there are two classes of caspases: initiators and effectors (Salvasen, Dixit, 1999 and Hengartner, 2000) .The two initiator caspases are 8 and 9. Caspase 8 detects specific cell surface receptor-ligand interactions, i.e., the death receptor pathway, such as the well known interaction between Fas and Fas-ligand. Caspase 9 is activated by events that cause intracellular damage to DNA, damage to proteins, and eventually, alterations in mitochondrial membrane potential (i.e., the mitochondrial pathway). By limited protease action, activated caspases 8 and 9 then activate another class of effectors or executioner caspases like 3 and 7, which actually perform the proteolytic steps leading to cell death.

2.4.3Control of erythropoiesis

Normal erythropoiesis is a tightly controlled process requiring erythropoietin, which enhances proliferation and differentiation and protects against apoptosis by increasing the expression of Bcl-XL, an antiapoptotic gene. Immature erythroid precursors are susceptible to apoptosis because they have low levels of antiapoptotic genes, and these erythroid precursors express the membrane receptor, Fas (Stanley, 2002).

It now appears that Fas-ligand is also expressed on erythroid precursors, primarily on the more mature ones. Therefore, it is proposed that in marrow erythroid is-lands, the Fas–Fas-ligand interaction can produce a negative feedback on erythropoiesis, particularly when erythropoietin levels are low (DeMaria, Testa and Luchetti *et al*, 1993). It was shown that this negative feedback loop is probably caused by the activation of caspases 3, 7, and 8, which in turn degrade the important transcription factor GATA-1 (DeMaria, Zeuner, Eramo *et al*, 1999), a factor required for erythroid differentiation. Paradoxically, further study showed that activation of caspase 3 via the mitochondrial pathway is required for normal terminal erythroid differentiation (Zermati, Garrido, Amsellem *et al*, 2001).

These two lines of experimentation showed that there was an intersection between apoptosis and normal erythroid development involving both the death receptor and mitochondrial pathway. The initial focus on the death receptor pathway showed that both Fas and Fas-ligand were unregulated, as measured by flow cytometry, on β thalassemic erythroid precursors. Fas-ligand mRNA was also increased as measured by reverse transcription polymerase chain reaction (Vrneris, Ma, Bai *et al*, 1999). These preliminary results do not explain how accumulation of α-globin leads to apoptosis, but it appears that the death receptor mechanism is involved.

2.4.4 Mechanism of anaemia

In severe untreated β-thalassemia, erythropoiesis may be increased by a factor of up to 10, more than 95 percent of which may be ineffective. Ineffective erythropoiesis, the hallmark of β-thalassemia, is a result of the myriad deleterious effects of a relative excess of α-globin chains (Nathan, Gunn, 1966). This relative excess interferes with most stages of normal erythroid maturation: both intramedullary death of red cell precursors through arrest in the G1 phase of the cell cycle and accelerated intramedullary apoptosis of late erythroblasts (Yuan, Angelucci, Lucarelli, 1993 and Schrier, 1997)have been demonstrated. Studies of the consequences of the accumulation of excess α-globin chains and their degradation products within the red cell membrane and its skeleton (Schrier, 1997 and Grinberg, Rachmilewitz, 1995) have also demonstrated abnormalities in the ratio of spectrin to band 3 and in the function of the band 4.1.This subject has been thoroughly reviewed recently (Schrier, 1997). The observation that the presence of excess membrane iron may aggravate membrane changes (Grinberg, Rachmilewitz, 1995) has led to interest in the red cell membrane as a potential therapeutic target in β-thalassemia. In a mouse model, increased cellular rigidity and decreased stability in connection with membrane-associated α-globin chains (Sorenson, Rubin, Polster, Mohandas, Schrier, 1990) have reportedly been ameliorated during exposure to agents that bind membrane iron (Shalev, Repka, Goldfarb *et al*, 1995). Further understanding of these processes may guide future therapies.

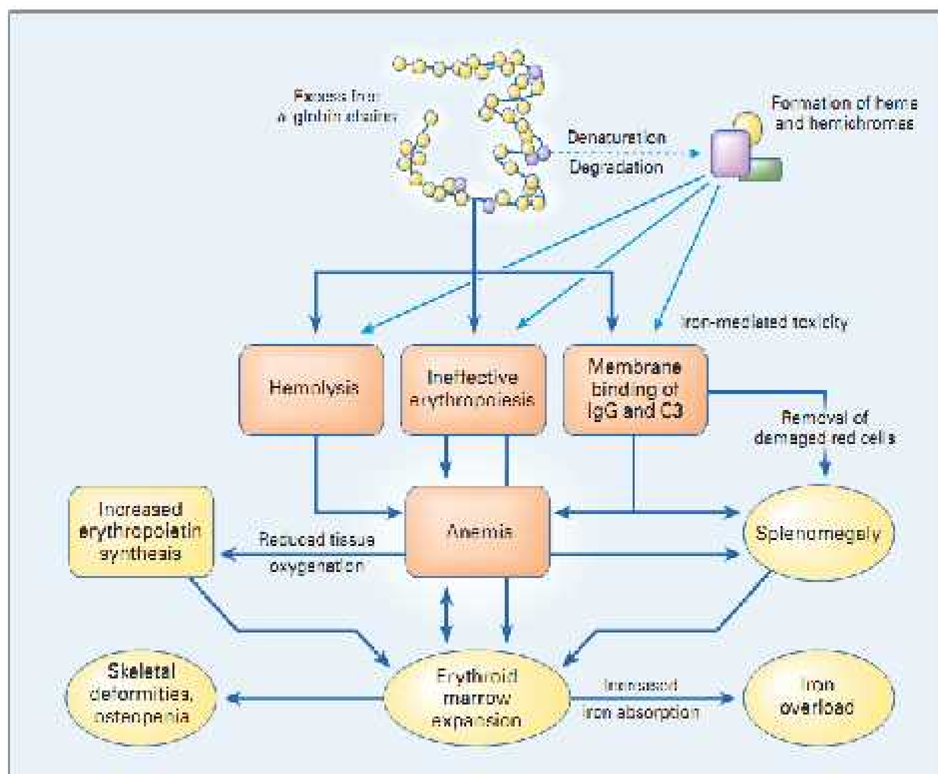


Fig.15; Effects of Excess Production of Free α-Globin Chains. (Source-Massachusetts Medical Society.)

Clinical consequences of Anaemia

The severe ineffective erythropoiesis results in erythroid marrow expansion to as much as 30 times the normal level. Both an increase in plasma volume as a result of shunting through expanded marrow and progressive splenomegaly exacerbate anemia (Fig. 15).Increased erythropoietin synthesis may stimulate the formation of extramedullary erythropoietic tissue, primarily in the thorax and paraspinal region. Marrow expansion also results in characteristic deformities of the skull and face, as well as osteopenia and focal defects in bone mineralization (Rioja, Girot, Garabedian, Cournot, 1990 and Orvieto, Leichter, Rachmilewitz, Margulies, 1992), and may aggravate a painful periarticular syndrome characterized histologically by microfractures and osteomalacia (Gratwick, Bullough, Bohne, 1978). Marrow hyperplasia leads ultimately to increased iron absorption and progressive deposition of iron in tissues.

2. 5 Mutations causing β-thalassemia

Nearly 200 different mutations have been described in patients with β-thalassemia and related disorders. Although most are small nucleotide substitutions within the cluster, deletions may also cause β-thalassemia (Weatherall, Clegg, 1994). All the mutations result in either the absence of the synthesis of β-globin chains (β^0-thalassemia) or a reduction in synthesis (β^+-thalassemia).

Mutations in or close to the conserved promoter sequences and in the 5'untranslated region down regulate transcription, usually resulting in mild β^+-thalassemia. Transcription is also affected by deletions in the 5' region, which completely inactivate transcription and result in β°-thalassemia. Both splicing of the messenger RNA (mRNA) precursor and ineffective cleavage of the mRNA transcript result in β-thalassemia. In some mutations, no normal message is produced, whereas other mutations only slightly reduce the amount of normally spliced mRNA. Mutations within invariant dinucleotides at intron-exon junctions, Critical to the removal of intervening sequences and the splicing of exons to produce functional mRNA, result in β°-thalassemia. Mutations in highly conserved nucleotides flanking these sequences, or in "cryptic" splice sites, which resemble a donor or acceptor splice site, result in severe as well as mild β^+-thalassemia. Substitutions or small deletions affecting the conserved AATAAA sequence in the 3' untranslated region result in ineffective cleavage of the mRNA transcript and cause mild β^+-thalassemia.

Mutations that interfere with translation involve the initiation, elongation, or termination of globin-chain production and result in β°-thalassemia. Approximately half of all b-thalassemia mutations interfere with translation; these include frame-shift or nonsense mutations, which introduce premature termination codons and result in β°-thalassemia. A more recently identified family of mutations, usually involving exon 3, results in the production of unstable globin chains of varying lengths that, together with a relative excess of α-globin chains, precipitate in red-cell pre-cursors and lead to ineffective erythropoiesis, even in the heterozygous state. This is the molecular basis for dominantly inherited (β^+) thalassemia. In addition, missense mutations, resulting in the synthesis of unstable β-globin chains, cause β-thalassemia (Nancy, Massachusetts Medical Society, 1999).

2.6 Clinical form

The β-thalassemias include four clinical syndromes of increasing severity: two conditions are generally asymptomatic, the silent carrier state and b-thalassemia trait, and usually result from the inheritance of one mutant b-globin gene, and two require medical management, thalassemia intermedia and thalassemia major. The more severe forms most often result from homozygosity or compound heterozygosity for a mutant b-globin allele and, occasionally, from heterozygosity for dominant mutations (Thein, Hesketh, Taylor, 1990). Homozygous or compound heterozygous b-thalassemia usually presents no diagnostic problems. The early onset of anemia, characteristic blood changes, and elevated fetal hemoglobin concentrations are found in no other condition. The diagnosis can be confirmed by the demonstration of the β-thalassemia trait in both parents. This condition is characterized by mild anemia, reduced mean cell volumes and mean cell hemoglobin concentrations (Weatherall, Clegg, 1999), and elevated concentrations of the normal minor adult component of hemoglobin (usually exceeding 3.5 percent), hemoglobin A2 ($\alpha 2\delta 2$).

Thalassemia major and thalassemia intermedia have no specific molecular correlate but encompass a wide spectrum of clinical and laboratory abnormalities (Camaschella, Cappellini, 1995). Patients referred to as having thalassemia major are usually those who come to medical attention in the first year of life and subsequently require regular transfusions to survive. Those who present later or who seldom need transfusions are said to have thalassemia intermedia (Cao, 1988). After thalassemia is diagnosed, patients who appear not to require immediate trans-fusion may benefit from a period of observation and folate repletion, particularly if the disease is diagnosed after the age of one year. This approach will allow the identification of patients in whom early growth and development are normal and whose well-compensated anemia may be exacerbated only by infection, folate deficiency, or increasing hypersplenism (Camaschella, Cappellini, 1995; Cao, 1988; Rund, Oron, Filon, 1997; Ho, Hall, Luo, 1998). With advancing age, even patients with mild forms may have serious complications, including osteopenia, iron loading in tissues, and ectopic marrow expansion. The classic changes of untreated thalassemia major are now regularly seen only in countries without resources to support long-term transfusion programs.

2.7 Presentation of β-thalassemia

Symptoms may include:

Anaemia, which may be mild, moderate or severe

Jaundice

Enlarged spleen

Fatigue (tiredness)

Listlessness

Reduced appetite

Enlarged and Fragile bones, including:

- Thickening and roughening of facial bones
- Bones that break easily

Teeth that don’t line up properly
Growth problems
Increased susceptibility to infection
Skin pallor than usual
Hormone problems such as:
Delayed or absent puberty
Diabetes
Thyroid problems
Heart Failure
Shortness of breath
Liver problems
Gallstones

Patients with the beta thalassemia trait generally have no unusual physical findings. In patients with beta thalassemia major, the physical findings are related to severe anemia, ineffective erythropoiesis, extramedullary hematopoiesis, and iron overload resulting from transfusion and increased iron absorption.

The skin may show pallor from anemia and jaundice from hyperbilirubinemia, and the skull and other bones may be deformed secondary to erythroid hyperplasia with intramedullary expansion and cortical bone thinning. The extremities may demonstrate skin ulceration.

Heart examination may reveal findings of cardiac failure and arrhythmia, related to either severe anemia or iron overload.

Abdominal examination may reveal changes in the liver, gallbladder, and spleen. Hepatomegaly related to significant extramedullary hematopoiesis typically is observed. Patients who have received blood transfusions may have hepatomegaly or chronic hepatitis due to iron overload.

The gallbladder may contain bilirubin stones formed as a result of the patient's lifelong hemolytic state. Splenomegaly typically is observed as part of the extramedullary hematopoiesis or as a hypertrophic response related to the extravascular hemolysis.

In addition to cardiac dysfunction, hepatomegaly, and hepatitis, iron overload can also cause endocrine dysfunction, especially affecting the pancreas, testes, and thyroid. Transfusion-associated viral hepatitis resulting in cirrhosis or portal hypertension also may be seen.

However, the signs and symptoms you experience depend on the type and severity of thalassemia you have. Some babies show signs and symptoms of thalassemia at birth, while others may develop signs or symptoms during the first two years of life. Some people who have only one affected hemoglobin gene don't experience any thalassemia symptoms.

2.8 Complications

One of the most challenging aspects of living with and treating beta thalassaemia major BTM) is the number of possible complications that can occur.
People with BTM (and some people with moderate-to-severe BTI) will need frequent check-ups, so the risk of possible complications can be regularly assessed.
Some common complications of BTM are outlined below.

a) Enlarged spleen (hypersplenism)

One of the functions of the spleen (an organ found behind the stomach) is to recycle red blood cells. In people with BTM, the blood cells are often abnormal in shape, so the spleen has problems recycling them. The result is that an increasing amount of blood stays in the spleen, making it grow larger.
This can lead to the spleen becoming overactive, when it starts to destroy healthy blood cells received during blood transfusions, making effective treatment for BTM difficult. In these circumstances, the only treatment is to remove the spleen using a procedure known as a splenectomy.
The spleen also plays an important part in fighting infections. Therefore, if your child has their spleen removed, it is likely that vaccinations against potentially serious infections, such as meningitis and flu, will be recommended.
Encourage your child to be alert to possible symptoms of infections, such as muscle pain or fever, and report them as soon as possible. This is because infections could have a more serious effect on them than most people.

b) Hormone complications

One of the glands that regulate the hormone system (the pituitary gland) is very sensitive to the effects of iron. It can, therefore, become damaged in some people with BTM, even if they stick to their chelation therapy. Damage to the pituitary gland can result in a number of hormonal conditions, including delayed puberty and restricted growth. Hormone replacement therapy may be needed to correct these conditions. Other complications that can appear after puberty include diabetes and an underactive thyroid gland or overactive thyroid gland (Kaye, Todd, Guay, 1993).Children with BTM will need their height and weight checked every six months to make sure they are developing normally. Teenagers who have begun puberty will need their development assessed every year.

c) Heart complications

Iron overload can cause damage to the heart, leading to:

- an irregular or disturbed heartbeat (arrhythmia)
- impaired pumping of the heart (systolic dysfunction)
- build-up of fluid in the tissue of the heart (pleural effusions)

- heart failure

If you have BTM, you will need a check-up every six months to determine how well your heart is functioning. Every year, you will also need a full examination, carried out by a cardiologist (heart specialist), using an electrocardiogram (ECG) test to measure the electricity of your heart (Mayoclinic, 2011).

If damage to your heart is detected, it can be stopped and possibly reversed using more extensive chelation therapy. Medication, such as angiotensin-converting enzyme (ACE) inhibitors, can also be used to improve the functioning of your heart (Tanner, Mark, 2008).

d) Liver complications

Iron overload can also cause damage to the liver, resulting in:

- hepatitis (swelling of the liver)
- an enlarged liver (fibrosis)
- cirrhosis (a progressive disease where the liver is increasingly damaged by scarring)

Chelation therapy can prevent further damage to the liver and antiviral medicines can be used to prevent further liver infection. Liver tests are recommended every three months to monitor the condition of the liver (Stickel, Felix, 2010).

e) Bone complications

If your body is not receiving enough healthy red blood cells, it will try to compensate by expanding the bone marrow, which in turn will expand the bones. This can lead to skeletal deformities, bone and joint pain and osteoporosis (a condition where the bones become thin and brittle.)

Low bone density is common, even in people who have been receiving regular blood transfusions. Those with low bone density are at increased risk of fracturing (breaking) their bones.

People with BTM are encouraged to eat a diet high in calcium and vitamin D, both of which help strengthen the bones. Foods high in calcium include:

- beans
- tofu
- sardines
- salmon
- broccoli
- oranges
- oats
- dairy products, such as milk and cheese

Foods that are high in vitamin D include:

- milk

- orange juice
- eggs
- fish
- liver

You may also be advised to take vitamin D and calcium supplements.
Regular exercise can also help strengthen bones. Adults should do at least 150 minutes (2 hours and 30 minutes) of moderate-intensity aerobic activity (i.e. cycling or fast walking) every week. Two types of activity particularly important in improving bone density and helping prevent osteoporosis are weight-bearing exercises, such as running and aerobics, and resistance exercises, such as weight training and press-ups.
Osteoporosis can be treated using medicines called bisphosphonates, which help maintain bone density and reduce the chances of fracture. However, bisphosphonates are not recommended for children and teenagers because they can interfere with normal bone development.

2.9 Risk factors

- **Family history of thalassemia.** Thalassemia is passed from parents to children through mutated hemoglobin genes. If you have a family history of thalassemia, you may have an increased risk of the condition.
- **Certain ancestry.** Thalassemia occurs most often in people of Italian, Greek, Middle Eastern, Asian and African ancestry.

2.10 Diagnosis

Thalassaemia can be diagnosed using a blood test. Further DNA testing of the blood may be required so the exact type of thalassaemia can be determined.

a) Antenatal screening

The purpose of antenatal screening (screening that is carried out during pregnancy) is to check for inherited disorders such as sickle cell anaemia and to provide parents with information they need to make informed decisions.
Antenatal screening for the thalassaemia trait is available in areas where the condition is most common. These are typically cities and towns with large South Asian communities. In areas where thalassaemia is uncommon, a questionnaire on family origin is used as an initial screening tool to assess the risk of thalassaemia (Leung, Lau, Chung, 2005).
Pregnant women are routinely screened for the thalassaemia trait. If they test positive their partner will also be offered the test. If both parents have the thalassaemia trait, there is a 1 in 4 chance their baby will have thalassaemia.

Further testing is available (if you want it) to confirm whether your baby will definitely be born with thalassaemia. There are three methods of doing this:

- chorionic villus sampling (CVS) - during CVS, a small sample of placenta (the organ that is attached to the womb lining during pregnancy) is removed from the womb and tested
- amniocentesis - a small sample of the fluid inside the womb (amniotic fluid) is taken for testing
- foetal blood sampling (FBS) - under local anaesthetic, a small sample of blood is taken from your baby's umbilical cord, or from the umbilical vein as it passes through their liver

b) Counselling

Being told your baby will be born with thalassaemia can be traumatic and upsetting. You will be offered counselling to give you and your partner the opportunity to express your feelings and to ask questions about how the diagnosis may affect you.
The counsellor will inform you of different options available, allowing you to make a more informed decision about how to proceed with the pregnancy (Orkin, Stuart, Nathan, 2009).

Pre-implantation genetic diagnosis (PGD) is an option for couples who do not want to give birth to a child with thalassaemia but are unwilling to consider terminating a pregnancy.
PGD is similar to in-vitro fertilisation (IVF). IVF is a method of helping infertile couples conceives by surgically removing an egg from the woman's ovaries and fertilising it with the man's sperm in a laboratory.

As with IVF, PGD involves removing eggs from a woman's ovaries, which are fertilised using a sample of sperm taken from her partner. The fertilised embryo can be tested for thalassaemia. If the results are negative, the embryo can then be implanted into the woman's womb.

PGD is a new procedure only available at a number of specialist thalassaemia centres. A list of sickle cell centres in England is available on the website of the Brent Sickle Cell and Thalassaemia Centre.

c) After birth

Unlike the related disorder sickle cell anaemia, newborn babies are not regularly screened for thalassaemia (Medical care open publishing, 2011). There are two reasons:

- blood tests are usually unreliable during the first six months of life
- unlike sickle cell anaemia, thalassaemia does not present an immediate threat to a baby's life (babies born with sickle cell anaemia have a high risk of catching serious infections and need immediate treatment with antibiotics)

If your baby does begin to develop symptoms as they grow older, the diagnosis can be confirmed using a blood test (Orkin, Stuart, 2009).

d) Assessing iron levels

People with beta thalassaemia major (BTM) will require regular blood transfusions, which will increase the level of iron in their body (Samavat, Model 2004).

To remove the excess iron, they must have treatment called chelation therapy. This helps prevent serious complications from excess iron, such as heart or liver disease. Regular tests to measure iron levels are required to monitor the effectiveness of chelation therapy.
There are three main ways of assessing iron levels:

- blood tests
- magnetic resonance imaging (MRI) scans
- liver biopsy

e) Blood tests

Blood tests provide a convenient way of measuring the amount of iron in your blood, although they do not provide a detailed assessment of how much iron may be collecting in certain organs, such as in your brain. The measurements provided by blood tests can also be distorted by other factors, such as infection (Samavat, Modell, 2004).

Blood tests are used to provide a general overview of how well your chelation therapy is working, but cannot be used in isolation. It is usually recommended that a person with thalassaemia receives a blood test at least every three months (Burdick, Ntaios, 2009).

f) MRI scans

A magnetic resonance imaging (MRI) scan uses powerful magnetic waves to build up a detailed picture of the inside of your body. MRI scans are able to detect and then measure any iron in your organs.

The two organs known to be most vulnerable to the effects of iron are the liver and the heart. It is usually recommended that you have an MRI scan of your liver at least once a year and an MRI scan of your heart at least once every two years. More frequent scans may be required if high levels of iron are found in your heart and liver (Tanner, Mark, 2008).

g) Liver biopsy

A liver biopsy uses minor surgery to remove a tiny section of your liver to test it for the presence of iron. An MRI scan is usually preferred to a liver biopsy as it is more convenient (for both the doctor and the person having the scan). However, if a detailed assessment of

the level of iron in the body is required, a liver biopsy may be necessary (Valenti, Luca, 2010).

2.11 Management and treatment

It is important to be able to make a definitive diagnosis of these causative mutations to inform management and counselling. Couples at risk of having an affected child can be offered genetic counselling and reproductive options including prenatal diagnosis which involves foetal sampling to determine the fetal genotype. Screening programs, aimed at prevention of the disease, and prenatal diagnosis have resulted in a marked reduction in the birth rate of affected children in Greece, Cyprus, continental Italy, and Sardinia. Widespread use of similar programs in other areas of the world has not yet been possible. Screening for carriers is performed most efficiently by measurement of the red-cell indexes and, in samples from persons with reduced mean cell volumes and mean cell hemoglobin concentrations, estimation of the hemoglobin A2 concentration. The practical problems associated with screening for rarer forms of β-thalassemia and the effect of coexistent a thalassemia on the red-cell indexes have been reviewed recently. Prenatal diagnosis, first carried out by fetal blood sampling and assessment of globin chain synthesis in fetal blood, more recently has involved direct analysis of fetal DNA obtained by chorionic-villus sampling. This approach is associated with a very slightly increased risk of fetal loss and an error rate in experienced laboratories of less than 1 percent. The practical aspects of fetal DNA analysis have also been recently reviewed (Lozano, Naghavi, Mohen, 2010).

a) Blood transfusion (Medical Therapy)

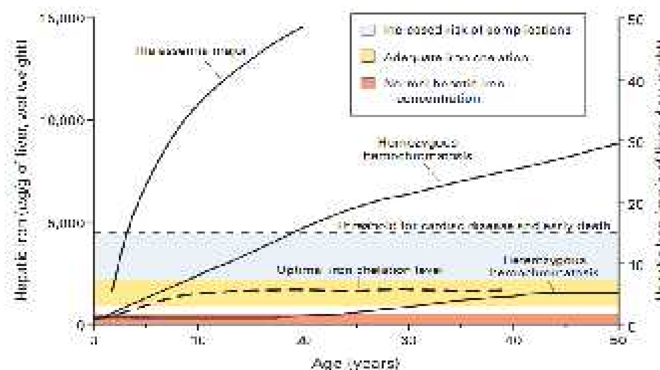
A decision to initiate regular transfusions in patients with β-thalassemia may be difficult and should be based on the presence and severity of the symptoms and signs of anemia, including failure of growth and development (Cianciulli, 2008). Only rarely is genotyping helpful in this decision. The goals of transfusion include correction of anemia, suppression of erythropoiesis, and inhibition of increased gastrointestinal absorption of iron. “Hypertransfusion” and “supertransfusion” regimens, which achieve these goals but are associated with substantial iron loading (Olivieri, Brittenham, 1997 and Fosburg, Nathan, 1990), have been supplanted by regimens in which the hemoglobin concentration before transfusion does not exceed 9.5 g per deciliter (Cazzola, Bornga, 1997). These newer regimens are associated with both adequate marrow suppression and relatively lower rates of iron accumulation.

The beneficial effects of iron-chelating therapy with parenteral deferoxamine, the only chelating agent widely available for clinical use, on the complications of iron loading have recently been reviewed (Olivieri, Brittenham, 1997). As a result of programs of deferoxamine therapy, the prognosis for patients in countries able to afford this therapy has greatly improved, in contrast to the prognosis for patients in developing countries, where wide-spread implementation of this regimen is still awaited.

Adequate deferoxamine therapy prevents early death from cardiac disease: maintenance of body iron bur-dens corresponding to hepatic iron concentrations of less than 15 mg per gram, dry weight, greatly decrease the risk of clinical disease. Nearly normal concentrations

of hepatic iron can be maintained with modern regimens of deferoxamine. Moreover, deferoxamine arrests the progression of hepatic fibrosis to cirrhosis, even when administered in regimens that stabilize, rather than reduce, the body iron burden. The importance of this finding in the seminal study that ushered in the modern era of deferoxamine therapy is highlighted by evidence that in another form of iron overload, hereditary hemochromatosis, progression of hepatic fibrosis is a critical event associated with an increased risk of death (Niederau, Fischer, 1996). A favourable effect of a sustained reduction in body iron is also suggested by the relatively low prevalence of thyroid, parathyroid, and adrenal abnormalities in the modern era (Bronspiegel, Olivieri, 1990). In parallel, early and intensive deferoxamine therapy may increase the incidence of normal sexual maturation (Bronspiegel, Olivieri, 1990), but it apparently does not reverse established abnormalities (Olivieri, Brittenham, 1997). Similarly, although deferoxamine prevents diabetes mellitus (Brittenham, Griffith, 1994) there is no evidence that it can reverse this complication. In summary, modern regimens of subcutaneous deferoxamine may extend survival free of many complications of iron overload, if body iron is reduced or maintained below critical concentrations (Olivieri, Brittenham, 1997).

A balance between the effectiveness of deferoxamine and its toxicity the latter observed primarily in the presence of relatively low body iron burdens can be maintained through regular determinations of body iron burden (Maggio, Aurelio, 2002). In clinical practice, the serum ferritin concentration is commonly used to assess the effectiveness of treatment. It is increasingly recognized that reliance on this test may lead to errors in management; changes in body iron account for little more than half the variation in serum ferritin concentrations (Brittenham, Cohen, McLaren et al, 1993). By contrast, the measurement of hepatic iron stores, whose concentrations are highly correlated with total body iron stores (Angelucci, Giardini, 1997), provides the most quantitative, specific, and sensitive method of evaluating iron burden in patients with thalassemia. Determination of hepatic iron concentrations in liver-biopsy specimens obtained with ultrasonographic guidance is safe and permits rational adjustments in iron-chelating therapy (Olivieri, Brittenham, 1997). Magnetic susceptometry provides a direct measure of hepatic iron stores that is quantitatively equivalent to that determined by biopsy of at least 0.6 mg of liver, dry weight (Olynyk, Britton, Bacon 1994) over a range of iron concentrations (Brittenham, Farrell, 1982). Magnetic susceptometry is currently available in only two centers worldwide. By contrast, the more widely available technique of magnetic resonance imaging fails to provide accurate quantitation of hepatic iron concentrations in patients with severe iron overload, hepatic fibrosis, or both (Angelucci, Giovagnoni, 1997).



Source-Massachusetts Medical Society

Fig.16; Hepatic iron burden over time and the effect of various hepatic iron concentrations in patients with thalassemia major, homozygous hemochromatosis, and heterozygous hemochromatosis (Stickel, Felix, 2010).

b) Bone Marrow transplantation

Bone marrow transplantation from HLA-identical donors has been successfully performed worldwide in over 1000 patients with severe β-thalassemia (Giardini, 1997). Out-comes after transplantation are greatly influenced by the presence of hepatomegaly, portal fibrosis, and in-effective chelating therapy before transplantation (Lucarelli, Giardini,1997). Children without any of these risk factors have rates of survival and disease-free survival exceeding 90 per-cent three years after transplantation. In those with all three risk factors, and in most adults, the rates are approximately 60 percent. Lower success rates are re-ported at smaller centers (Lucarelli, Giardini, 1997). Complications include a rate of chronic graft-versus-host-disease ranging from 2 to 8 percent and a variable incidence of mixed chimerism (Giardini, 1997). Post-transplantation management of pre-existing hepatic iron overload, iron-induced cardiac dysfunction, and viral hepatitis may prevent progression of these processes (Giardini, 1997). There is interest in experimental approaches to bone marrow replacement in patients with thalassemia, including cord-blood transplantation (Issaragrisil, Visuthisakchai, 1995), the use of unrelated phenotypically matched donors (Contu, La Nasa, 1994), and in utero transplantation (Westgren, Ringden 1996).

c) Gene therapy

Permanent correction of genetic deficit of the hematopoietic system requires the transfer of genes into stem cells and long-term, high-level, lineage-specific expression of these cells after autologous trans-plantation (Sodani, Isgro, 2011); mature cells and committed progenitors do not have the proliferative capacity to reconstitute the entire hematopoietic system (Chin, Joanna, 2008). Over the past decade, there has been progress in the development of transduction methods and vectors (Verma, Somia, 1997). Remaining problems include the identification of all sequences required for stable, high-level expression of the genes and the development of more effective and safe vectors for the transfer of genes (Higgs, Sharpe, 1998). Another approach correction of the defective gene by site-directed re-combination is feasible, but current methods lack the degree of efficiency required (Shesly, Kim, 1991).

2.12 β-thalassemia in Nepal.

There is no concrete data on the beta- thalassemia and its cases. Nepal Government does not have databases on thalassemia around the Nepal. But they claim to have only some regional thalassemic database.

There is only one organisation dealing with thalassemia on its own. From the private sector and contribution from thalassemic patients themselves, they have established an organisation named Nepal Thalassemia Society. According to the society, they have more than 250 thalassemic cases recorded in Kathmandu and Chitwan and the rate is increasing. Nepal Thalassemia society is helping patient with providing blood transfusion in cheaper

rates with collaboration of Nepal Red Cross, providing iron chelating agent and better counselling for the management of the disease.

Nepal government and regional health centres claim that thalassemia is common in the Terai region of Nepal. The chaudhary and Mandal communities are mostly affected by this disorder. The Nepal government has listed thalassemia under the non-communicable disease, but there is not a single programme to address the diseases till date (The Himalayan Times.18/1/13).

Chapter 3
MATERIALS AND METHOD

3.1 Selection of study site

Nepal Government has rough calculation of thalassemic cases. They report; thalassemic cases are prevalent in people living in Terai region and common in malaria prevalent areas. This statement reveals, chaudhary ethnic community are more prone to this disorder. Since there is no solid data of occurrence and distribution of thalassemia in Government level, we found difficulty in finding the study site.

We have come to know about a private organisation serving the thalassemic patients on its own with aid from various personnel in and out of the Nepal. This organisation is named as **"Nepal Thalassemia Society".** The society had been established by the energetic and enthusiastic thalassemic patients with help of some of the social worker several years earlier. They have claimed that they have been providing the service to thalassemic patients from all over the nation as well as from the India near to the border of Nepal.

We have seen the data provided by society and found their claim is true. Even we have directly met the patients from different region of Nepal. Hospital is not giving proper care to them. I must say, there is no such hospital particularly designed for the thalassemic patients till date.

Nepal Thalassemia Society is providing the simple services. Like, they provide blood transfusion in very cheaper rate with best care, haemoglobin estimation, iron chelating agents and counselling for the better management and care of these patients.

We have collected the blood samples from 61 patients and 46 one of the immediate family member with their written consent on approval from NHRC. These patients were from all over the Nepal with almost all of the ethnic group. Society has help in many ways with their deep and warm regard in this research.

3.2 Collection and transport of blood samples

For blood collection, the research participants satisfying the inclusion criteria (healthy donors and family member) and exclusion criteria (patients having HIV, tuberculosis, other genetic or congenital diseases) of age group from 2 years and above of all genders.

On approval of consent from respective patients and immediate family member, 3ml blood was withdrawn from the inclusion criteria satisfied patients by the help of the expert phlebotomist using the sterile syringe and transferred to BD vaccutainer followed by gentle shaking for proper mixing with anticoagulant. Collected blood samples were transported in cold chain box to the Central Department of Biotechnology Lab and stored at 4°C.

3.3 DNA extraction from blood sample by using Proteinase K digestion method (*Sambrook et. al*., 1989)

i) 500ul of blood sample was transferred into 1.5 ml eppendrof tube.

ii) TEN buffer was added to it.

iii) 5ul Proteinase K (100ug/ml) was added and again 20% SDS was added to the mixture.

iv) The mixture was vortexed for proper mixing and kept at 37°C in shaker incubator overnight.

v) Then equal volume of Tris –phenol was added to the mixture and mixed well.

vi) The mixture was centrifuged at 13000 rpm for 10 mins.

vii) The aqueous phase was transferred into the fresh ep. tube using wide mouthed microtip so that shearing is minimized and the interphase is not disturbed.

viii) Equal volume of chloroform-isoamyl (24:1) was added and mixed thoroughly.

ix) The tube with mixture was centrifuged at 13000rpm for 10 mins.

x) Again aqueous phase was collected in fresh ep. tube and $1/10^{th}$ volume of 3M sodium acetate (ph 5.2) was added.

xi) 2.5 times volume isopropanol was added and mixed by inverting gently few times. This step is done for DNA precipitation.

xii) Again it was spinned at 13000rpm for 10 mins to pellet the DNA and supernatant was discarded.

xiii) The pellet was washed 2 times with 70% ethanol to wash out the salt and once with absolute alcohol to remove traces of 70% ethanol. (Each time spinning at 13000 rpm for 2-3 mins. and discarding the supernatant.)

xiv) The pellet was vacuum dried and dissolved in 100ul TE buffer and stored at 4°C in cold room.

3.4 Quantification of DNA

The concentration of DNA of the given sample was determined because subsequent PCR requires particular amount of DNA for optimal performance. There are different methods for quantification of DNA like spectrophotometric quantification, UV florescence in presence of DNA dye etc.

In the research study, we initially quantify DNA by running the gel electrophoresis and look for the precise band. But this does not provide the accurate quantity and purity of the DNA to study. We prefer to quantify the DNA through nanodrop.

Figure 17, Quantification of DNA done by Nanodrop.

Introduction

Absorbance measurements made on a spectrophotometer, including any Thermo Scientific Nano Drop Spectrophotometer, will include the absorbance of all molecules in the sample that absorb at the wavelength of interest. Since nucleotides, RNA, ssDNA, and dsDNA all absorb at 260 nm, they will contribute to the total absorbance of the sample. Therefore, to ensure accurate results when using a nanodrop Spectrophotometer, nucleic acid samples will require purification prior to measurement.

260/280 Ratio

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Some researchers encounter a consistent 260/280 ratio change when switching from a standard cuvette spectrophotometer to a Nano-Drop Spectrophotometer.

260/230 Ratio

This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.

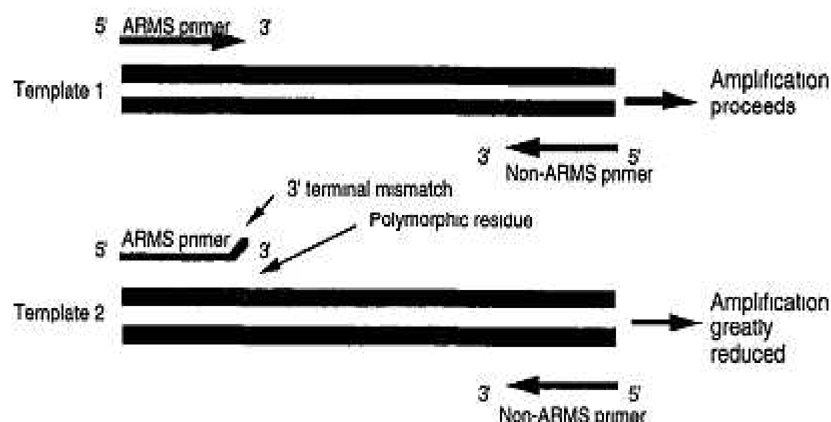
For DNA quantification, we take 1 O.D of double stranded DNA at 260 nm = 50ug/ml (Sambrook and Russell, 2001)

Therefore, DNA concentration (ug/ml) = A_{260} x dilution factor x 50

3.5 Multiplex ARMS (Amplification Refractory Mutation System) PCR.

The amplification refractory mutation system (ARMS) is an amplification strategy in which a polymerase chain reaction (PCR) primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue (Newton, Graham, Summers, 1989 and Bottema, Cassady, 1993). ARMS has also been termed allele-specific PCR (Wu, Ugozzoli, 1989) or PCR amplification of specific alleles (PASA)(Bottema, Cassady, 1993). Thus, an ARMS primer can be designed to amplify a specific member of a multi-allelic system while remaining refractory to amplification of another allele that may differ by as little as a single base from the former. The main advantage of ARMS is that the amplification step and the diagnostic steps are combined, in that the presence of an amplified product indicates the presence of a particular allele and vice versa. For routine diagnosis, this characteristic of ARMS means that it is a very time efficient method. However, this combination of the amplification and diagnostic steps has resulted in a system that may not be as robust as some of the other methods in which these two important steps are separated, e.g., PCR followed by restriction enzyme analysis.

ARMS is based on the principle that the *Thermus aquaticus* (Taq) polymerase, the DNA polymerase commonly used in PCR, lacks a 3’ to 5’ exonuclease activity and thus a mismatch between the 3’ end of the PCR primers and the template will result in greatly reduced amplification efficiency (Fig. 18). Thus, an ARMS typing system can be designed by constructing primers with their 3’ nucleotide overlying the polymorphic residue. Hence, one ARMS primer can be constructed to specifically amplify one allele of a multiallellic system. For typing a system with n alleles, n ARMS primers will be required with the typing achieved in n reactions. For many diagnostic applications, a second set of control primers are also included in the PCR, and act as an internal amplification control.



Fig, 18 showing ARMS PCR working

Multiplex ARMS is developed to facilitate the application of ARMS to genotyping multiallellic systems. In multiplex ARMS, a number of ARMS primers, each specific for a particular allele, are included in a single reaction in a single tube. PCR products corresponding to different alleles can then be distinguished by physical characteristics, such as length. A number of formats for multiplex ARMS are potentially possible (Fig.19).

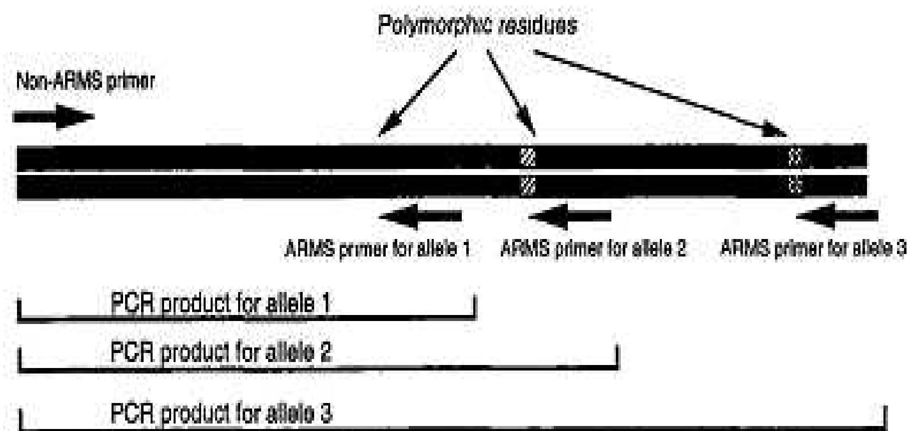


Fig. 19, Multiallellic genotyping by Multiplex ARMS PCR

Primer design is the most important aspect in creating a working ARMS based typing system. The ARMS concept requires that the nucleotide or nucleotides distinguishing the various alleles to be placed at the 3' end of the ARMS primer (Newton, Graham, Summers, 1989). Different authors have slightly different views as to the type of mismatches that are most discriminatory for ARMS analysis (Lo, Fleming, 1994 and Kwok, Kellogg, 1990). The reason for this discrepancy between the observations of different groups is unclear but could be related to differences in reaction conditions used by different investigators. In our hands, A-G or G-A mismatches are the most discriminatory for ARMS analysis. For other types of nucleotide mismatches, we routinely introduce an artificial mismatch at the residue 1 or 2 bases from the 3' end of the primer to further enhance the specificity of the primer (Newton, Graham, Summers, 1989). Primers ranging from 14 to 30 bases have been used for ARMS analysis (Kwok, Kellogg, 1990). Newton *et al*. recommended the use of relatively long primers of 30 bases for this purpose and we have found that primers of this length work well. For a given polymorphism or mutation, an ARMS primer can be designed to prime in the sense or the antisense direction. Hence, if a given ARMS primer is found to be nonspecific m one direction, it is worthwhile constructing another one in the opposite direction.

Allele-specificity is dependent on the annealing temperature during amplification. We routinely start our optimization with an annealing temperature of 55°C and vary the temperature in 3°C steps. Although optimization of the $MgCl_2$ concentration is also important in certain primer combinations, we find that a concentration of 1.5 mM works well for most of our applications. The hot start technique (Chou, Russell, 1992) is useful for certain "problematic" primers, but for most primer-template combinations, conventional non-hot start PCR appears to be adequate. ARMS specificity is also affected by dNTP concentrations. Hence, a low deoxynucleoside triphosphate (dNTP) concentration has been found to result in a more specific reaction, although sometimes with reduced

sensitivity (Kwok, Kellogg, 1990). We find that a dNTP concentration of 100 µM for each of dATP, dCTP, dGTP, and dTTP works well for most ARMS primers.

3.5.1 Multiplex ARMS PCR protocol

According to the protocol generated by Institute of Medical Research Centre, Kualalumpur, Malaysia; we ran the multiplex ARMS PCR differentiated into various panels like MARMS A to F, for the analysis of different types of mutations. The MARMS was done in Eppendrof Mastercycler Gradient, 5531 Eppendrof version PCR.The master mixed was Qiagen (Hotstar Taqplus Master Mix Kit). The protocols for each MARMS panel are as follow;

Master mix preparation (5% overage)

MARMS- A

Components		Total Vol./Master mix tube(ul)	
HSTaq Plus		10.5	
M primer	IVS1-5	0.63	
	Cd 41/42	0.08	
	Cd 17	0.11	
	Cd26	0.11	
	Ctrl A	0.380	
	Ctrl B	0.280	
	Ctrl E	0.63	
Coral Load		2.1	
RNAse free H2O		4.08	
DNA Template		2.0	

Master mix preparation (5% overage)

MARMS- B

Master mix preparation (5% overage)

Master mix preparation (5% overage)

Components		Total ol./Master mix tube (ul)	
HSTaq Plus		10.5	
M primer	-86	0.08	
	Cd 19	0.32	
	Cap +1	0.08	
	Ctrl A	0.38	
	Ctrl B	0.28	
	Ctrl F	0.42	
Coral Load		2.1	
RNAse free H2O		4.74	
DNA Template		2.0	

MARMS- C

Components		Total Vol./Master mix tube (ul)	
HSTaq Plus		10.5	
M primer	-88	0.08	
	Ini Cd	0.32	
	Cd 15	0.08	
	-29	0.06	
	Ctrl A	0.380	
	Ctrl B	0.280	
	Ctrl F	0.42	
Coral Load		2.1	
RNAse free H2O			
DNA Template		2.0	

Components		Total Vol./Master mix tube (ul)	
HSTaq Plus		10.5	
M primer	IVS 1-1 (G-A)	0.63	
	Cd 43	0.150	
	Cd 16	0.42	
	Poly A	0.11	
	Ctrl A	0.380	
	Ctrl B	0.280	
	Ctrl E	0.63	
Coral Load		0.11	
RNAse free H2O		5.70	
DNA Template		2.0	

MARMS-D

Master mix preparation (5% overage)

Master mix preparation (5% overage)

Components		Total Vol./Master mix tube (ul)	
HSTaq Plus		10.5	
M primer	IVS 1- 1 (G-T)	0.63	
	Cd 8/9	0.340	
	-28	0.32	
	Cd 71/72	0.21	
	Ctrl A	0.380	
	Ctrl B	0.28	
	Ctrl E	0.63	
Coral Load		2.10	
RNAse free H2O		3.520	
DNA Template		2.0	

MARMS F **MARMS E**

Table 2; the multiplex ARMS PCR protocol (Table A, B, C, D, E and F).

Vol. /reaction tube: 20ul
Unit of polymerase: 5U/ul

The thermocycler programme for all the MARMS panels A-E is same.

Components		Total Vol./Master mix tube (ul)	
HSTaq Plus		10.5	
M primer	IVS 2-654	0.42	
	Ctrl C	0.21	
	Ctrl D	0.11	
	Crtl F	0.63	
Coral Load		2.1	
RNAse free H2O		4.94	
DNA Template		2.0	

Thermocycler programme

Cycling steps	Temp	Duration	Cycles
Enz.Activation	95°C	5min	1
Denaturation	94°C	45secs.	30
Anneal/Extent	64°C	45secs.	30
Extension	72°C	1:30min	30
Final extension	72°C	7min	1

Table 3; PCR cycle for MARMS A-E

3.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to confirm the amplification .For this, 2.5 – 3 % DNA grade agarose gel was prepared in 1% TBE buffer. Then it was casted on gel electrophoresis tank.3 ul of PCR products stained with Syber green dye were loaded in the gel with suitable molecular weight ladder (100bp, First base).The gel was run at 70 V for 1 hour. Finally the gel was observed under the gel doc apparatus (Alpha DigiDoc^{TM}1201) under the UV illuminator to see the various amplified PCR product and tally with the molecular ladder and accordingly analyse the respective mutations. The photograph was taken, printed and also saved in computer database for future use.

CHAPTER 4
RESULT AND DISCUSSION

4.1 DNA purity and quantity measurement

The DNA from the blood was extracted using Proteinase K digestion followed by phenol-chloroform method. The DNA samples purity and quantity measurement was carried out using the nano-drop spectrophotometry. The quantity of the DNA was found to be from 6.04ng/ul to 691.95 ng/ul and purity ranges from 0.99 to 1.85. Following is the table showing the DNA concentration and purity (**Table 4**).

SN	Absorbance 260nm	Absorbance 280nm	Concentration ng/ul	Purity
1	0.998	0.704	49.90	1.42
2	0.262	0.170	17.67	1.21
3	1.881	1.079	94.07	1.74
4	0.460	0.306	22.99	1.5
5	0.673	0.504	33.65	1.34
6	0.856	0.585	42.81	1.46
7	0.435	0.270	58.25	1.61
8	0.858	0.563	42.92	1.53
9	1.125	0.729	56.26	1.54
10	0.761	0.456	38.05	1.67
11	0.933	0.763	46.67	1.22
12	1.025	0.625	51.25	1.64
13	0.566	0.329	28.32	1.72
14	13.839	7.494	691.95	1.85
15	0.984	0.649	49.21	1.52
16	3.077	1.709	153.87	1.80

17	0.480	0.316	24.00	1.71
18	0.377	0.240	18.85	1.57
19	1.37	0.939	68.51	1.46
20	1.200	0.868	59.99	1.38
21	0.499	0.390	24.97	1.28
22	0.251	0.186	12.55	1.35
23	0.547	0.344	27.35	1.59
24	0.414	0.272	20.69	1.52
25	1.747	1.023	87.37	1.71
26	0.543	0.371	27.16	1.46
27	0.383	0.261	19.15	1.47
28	0.727	0.590	36.35	1.23
29	0.26	0.2	13.00	1.3
30	0.352	0.251	17.62	1.4
31	0.65	0.471	32.51	1.38
32	0.364	0.268	18.18	1.36
33	1.168	0.746	58.38	1.56
34	1.105	0.651	55.24	1.7
35	0.792	0.568	39.62	1.39
36	0.738	0.509	36.89	1.45
37	0.378	0.247	18.90	1.53
38	0.467	0.308	23.35	1.52
39	1.003	0.635	50.14	1.58
40	0.709	0.718	35.46	0.99
41	8.058	4.709	402.72	1.71

42	0.878	0.49	43.88	1.79
43	0.203	0.160	10.17	1.27
44	0.255	0.196	16.43	1.30
45	0.493	0.346	24.67	1.66
46	0.192	0.171	9.59	1.12
47	0.358	0.315	17.88	1.13
48	2.367	1.524	118.34	1.55
49	0.639	0.419	31.95	1.53
50	1.859	1.227	92.94	1.52
51	0.360	0.239	18.43	1.54
52	1.320	0.771	65.98	1.71
53	1.093	0.615	54.67	1.78
54	0.377	0.327	18.85	1.15
55	0.16	0.14	7.98	1.14
56	0.238	0.198	11.92	1.2
57	0.121	0.118	6.04	1.03
58	0.216	0.189	10.82	1.15
59	0.595	0.375	29.76	1.59
60	0.475	0.371	23.76	1.28
61	0.307	0.231	15.35	1.33
62	0.509	0.385	25.44	1.32
63	0.917	0.528	45.85	1.74
64	0.372	0.258	18.58	1.44
65	0.407	0.349	20.34	1.17
66	0.894	0.875	44.71	1.02

67	0.183	0.126	9.14	1.45
68	0.203	0.177	10.16	1.15
69	0.433	0.284	21.64	1.52
70	0.533	0.315	26.63	1.69
71	0.242	0.195	12.1	1.24
72	0.400	0.309	20.00	1.29
73	0.726	0.492	36.30	1.47
74	0.358	0.294	17.89	1.22
75	1.508	0.882	75.38	1.71
76	0.959	0.609	47.97	1.58
77	0.220	0.194	11.00	1.14
78	0.189	0.155	9.43	1.22
79	0.475	0.392	23.75	1.21
80	0.551	0.348	27.53	1.58
81	1.310	0.908	65.52	1.44
82	0.850	0.552	42.48	1.54
83	1.333	0.913	66.63	1.46
84	0.326	0.227	16.30	1.43
85	0.256	0.212	12.82	1.21
86	0.172	0.137	8.59	1.25
87	0.086	0.8	4.29	1.07
88	0.280	0.246	14.01	1.14
89	0.558	0.329	27.91	1.7
90				
91	1.049	0.735	52.24	1.42

92	0.632	0.442	31.61	1.43
93	0.744	0.473	37.21	1.57
94	0.644	0.460	32.21	1.40
95	1.241	0.734	62.06	1.69
96	0.262	0.210	13.11	1.25
97	0.317	0.268	15.85	1.18
98	0.199	0.151	9.94	1.32
99	0.406	0.267	20.30	1.52
100				
101	1.985	1.090	99.25	1.82
102	1.701	0.997	85.05	1.71
103	1.598	0.916	79.92	1.71
104	0.574	0.321	28.72	1.70
105	0.365	0.292	18.25	1.25
106	1.276	0.818	63.80	1.56
107	1.267	0.703	63.34	1.8

Table 4; DNA concentration and purity.

4.2 Agarose gel electrophoresis

The respective PCR products were run on the agarose gel. The 2%, 2.5% and 3% agarose gel (Agarose biotechnology Grade (1st Base ptel_th) was prepared. The gel was casted in electrophoretic tank. The 1% TBE buffer was used as running buffer. The required volt of electricity was applied for 1 hour. After electrophoresis, the gel was observed under gel doc to analyse the respective mutations.

The amplicons produced after the MARMS were stored in 4°C before gel run. The amplicon were mixed with syber green in 1:1 ratio for the visualisation under gel doc.The the mixture was loaded in respective wells in gel. The 100 bp DNA ladder was used to interpret the result. Various types of mutation were analysed after gel electrophoresis, when the bands were tallied with the 100bp first base molecular ladder.

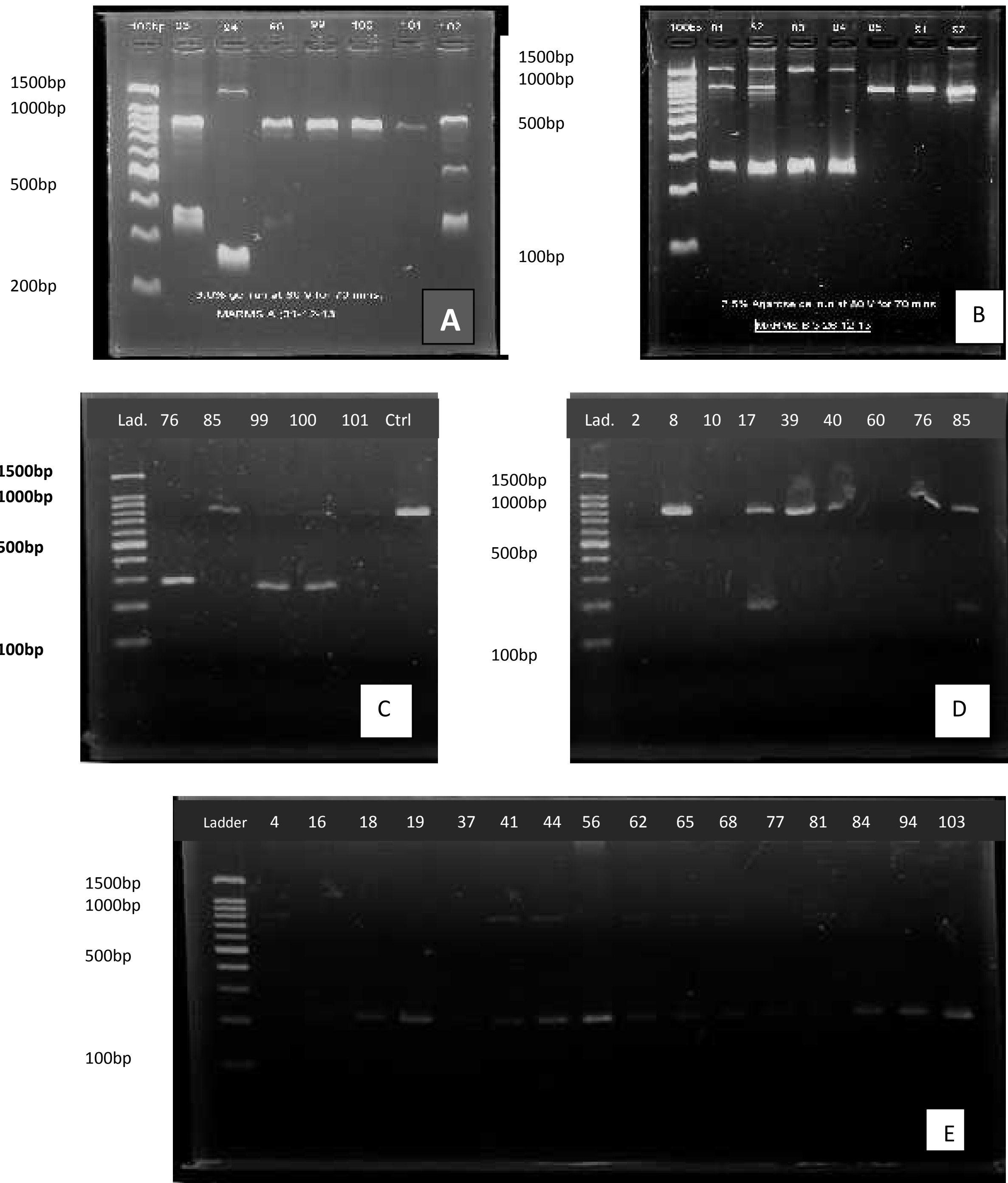


Figure 20, **A)** agarose gel electrophoresis of MARMS A amplicons showing various mutations like IVS 1-5, Cd 26 in sample with patients 93 and 619 del. in sample with ID 94. **B)** Agarose gel electrophoresis of MARMS B amplicon showing mutation Cd 8/9 in sample with ID 81, 82, 83 and 84. **C)** Agarose gel electrophoresis of MARMS C amplicon showing Cd 16(-C) mutation in sample ID 76, 99 and 100. **D)** Agarose gel electrophoresis of MARSM D amplicons showing mutation Cd 19 in sample ID 17 and 85 **E)** 619 deletion confirmation of the sample ID 4, 16, 18, 19, 37, 41, 44, 56, 62, 65, 68, 77, 81, 84, 94 and 103.Molecular ladder of 100 bp was used in all the procedures above.

4.3 Molecular diagnosis

The collected blood samples were subjected to the DNA extraction. The DNA was then quantified and the purity was measured for the molecular diagnosis. The molecular diagnosis includes the mutation analysis and confirmation of mutation.

General analysis of mutations was done by running the multiplex ARMS-PCR and the amplicons were run on the agarose gel. The bands were analysed to screen the mutations with the help of the molecular size of the band which were compared with the molecular 100 bp ladder.

There are different types of primers designed for mutations. These primers were used at once during the reaction mixture preparation. Like we use four different mutation primers in MARMS –A, B, C, and D panel. Each primer is 30 bp long and their sequence was provided in the table below. All these primers were designed by Institute of Medical Research (IMR) Kualalumpur. The primers control A and B were used in MARMS A to E. These primers were used as the internal control and produce a band of 861 base pair. Similarly, primers control C and D were used as internal control for MARMS F only that produce a band of 493 bp because of the mutation location at farther 3' region of beta globin gene. Control E primer was used as common primer (reverse) for MARMS A, B and C but control F is used as common reverse primer for MARMS D, E and F. During the analysis, the bands of respective molecular weight were tallied with the ladder. Following table was used during the mutation analysis after gel electrophoresis.

Panel	Primer sequence	Amplicon size(bp)
MARMS –A		
IVS 1-5 (G-C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	319
Cd 41/42 (-TTCT)	GAGTGGACAGATCCCCAAAGGACTCAACCT	476
Cd 17 (A-T)	CTCACCACCAACTTCATCCACGTTCAGCTA	275
Cd 26 (G-A)	TAACCTTGATACCAACCTGCCCAGGGCGTT	301
MARMS –B		
IVS 1-1 (G-T)	TTAAACCTGTCTTGTAACCTTGATACGAAA	315
Cd 8/9 (+G)	CCTTGCCCCACACGGCAGTAACGGCACACC	250
-28 (A-G)	TAAGCAATAGATGGCTCTGCCCTGAGTTC	145
Cd 71/72 (+A)	GGTTGTCCAGGTGAGCCAGGCCATCAGTT	569

MARMS –C		
IVS 1-1 (G-A)	TTAAACCTGTCTTGTAACCTTGATACGAAT	315
Cd 43 (G-T)	ATCAGGGAGTGGACAGATCCCCAAGGAGTA	482
CD 16 (-C)	TCACCACCAACTTCATCCACGTTCACGTTC	273
Poly A (AATAAA-AATAGA)	GGCCTTGAGCATCTGGATTCTGCCTATTAG	393
MARMS -D		
-86(C-G)	ACTTAGACCTCACCCTGTGGAGCCACTCCG	367
Cd 19 (A-G)	TGCCGTTACTGCCCTGTGGGGCAAGGAGAG	173
CAP +1 (A-C)	AAAAGTCAGGGCAGAGCCATCTATTGGTTC	281
MARMS-E		
-88(C-T)	TCACTTAGACCTCACCCTGTGGAGCCTCAT	369
Initiation Cd	TGTTCACTAGCAACCTCAAACAGACAGCAG	248
Cd 15 (G-A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	203
-29 (A-G)	CAGGGAGGGCAGGAGCCAGGGCTGGGTATG	310
MARMS-F		
IVS 2-654 (C-T)	GAATAACAGTGATAATTTCTGGGTTAACGT	826
Control A	CAATGTATCATGCCTCTTTGCACC	861
Control B	GAGTCAAGGCTGAGAGATGCAGGA	
Control C	CAACTTGCTCAAGCATACACT	493

Control D	AATAATAGGCATAGTGACAAGTGC	
Control E	TGAAGTCCAACTCCTAAGCCAGTG	
Control F	CAATAGGCAGAGAGAGTCAGTGCCTATCA	

Table 5; Primers used, their sequence and molecular size.

During the research study, MARMS panel A to F were only implied for the detection of the mutations. 20 different mutations which were most prevalent in Malaysia were covered in these panels. These panels were designed after series of the research carried out by number of scientists around the world. The protocol for our study was produced by Institute of medical Research in Kualalumpur. The primer sequences are identical but the PCR programming was altered according to the mutation pattern they possess. 61 different thalassemic patients from Nepal were also analysed using the same protocols. Fortunately, 57 Nepalese cases showed the mutation and remaining 4 cases cannot accommodate their mutation within these panels. All the 61 cases from Nepal were clinically diagnosed and phenotypically distinguished. The clinical data of all the 61 patients were rigorously monitored and mutation were defined and confirmed. Similarly, 46 cases of the family member of patients were also analysed and the mutation were confirmed agarose gel electrophoresis after MARMS-PCR.

The exact locations of the mutations in the beta globin gene are shown in the figure below. This figure also shows the location of different relative position of all the primers and MARSM–PCR.

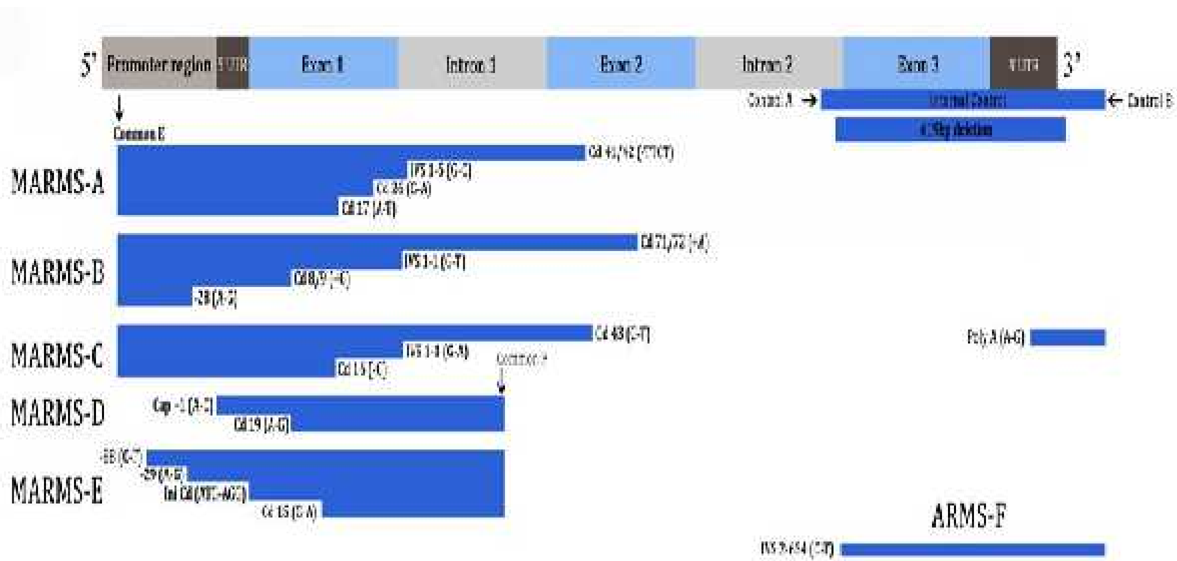


Fig 21; S Beta globin gene with relative position of primers, MARMS-PCR and mutation sites

The 619 deletion mutation was not included in MARMA panels. The presence of 242 bp band after the gel electrophoresis can show 619 deletion mutation in patient. But the result may not be true because other mutations like Cd 17 (A-T) 275bp, Cd 8/9(+G) 250 bp, Cd 16

(-C) 273 bp, and initiation codon 248bp were almost similar size when seen under the gel doc. The panel where we use the control A and B can show the 242bp amplification, which can create confusion in confirmation of 619 deletion. For this, the master mix was prepared using the control A and B primers along with template DNA only. If band is present, then it should be 619 deletion.

Similarly, the heterozygosity and homozygosity of the allele specific mutation can also be determined. The presence of internal band along with respective mutation band in gel can reveal heterozygosity and only presence of mutation band with absence of internal control band shows homozygosity of that mutation.

4.4 Mutation found during analysis.

Different types of mutations were found after the agarose gel electrophoresis was completed. According to the data provided by Nepal Thalassemia Society, 61 cases were clinically diagnosed and blood transfusion dependent patients and 46 cases were one of their immediate family member.

The mutations were screened according to the protocol provided by the International Centre for Medical Research (IMR). The band size and the location can confirm the respective mutation in patients. The same protocol was developed by many of the scientists and researcher around the world but there are slight modifications in master mix preparation methods and thermocycler programme. But the sequences of the primers were similar in other studies conducted in different nations.

The mutations were categorised as compound mutation and single mutation. The data of mutation patterns were again separately categorised according to age, gender and ethnic groups in both clinical 61 cases and their family member. And the Nepalese mutations were also compared with the Chinese, Indian and Malaysian.

4.4.1 Mutations in Thalassemic patients

Based on the protocol provided by IMR, our study screened the different mutations in Nepalese population. The result provided using above protocol can confirm respective mutation because of the standardisation of the protocol several times around the world. The mutation detection is simple and reliable and cost effective.

57(93.44%) of thalassemic cases show the different mutations on screening using multiplex ARMS panel from A to F and remaining 4 (6.55%) cases were still on research for detection of the mutation. The table 6 shows the distribution of compound mutation, no of patients affected and its frequency in whole population of 61 clinically diagnosed, transfusion dependent thalassemia patients.

Compound mutations	No. Of Patients (n), Frequency (%)
Het.Cd26(G-A)& IVS 1-5 (G-C)	4(6.55%)

Het.619 del. & Cd 8/9 (+G)	3(4.91%)
Homo. 619 del. & Cd 8/9 (+G)	5(8.19%)
Het.IVS 1-5(G-C) & Cd 41/42 (-TTCT)	1(1.63%)
Homo.IVS 1-5(G-C) & Cd26(G-A)	1(1.63%)
Het. Cd 17 (A-T) & Cd 8/9 (+G)	1(1.63%)
Homo. Cd 17 (A-T) & Cd 8/9 (+G)	1(1.63%)
Het. Cd26 (G-A) ,Cd 8/9 (+G)& IVS 1-5 (G-C)	1(1.63%)
Het. IVS 1-5 (G-C), 619 del. & Cd 8/9 (+G)	1(1.63%)
Het. IVS 1-5 (G-C), 619 del. & IVS 1-1(G-T)	1(1.63%)

Table 6; Compound mutations detected in 61 thalassemia cases.

Similarly, table 7 shows the single mutation, no of patients and frequency in 61 clinically diagnosed, transfusion dependent thalassemia patients. This result shows Cd 26(G-A) is more common mutation followed by IVS 1-5 (G-C), 619 del. and others. The least frequency is shown by IVS 1-1 (G-T) with 1.63 prevalence percentile.

Mutations	No. Of Patients (n), frequency (%)
Cd26(G-A)	11(18.03%)

IVS 1-5 (G-C)	9(14.75%)
619 del	7(11.47%)
Cd 16(-C)	6(9.83%)
Cd 41/42(-TTCT)	2(3.27%)
Cd 19(A-G)	2(3.27%)
IVS 1-1(G-T)	1(1.63%)

Table 7; Single mutation in 61 thalassemic cases.

There were 9 different mutations detected in research study. The relation between total number of particular mutations in total participants shows perfect picture of mutations in Nepalese population. The most common mutation were found to be IVS 1-5(G-C), Cd 26 (G-A) and 619 del. and the least is Cd 17 (A-T).The figure 22 shows the distribution of different mutations in participants.

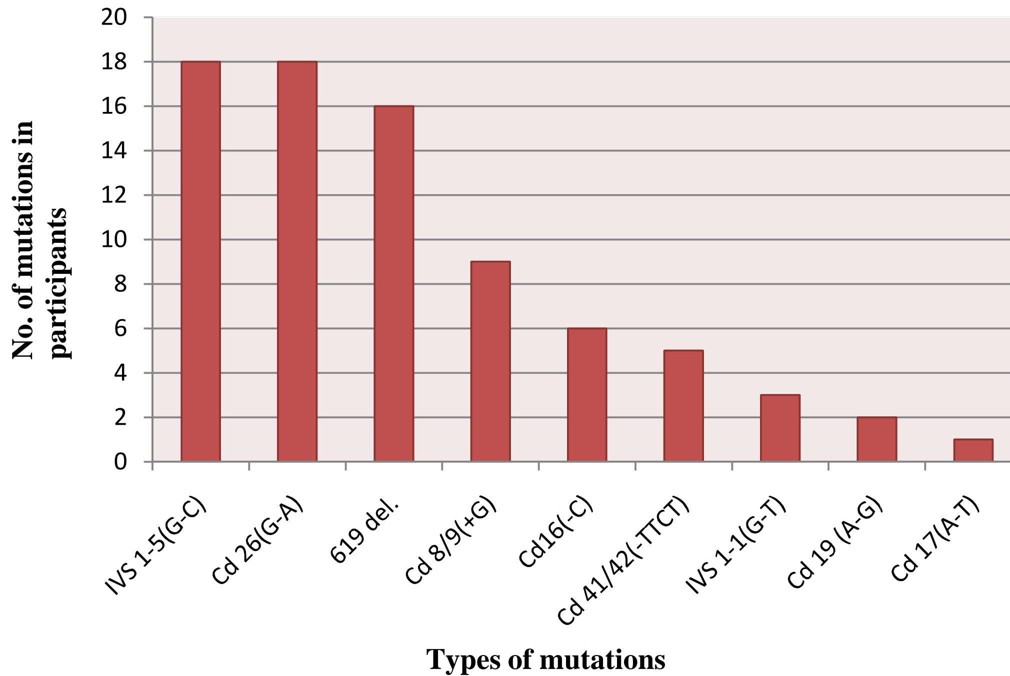


Fig. 22; Distribution of mutations in 61 thalassemia patients

The figure 23 shows the similar result shown by the above figure but the calculated in percentile. This shows the 23% occurrence of IVS (G-C) and Cd 26 (G-A) followed by 619 del. (20%) and the least was found to be Cd 17(A-T) 1%.

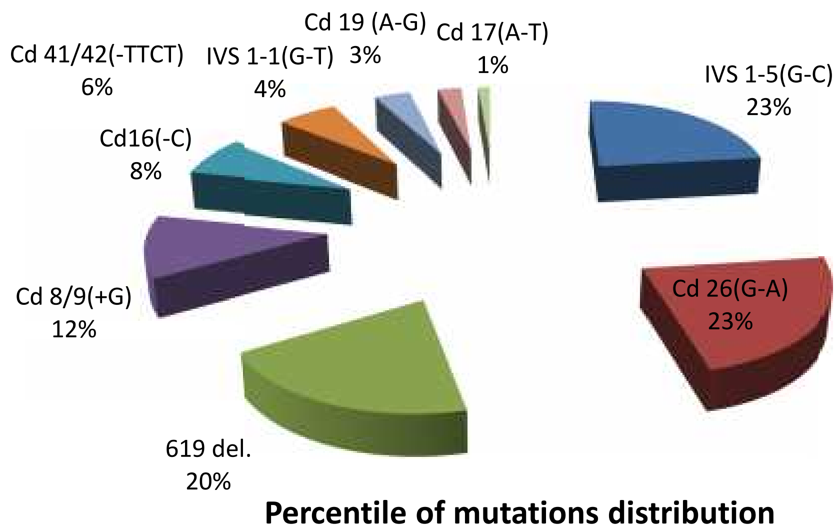


Fig. 23; percentile of different mutations in thalassemia patients

4.4.2 Mutation in one of their family member

One of patient's immediate family members was phenotypically and clinically healthy. This study was done to trace out the relation between the family members. The cases studied do not represent the whole family member. The cases represent either mother or father or brother or sister. The 25 (54.34%) of member possess mutation but they were healthy and

remaining 21 (45.65%) cases do not show mutation. This study may not show complete lineage of thalassemia among the family member but help to trace out relation of disease in unaffected carrier parents and affected offspring.

Compound Mutation	No. Of Family member (n), frequency (%)
IVS 1-5(G-C) and Cd 8/9(+G)	1(2.22%)
IVS 1-5(G-C) ,619 del.and Cd 8/9(+G)	1(2.22%)
619 del. and IVS 1-1(G-T)	1(2.22%)
619 del. and Cd8/9(+G)	6(13.33%)
619 del.,Cd 26(G-A) and Cd8/9(+G)	1(2.22%)

Table 8; compound mutation occurrence and frequency family member

Mutations	No. Of Patients (n), frequency (%)
619 del.	4(8.69%)
Cd 26 (G-A)	4(8.69%)
Cd 41/42 (-TTCT)	3(6.52%)
IVS 1-5 (G-C)	2(4.34%)
Cd 19 (A-G)	1(2.17%)
Cd 16(-C)	1(2.17%)

Table 9; Single mutation occurrence and frequency in family member.

The mutations which were detected in patients and their family member were deeply analysed. Among the 46 family members, only 25 showed the mutation and remaining did not. This means most of the family member under study were unaffected healthy carrier where as remaining portion were genotypically and phenotypically health with the mutation in beta- globin gene.

The relation among the family member and the patients were correlated according to the type of mutation they possess. During study, not whole complete family member were taken due to their own circumstances For example, there was some cases with father or mother only or both and even brother or sister only or both and vice versa. 4 cases in patients were undiagnosed in term of mutation but they are clinically and phenotypically

proven as thalassemic patients. The table in the appendix show the type of mutation possessed by patients and family member and their relation.

4.4.3 Age categorised mutation

In context of Nepal, there is less awareness and ideas about the thalassemia. Patients were left undiagnosed and managed even if they got diagnosed. This is the reason why, most of the study includes patients under the age of 15 years. We hardly find the patients over the age of 20 years old. Following table shows the participation of patients according to their age factor.

Age group (years)	No. of Participation	Participation %
1-5	12	19.67%
6-10	22	36.06%
11-15	18	29.50%
16-20	5	8.19%
Above 20	4	6.55%

Table 10; Age group of the patients under study

4.4.4 Gender categorised mutation

Thalassemia disease does not discriminate gender. It affects equally to both of the sexes. There is not data till date mentioning the particular sex more prone to the thalassemia disease. Among 61, 39 (63.93%) male and 22 (36.06%) female patients were studied under this research.

Gender	Number of participants	Mutation found	Percentage	Mutation not found	Percentage
Male	39	37	60.65%	2	3.27%
Female	22	20	32.78%	2	3.27%

Table 11; Gender of participated cases under study.

4.4.5 Ethnic group categorised mutation

Many of the Government officials have said that thalassemia is endemic in Terai (low land near border of India in south) region of Nepal only. But our study suggests that β-

thalassemia is found in all the ethnic community living in all geography of the country. According to the study conducted, the thalassemia is common in malaria endemic areas for its ability to counter disease but the consequences are disastrous.

In study, there was overwhelming participation of all the ethnic groups from different parts of Nepal. They were excited about their disease so that this study will draw attention of Government as well as private sectors to help their children for better care and treatment. After months of research, our study suggests that thalassemia is now found all over the Nepal. This may be due to the migration of people to and from these geographical areas for various purposes. The table below indicates our result of mutation incidence in different ethnic groups of Nepal.

Ethnic Group	Mutation detected	Participants/percentage
Brahmin	619 del., IVS 1-5(G-C), Cd 26(G-A), Cd 8/9(+G)	11(19.29%)
Chhetri	619 del., Cd 8/9(+G), Cd41/42(-TTCT), Cd16(-C), Cd 17(A-T), Cd 26(G-A)	10(17.54%)
Rai/Limbu	619 del., IVS 1-5 (G-C), Cd 26(G-A), Cd16(-C), Cd 8/9(+G)	7(12.28%)
Chaudhary	Cd 41/42(-TTCT), Cd 8/9(+G), IVS 1-5 (G-C), 619 del., Cd 26(G-A)	7(12.28%)
Newar	619 del., Cd41/42(-TTCT), Cd 19 (A-G), Cd 8/9(+G), Cd 26(G-A)	5(8.77%)
Tamang	Cd 16(-C), Cd 26(G-A)	5(8.77%)
Muslim	IVS 1-5(G-C)	2(3.50%)
Magar	IVS 1-5(G-C),	1(1.75%)
Mandal	IVS 1-5(G-C),	1(1.75%)
Misra	Cd 19 (A-G)	1(1.75%)
Rauniyar	IVS 1-1(G-T)	1(1.75%)
Mahato	619 del., IVS 1-5 (G-C)	1(1.75%)
Thakur	Cd 26(G-A), Cd 41/42(-TTCT)	1(1.75%)
Majhi	619 del.	1(1.75%)

Jaiswal	Cd 26(G-A)	1(1.75%)
Bhaskar	IVS 1-5(G-C), Cd 26(G-A)	1(1.75%)
Shah	Cd 26(G-A)	1(1.75%)

Table 12; Distribution of mutations among ethnic groups.

Total 57 cases were included in the above table where we can find the mutations in research study. And remaining 4 cases (3 Chhetri and 1 Newar) were excluded because mutation was not detected in our study panel.

Taking this data in consider, the study reveals that most of the major ethnic group of Nepal were found to be affected and susceptible to produce more new thalassemic patients. The most affected was Brahmins with 19.29%, followed by Chhetri 17.54%, Rai 12.28%, Chaudhary 12.28%, Newar and Tamang sharing same frequency of 8.77%.

4.4.6 Comparison of Nepalese mutation to Malaysian population.

Our research was carried out in Universiti Sains Malaysia and according to the paper published by Syahzuwan in 2012, the mutation pattern found in Malaysia were shown in table 13 below. Our sample size was smaller than in paper but it reflects the mutations were similar to different ethnic Nepalese group. The result came from my research was then compared with the result of Malaysian population. This comparison shows our nine different mutations were also found in Malaysians, more common in Chinese and Malay Malaysians.

Mutation	**Chinese**	**Malay**	**Indians**	**Others**	**Total**	**Nepalese**
–88 (C>T)	0	0	0	0	0	0
Cap+1 (A>C)	0	2	0	0	2	0
–28 (A>G)	2	0	0	0	2	0
–29 (A>G)	0	2	0	0	2	0
Ini.Cd (T>G)	0	0	0	0	0	0
Cd 8/9 (+G)	0	4	0	0	4	9(11.53%)
Cd 15 (G>A)	0	0	1	0	1	0
Cd 16 (–C)	0	0	0	0	0	6(7.69%)
Cd 17 (A>T)	0	3	0	0	3	1(1.28%)

Cd 19 (A>G)	0	8	0	0	8	2(2.56%)
Cd 26 (G>A) Hb E	2	36	0	1	39	18(23.07%)
IVS1–1 (G>T)	0	27	0	0	27	3(3.84%)
IVS1–1 (G>A)	0	1	0	0	1	0
IVS1–5 (G>C)	1	37	0	1	39	18(23.07%)
Cd 43 (G>T)	0	0	2	0	2	0
Cd 41/42 (–TCTT)	13	14	0	0	27	5(6.41%)
Cd 71/72 (+A)	0	0	0	0	0	0
IVS 2–654 (C>T)	6	1	0	0	7	0
Poly A (A>G)	0	5	0	0	5	0
619 Deletion	0	0	0	0	0	16(20.51%)
Total	**24**	**140**	**3**	**2**	**169**	**78**

Table 13; Comparison of mutations in Malaysian and Nepalese population data Source from Syahzuwan, Rahimah,2012

This table was cited from the article published on 2012 by Shyazuwan and Colleagues. The data provides the mutation pattern found in Malaysia. Since the study was carried out in Malaysia and many similiarities of Nepalese mutations with them, our final data was tallied with theirs. The mutation IVS 1-5 (G-C) and Cd 26 (G-A) were found most common among Nepalese population and also in Malay population which accounts for 60% population of Malaysia. Similarly Cd 17 (A-T) the least common mutation in Nepalese was also among the less occuring mutation in all the ethnic group of Malaysian.

Chapter 5
DISCUSSION

Beta- thalassemia is a group of genetic disease affecting the blood of the person. This disease has a distinct feature with total or partial loss of formation of the beta globin chain inside the haemoglobin. This makes haemoglobin unstable and cannot bind with oxygen molecules properly and hence the RBCs get lyse prematurely. Patients may be asymptomatic to severely anaemic according to the hemoglobin variants they possess (Galanello, Renzo, 2010).The beta-globin gene is present in chromosome 11 and mutation in any nucleotide or sequence leads to this disorder (Galanello, Renzo, 2010). Thalassemia originates in the mediterranean geography on this planet according to various scientific evidences. In Greek thalassa means sea and haima is blood; literally named as sea in blood. The historical background of thalassemia and malaria is related to each other. Many scientists believe that plasmodium (malarial parasites) cannot survive in thalassemic RBCs hence it shows a clear evidence of development of thalassemia in malaria endemic areas (Iandola, Ortner, 2003). The first person to deal with thalassemia is said to Dr. Cooley in 1925.There are more than 200 mutation detected till the date in beta globin gene. The majority of the beta- thalassemia mutations are nucleotide substitutions, frame shifts (Huisman, Carver, 1997) minor deletion and rarely, large deletions are also reported (Higgs, Thein, 2001). Blood transfusions and iron chelator treatment for patients with beta-thalasemia major are expensive and beyond the economic reach of many families and therefore, the provision of genetic counselling even before marriage is important (Syazuwan, Rahimah, 2012). Different geographical sites have their own trademark of mutations. But many researchers have found mutations originally developed in different areas of the world. This is because of the migration of people from one place to other for various reasons like trade, farming and for security (Rosatelli et al., 1992).

According to a WHO report on Global distribution of haemoglobin disorders there are 0.2-0.99 birth per 1000 infants with a major hemoglobinopathy in Nepal (Genome resource centre, 2012). There was no study to characterise the mutations in the beta- globin gene from Nepalese thalassemic cases that is why selection of the sampling site is one of the strenuous step. The DNA extraction from blood is another crucial part of the study. The DNA was extracted by Proteinase K digestion followed by phenol–chloroform method (Sambrook, *et al* 1989). Therefore DNA extraction is done manually. The quantification of the DNA was done by agarose gel electrophoresis, and further confirm by nanodrop spectrophotometry. The band was distinct on gel visualisation but quantification is required for the proper scientific study. The absorbance at 260nm and 280nm are taken and calculation of purity and amount of the DNA are done by nano spectrophotometer very efficiently.

The main purpose of this research is application of multiplex ARMS PCR for the mutation screening of the beta-thalassemia. To evaluate the working capacity and efficiency of this molecular tool in the mutation detection. In addition to that, to make it one of the leading

molecular tools for the mutation detection in developing country like Nepal because it has already proven its reliability, convenient and cost effectiveness. Moreover, this study help us to design and use a rapid, inexpensive, and simple PCR approach to update, and characterise the spectrum of β-globin gene mutations among Nepalese . Based on earlier reported studies of β-thalassaemia heterogeneity among Malaysians (Tan, Wong, 2001; Thong, Tan, 2005; Tan Yap, 1998), IMR modified the MARMS developed by Bhardwaj *et al.*, (Bhardwaj, Zhang, 2005) by adding primers, suitable for the local mutation heterogeneity(Syahzuwan, Rahimah, 2012). Five sets of MARMS and one single ARMS were used to detect 20 different mutations of the β-globin gene. Direct sequencing was used for mutation confirmation.

Selection of the primers and the standardisation of the protocol were not needed. The protocol was developed after the numerous researches and used as diagnostic protocol for the analysis of the β-thalassemia in Malaysia. The primers were ready made and the master mix was Qiagen brand with label as Hotstar TaqPlus master Mix Kit. The amplicon were analysed after agarose (Agarose biotechnology grade, 1st base ptel_th) gel electrophoresis.

There have been numerous studies done on β- thalassemia in many countries. Our neighbouring countries China and India have already research in this sector for many times. They have their own clinical database for this disease. Similarly, Thailand, Pakistan, Srilanka and other South Asian nations are far away in comparison to Nepal in research in thalassemia. The research methodology is different but the Multiplex ARMS PCR is the most used molecular tool for the research purpose as well as for diagnosis.

The study conducted in Thailand (Anchalee, Sumalee, Suporn, Punnee, 2011), the mutation Cd 41/42 (-TCTT) 42.5% was more common in Thailand which is also found in Nepal with 6.41%. Similarly Cd 17(A-T), IVS 1-5 (G-C), IVS 1-1 (G-T) were also seen in Thailand as also seen in Nepalese population. A study conducted in Nepal (Mishra et al. 2012) shows the result as Cd 41/42 (-TCTT) 31.71%, IVS 1-5 (G-C) 17.07%, IVS 1-1 (G-T) 14.64%, Cd 8/9 (+G) 9.76%, 619 del. 14.64%, -88 (C-T) 7.32%, Cd 15 (G-A) and Cd 16 (-C) with 2.43%. This result also resembles to our study with some few dissimilarities. Perhaps this is due to the sample size and place where they belong.

There have been numerous giant projects running in India for the thalassemia. Being the largely populated country, the data obtained from the India can be correlated to Nepalese. The open border and the inter-nation marriage can be the cause behind. The study in India (Chitra, 2012) has done the research on thalassemia in almost all states. The screening result is surprising. Those mutations in India were also seen in Nepal. Like IVS 1-5 (G-C) and 619 del. were the commonest mutation in India and our study shows the similarity in mutation. This is perhaps due to geographical location alike between these countries. The mutation Cd 41/42 (-TCTT) is the commonest among the Chinese population (Zeng, Huang, 2001) and this mutation was also found in Nepal with frequency of 6.41%.

In all the above reports, MARMS plays an important role in analysis and screening of the β-thalassemia. Nepal is still in preliminary stage in using molecular diagnosis. On the other hand, the thalassemia prevalence is increasing not only in Terai region but also in other areas of Nepal affecting almost all the ethnic groups. This situation can be stopped or preventable. Early diagnosis is only solution for the prevention of the thalassemia. MARMS is taken as the cheapest method with best reliability and easy for operation, adds up the point for the use of MARMS in context of Nepal.

Developing countries like Nepal can do better in the field of thalassemia. Clinical awareness and easy access to health service for diagnosis can pay out the result. Thalassemia can be burden to family, community, society and to nation for country like Nepal. Treatment is far behind the limit and cross the capacity of general Nepalese people. So there is an only way out for the management of thalassemia. The pre-diagnosis is the only option for the diagnosis. There are number of ways of diagnosing thalassemia. Pre-marital diagnosis is the most convenient way to push back the number of thalassemia. This diagnosis can be done in the areas where the thalassemia and malaria is endemic. This test also can be done in suspected couple before marriage.

Similarly, fetal diagnosis is second option for the testing of thalassemia. The foetus can also be diagnosed after genetic evaluation. An amniocentesis is done for the sample collection from the foetus. The DNA will be extracted and analysed for the presence or absence of mutation in beta thalassemia gene. Besides this, there is no other option to prevent from the thalassemia. Treatment cost is very big and cannot afford that much cost by Nepalese citizens.

Hence it is requested that pre-marital diagnosis before marriage and foetus diagnosis should be carried out in the most risk population. And also strictly advised for the early consultation with medical doctors for the treatment for prolong healthy life of the patients.

Chapter 5
SUMMARY

Thalassemia is a genetic disease which inherited in autosomal recessive pattern. This disease affects the globin chain synthesis partially or complete. Concerning the β–thalassemia, there is loss or absent of the beta globin gene in the haemoglobin. This leads to the unstable haemoglobin that cannot bind properly with the oxygen molecules hence RBC breaks down prematurely. Thalassemia was historical disease in the Mediterranean areas of the Eastern Europe particularly in Ancient Greece according to the many scientific evidence and artefacts. Many patients were diagnosed as severely anaemic by the Cooley in the year of 1925, mostly immigrants from Greece and Italy. Later, they named it as Thalassemia by G.Whipple and W.Bradford in 1933.

The origin of thalassemia is directly related to the malarial plasmodium. Many historical evidence proved that thalassemia is more common in the malaria endemic region. This directly shows the genetic modification of human against the plasmodium, the causative agent of malaria but with the lethal fates. The modification in chromosome 11 in beta globin gene can prevent from malaria but they are more vulnerable towards thalassemia. There are more than 200 mutations known till date for thalassemia. The beta globin gene is 1606 bp long with 3 exons and 2 introns. The length of exon 1 is 142 bp with 50 bp UTR and ATG with start codon. The exon 2 and 3 are 223bp and 261 bp long, similarly, intron 1 and 2 are 130 bp and 850 bp long respectively.

The blood samples were collected from the thalassemic patients with kind coordination of Nepal Thalassemia Society. The samples were preserved in 4°C till the number of the samples reaches more than 50. The DNA was isolated from the blood samples using Proteinase K digestion. The DNA were quantifies both by gel electrophoresis and nano drop spectrophotometry. The quantified DNA were then stored at 4°C for the further analysis.

The study was done for the β thalassemia mutation detection in Nepalese population. There was use of only one technique for the detection of thalassemia in this study. The technique we follow for the research was very reasonable in terms of operation cost, reliability, accuracy and handling. The most widely used method for the detection of the thalassemic mutation is multiplex ARMS (Amplified refractory mutation system) PCR. This molecular tool can detect number of mutations in single reaction. Hence we do not have to waste much time for the analysing mutations. The different primers can be used at once in reaction mixture. The optimum concentration and volume of the primers and other ingredients should be carefully optimised for the proper functioning of the MARMS.

The treatment cost and other miscellaneous cost for the management of thalassemia is too high for the Nepalese. There is an only way for not making a single new thalassemic patient. Diagnosis before marriage in high risk population and fetal examination for the susceptible

cases can prevent the prevalence of thalassemia. These all procedure can be done only by MARMS PCR with maximum accuracy within the budget of general Nepalese people.

MARMS is a simplest and most widely used method of doing PCR for the amplification of numerous primers in single reaction. The primers are designed in such a way that they cover the sequence and the common reverse primer is used for all the primers used before. Like in MARMS panel A, there are four allele specific primers for IVS 1-5(G-C), Cd 41/42(-TTCT), Cd 17 (A-T) and Cd 26 (G-A) and control A and B as internal control and control E acts as a common reverse primer for all of the primers used before in reaction mixture. Coral Load is a loading dye made from a certain species of coral which can be used during the preparation of reaction mixture or during loading amplicon for agarose gel electrophoresis.

In Nepal, there are number of unknown thalassemic cases who were remained ignored or undiagnosed. Their actual number is yet to be revealed but many studies claim that the thalassemic cases might exceed in the figure of thousand. The clinical services and facilities are far behind the reach of them and even they cannot address their disease. This is a critical situation in Nepal that makes the condition worse than the worst.

To counter the problem faced by the thalassemic patients in Nepal, health awareness is the most needed approach for the prevention of the disease. Without knowledge, they cannot interpret their symptoms of thalassemia; this step can help general people to understand about the disease and themselves. Secondly, Nepal Government has to establish a medical institution to deal with the thalassemic patients. This institute should have all the health requirements that are needed for proper management and indeed the survival of the cases.

Chapter 6
CONCLUSION

There have a lot of techniques using for the analysis of the β-thalassemia around the world. RFLP, melting curve method were also used for screening of the mutations in β-thalassemia. Most of the techniques used were tedious, costly and some with faulty result. All these drawbacks of those techniques can be overcome by the use of multiplex PCR. It can be used for the screening and diagnosis of the mutations thalassemia.
In MARMS, we can use number of allele specific primers specific to the mutations we want to detect. Many primers can be used at once as many as we want and can be modified according to our need. The PCR condition can also be changed as per our requirement. In our study we have used up to 7 primers in single master mix preparation. Among them 4 primers were allele specific, 2 were taken as internal control and last but not the least primer was used as common reverse primer for all of the above 6 primers.

The thermocycler programming was same for the entire MARMS panel A to E. The enzyme activation at 95°C single time for 5 minutes and denaturation, annealing and extension were at 94°C, 64°C and 72°C for 30 cycles continuously for 45 seconds,45 seconds and 1 and half minute respectively. The final extension phase was at 72°C for 1 cycle for 7 minutes. In ARMS F, the annealing temperature is 60°C and remaining parameters are same like other MARMS A to E. The amplicons were run in 2.5% and 3% agarose gel at 80V for 1 hour. The band were visualised under gel doc.The bands observed were categorised with DNA ladder (100bp). The relative size and position of the bands can reveal the type of mutation possessed by respective individual. The protocol has been already optimized many number of scientists and researchers and the result obtained from above procedures are reliable and worthy.

MARSMS is very simple to use. The running cost of this technique can be fit within the range of Nepalese income capacity. The result obtained can be considered as the most reliable with comparison among other technique. This technique takes less time and can be handled by general person with training from the institute. Thus this technique can be a very useful molecular tool in context of Nepal.

In near future, other Nepalese scientists can work using MARMS. There should be research in large Nepalese population from all over the nation. This study should produce a Nepal based database in thalassemia. We have to develop our own control for the thalassemia. This control can be use in various research projects. This database can be use for various purposes for the prevention, management and treatment of the thalassemic patients. Data base helps Nepal Government to separate budget for thalassemia and its patients. This database should be accessible worldwide.

REFERENCE

Advani R, Sorenson S, Shinar E, et al.:(1992), Characterization and comparison of the RBC membrane damage in severe human alpha and beta thalassemia. Blood 79:1058–1063.

Aessopos A, Farmakis D, Karagiorga M, et al.: (2001), Cardiac involvement in thalassemia intermedia: a multicenter study. Blood 2001, 97:3411–3416.This excellent clinical study shows a surprising frequency of pulmonary hypertension

Aessopos A, Stamatelos G, Skoumas V, Vassilopoulos G, Mantzourani M, Loukopoulos(1995), D. Pulmonary hypertension and right heart failure in pa-tients with b-thalassemia intermedia. Chest ;107:50-3.

Agrawal, S.; Naveed, M.; Gupta, U.R.; Kishor, P.; Agrawal, S.S. (1994), Characterization of beta Thalassemia mutations in 57 beta Thalassemia families seen at Lucknow. Ind. J. Med. Res.,100(3), 106-110.

Alana, K.; Bergstrome, J.; Annette, P. (2002), Evaluation of single tube multiplex polymerase chain reaction screen for detection of common Alpha Thalassemia genotypes in clinical laboratory. Am. J. Clin. Pathol., , 118(1), 18-24.

Alwan, A.; Modell, B (2002), Recommendations for introducing genetics services in developing countries. Nat. Rev. Genet., , 4(1), 61-68.

Analysis of beta globin mutations in the Indian population; presence of rare and novel mutations and region wise heterogeneity. Clin. Genet., 2008, 73(4), 331-33.

Angelini, V.:(1937), Primi risultati di ricercheematologiche nei familiari di am-malati di aneniia di Cooley. Minerva Illed. 2:331-332,

Angelucci E, Giardini C, Brittenham GM, Lucarelli G.(1997), Hepatic iron concentration and body iron stores determined by quantitative phlebotomy in patients cured of thalassemia major by bone marrow transplantation. Blood 90:Suppl 1:265a. abstract.

Angelucci E, Giovagnoni A, Valeri G, et al.(1997), Limitations of magnetic resonance imaging in measurement of hepatic iron. Blood ;90:4736-42.

Angelini, V. (1937): Primi risultati di ricerche ematologiche nei familiari di ammalati di aneniia di Cooley. MinervaIlled. 2:331-332.

Baig, S.M. Molecular diagnosis of Thalassemia by multiplex ARMS PCR: a cost effective method for developing counties like Pakistan. Prenatal. Diagnosis, 2007, 27(6), 580-581.

Balgir, R.S. (2000), The burden of hemoglobinopathies in India and the challenges ahead. Curr. Sci.,, 79(11), 1536-1547.

Banks, L. 0. and Scott, R. B. (1953), Thalassemia in Negroes; report of case of Cooley's anemia in a Negro child.Pediatrics 11:622-627,.

Banton, A. H.:(1951), Genetic study of Mediterranean anaemia in Cyprus. Am.J. Human Genet. 3:47-64,

Bhardwaj U, Zhang YH, Lorey F, McCabe LL, McCabe ER (2005),. Molecular genetic confirmatory testing from newborn screening samples for the common African-American, Asian Indian, Southeast Asian, and Chinese β-thalassaemia mutations. Am J Hematol.;78(4):249–255.

Bhardwaj, U.; and McCobe, E.R.B. (2005), Multiplex PCR Assay for the deletions causinghereditary Persistence of Fetal Hemoglobin. Mol. Diagnosis., , 9(3), 151-155.

Bottema, C. D. K., Sarkar, G., Cassady, J. D., Li, S., Dutton, C. M., and Sommer, S. S. (1993) Polymerase chain reaction amplification of specific alleles: a general method of detection of mutations, polymorphisms, and haplotypes. Methods Enzymol. 218,388-402.

Brittenham GM, Griffith PM, Nienhuis AW,(1994), et al. Efficacy of deferox- amine in preventing complications of iron overload in patients with thalassemia major. N Engl J Med 331:567-73.

Brittenham GM, Cohen AR, McLaren CE, et al.(1993), Hepatic iron stores and plasma ferritin concentration in patients with sickle cell anemia and thalassemia major. Am J Hematol ;42:81-5.

Brittenham GM, Farrell DE, Harris JW, et al (1982),Magnetic-susceptibility measurement of human iron stores. N Engl J Med 307:1671-5.

Bronspiegel-Weintrob N, Olivieri NF, Tyler B, Andrews DF, Freed-man MH, Holland FJ.(1990) Effect of age at the start of iron chelation therapy on gonadal function in b-thalassemia major. N Engl J Med 323:713-9.

Brumpt, L. C.: A propos de l' anaemie de Cooley: thalassemie ou sinemie?Bull. Acad. nat. med. 139:333-336,1955.

Burdick CO; Ntaios, G.; Rathod, D. (March 2009)."Separating thalassemia trait and iron deficiency by simple inspection". *Am. J. Clin. Pathol.* **131** (3): 444; author reply 445.

CagganaM, Conroy JM, PassKA. (1998), Rapid, efficient method for multiplex amplification from filter papers. Human Mutation ;11:404–409.

Camaschella C, Cappellini MD(1995), Thalassemia intermedia. Haematolog-ica 80:58-68.

Caminopetros, J (1938).: Researches on infantile erythroblastic anemia in people of eastern Mediterranean. Ann.med. 43:27-61.

Cao A. Diagnosis of b-thalassemia intermedia at presentation. In: Fu-charoen S, Rowley PT, Paul NW, eds. (1988), Thalassemia: pathophysiology and management. Part B. Vol. 23 of Birth defects: original article series. New York: Alan R. Liss,:219-26.

Cao A, Galanello R, Rosatelli MC.(1998), Prenatal diagnosis and screening of the hemoglobinopathies. Baillieres Clin Haematol 11:215-38.

Carr IM, Camm N, Taylor GR, Charlton R, Ellard S, Sheridan EG, et al.(2011), GeneScreen: a program for high-throughput mutation detection in DNA sequence electropherograms. J Med Gens.;48(2): 123–130

Cartwright GE, Edwards CQ, Kravitz K, et al.(1979), Hereditary hemochromatosis: phenotypic expression of the disease. N Engl J Med 301:175-9.

Celedon G, Rodriguez I, Espana J, et al.:(2001), Contribution of hemoglobin and membrane constituents modification to human erythrocyte damage promoted by peroxyl radicals of different charge and hydrophobicity. Free Radic Res 34:17–31.

Centis F, Tabellini L, Lucarelli G, et al.:(2000), The importance of erythroid expansion in determining the extent of apoptosis in erythroid precursors in patients with beta-thalassemia major. Blood 96:3624–3629.This article provides quantitative data on the extent of erythroid expansion and its impact on apoptosis.

Chakraborty D, Bhattacharyya M: (2001), Antioxidant status of red blood cells of patients with beta-thalassemia and E beta-thalassemia. Clin Chim Acta 305:123–129.

Chan YF, Tan KL, Wong YC, Wee YC, Yap SF, Tan JAMA (2001),. The use of the Amplification Refractory Mutation System (ARMS) in the detection of rare β-thalassaemia in the Malays and Chinese in Malaysia. Southeast Asian J Trop Med Public Health.;32(4):872–879.

Chang JG, Lin CP, Liu TC, et al. (1994), Molecular basis of beta thalassemia minor in Taiwan. Int J Hematol;59:267–272.

Chang JG, Liu HJ, Huang HM, Yang TY, Chang CP. (1997), Multiplex muta-genically separated PCR: diagnosis of b-thalassemia and hemoglobin variants. BioTechniques;22:520–527.

Chelation Therapy In Thalassemia Major For The Treatment Of Severe Myocardial Siderosis With Left Ventricular Dysfunction." Journal of Cardiovascular Magnetic Resonance 10.1 (2008): 12. Print.

Chin, Joanna Y.; Kuan, Jean Y.; Lonkar, Pallavi S.; Krause, Diane S.; Seidman, Michael M.; Peterson, Kenneth R.; Nielsen, Peter E.; Kole, Ryszard; Glazer, Peter M.

(2008). "Correction of a splice-site mutation in the beta-globin gene stimulated by triplex-forming peptide nucleic acids".

Chou, Q., Russell, M., Birch, D. E., Raymond, J., and Bloch, W. (1992) Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. 20, 17 17-1723.

Chui DH, Waye JS.(1998), Hydrops fetalis caused by alpha-thalassemia: an emerging health care problem. Blood 91:2213-22.

Cianciulli P(2008). "Treatment of iron overload in thalassemia". *Pediatr Endocrinol Rev* **6** (Suppl 1): 208–13.

Colah, R., Gorakshkar, A.; Nadkarni, A. (2010), Global burden, distribution and prevention of thalassemias and hemoglobin E disorders. Expert. Rev. Hematol., , 3(1), 103-117.

Colah, R.; Gorakshakar, A.; Nadkarni, A.; Phanasgaonkar, S.; Surve, R.; Sawant, P.; Mohanty, D.; Ghosh, K. (2009), Regional heterogeneity of -thalassemia mutations in the multi ethnic Indian population. Blood Cells Mol. Dis., , 42(3), 241-246.

Contu L, La Nasa G, Arras M, et al.(1994), Successful unrelated bone marrow transplantation in b-thalassemia. Bone Marrow Transplant 13:329-31.

Cooley TB, Lee P. (1925), A series of cases of splenomegaly in children with anemia and peculiar bone changes. Trans Am Paediatric Society ;37:29-30.

Dameshek, W. (1940): "Target Cell" anemia. An erythroblastic type of Cooley's erythroblastic anemia. Am.J.M.Sc. 200:445-454.

De, M.; Das, S.K.; Bhattacharya, D.K.; Talukder, G.(1997), The occurrence of beta Thalassemia mutations and it's interaction with hemoglobin E in Eastern India. Int. J. Hematol.,66(1), 31-34.

DeMaria R, Testa U, Luchetti L, et al.: (1999) ,Apoptotic role of Fas/Fas ligand in the regulation of erythropoiesis. Blood 93:796–803.

DeMaria R, Zeuner A, Eramo A, et al.: Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. Nature 401:489–493.Careful study showing how GATA-1 is cleaved by caspases 3, 7, and 8 to down regulate erythropoiesis.

Du ZD, Roguin N, Milgram E, Saab K, Koren A.(1997), Pulmonary hyper-tension in patients with thalassemia major. Am Heart J 134:532-7.52.

Edwards, M.C.; Gibbs, R.A.(1994), Multiplex PCR: Advantages, development, and applications. Genome Res., , (3), 65-75.

Fortina, P.; Dotti, G.; Conant, R.; Monokian, G.; Parrella, T.; Hitchcock, W.; Rappaport, E.; Schwartz, E.; Surrey, S. (1992), Detection of the most common mutations causing Thalassemia in Mediterraneans using a multiplex amplification refractory mutation system(MARMS). PCR Method Appl.,2(2), 163-166.

Gabutti V, Piga A.(1996) Results of long-term iron-chelating therapy. Acta Haematol 95:26-36.

Galanello, Renzo; Origa, Raffaella (2010). *Beta-thalassemia*. Orphanet Journal of Rare Diseases.PMC 2893117.

Garewal, G.; Fearon, C.W.; Warren T.C.; Marwah, N.; Marwah, R.K.; Mahadik, C.; and Kazazian, H.H., Jr. (1994), The molecular basis of Thalassemia in Punjabi and Maharashtran Indians includes a multilocus aetiology involving triplicated globin loci. Br. J. Hematol., , 86(2), 372-376. .

Giardini C.(1997), Treatment of b-thalassemia. Curr Opin Hematol 4:79-87.

Goldberg, NI. A. and Schwartz, S. C (1954),: Mediterranean anemia in Negro complicated by pernicious anemia of pregnancy; report of case. Blood 9:648-654.

Gratwick GM, Bullough PG, Bohne WHO, Markenson AL, Peterson CM. (1978),Thalassemia osteoarthropathy. Ann Intern Med 88:494-501.

Grinberg LN, Rachmilewitz EA. (1995),Oxidative stress in b-thalassemic red blood cells and potential use of antioxidants. In: Beuzard Y, Lubin B, Rosa J, eds. Sickle cell disease and thalassaemia: new trends in therapy. London: Colloque INSERM/John Libby Eurotext, 519-24

Hengartner M: (2000), The biochemistry of apoptosis. Nature 404:770–776.This is an excellent written and graphic view of apoptosis and its several pathways of induction.

Higgs DR(1993). a-Thalassaemia. Baillieres Clin Haematol ;6:117-50.

Higgs DR, Sharpe JA, Wood WG.(1998), Understanding a-globin gene expression: a step towards effective gene therapy. Semin Hematol;35:93-104.

Ho PJ, Hall GW, Luo LY, Weatherall DJ, Thein SL.(1998), Beta thalassaemia intermedia: is it possible consistently to predict phenotype from genotype Br J Haematol 100:70-8.

Huang, M. M., Arnhetm, N., and Goodman, M. F. (1992) Extension of base mis pairs by Taq DNA polymerase: implications for single nucleotide discrimination in PCR. Nucleic Acids Res. 20,4567-4573.

Human hemoglobins: current status(1958).. Am. Pract.& Digest Treat. 9:569-581, .

Issaragrisil S, Visuthisakchai S, Suvatte V, et al(1995),. Transplantation of cord-blood stem cells into a patient with severe thalassemia. N Engl J Med332:367-9.

Jacob, C. F. and Raper, A. B.: Hereditary persistence of foetal haemoglobin production, and its interaction with the sickle-cell trait. Brit.J.Haemat. 4:138-149, 1958.

Kattamis C, Kattamis AC (2001), Oxidative stress disturbances in erythrocytes of beta-thalassemia. Pediatr Hematol Oncol, 18:85–88.

Kazazian HH Jr, Dowling CE, Waber PG, Huang S, Lo WHY. (1986), The spectrum of b-thalassemia genes in China and Southeast Asia.Blood;68(4):964–966.

Kotze, M. J., Theart, L., Callis, M., Peeters, A. V., Thiart, R., and Langenhoven, E. (1995) Nonradioactive multiplex PCR screening strategy for the simultaneous detection of multiple low density lipoprotein receptor gene mutations. PCR Meth. Appl. 4,352-356.

Kunkel, H. C., Ceppellini, R., Muller-Eberhard, U. and Wolf, J. (1957),: Obser-vations on the minor basic hemo-globin component in the blood ofnormal individuals and patients with thalassemia. J.Clin.Invest. 36:1615-1625,.

Kwok, S., Kellogg, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C., and Sninsky, J. J. (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. l&999-1005.

Leung TN, Lau TK, Chung TKh (April 2005). "Thalassaemia screening in pregnancy". *Current Opinion in Obstetrics and Gynecology* **17** (2): 129–34.

Lozano, R; Naghavi, Mohsen; Foreman, Kyle; Lim, Stephen; Shibuya, Kenji; Aboyans, Victor; Abraham, Jerry; Adair, Timothy; Aggarwal, Rakesh; Ahn, Stephanie Y; Almazroa, Mohammad A; Alvarado, Miriam; Anderson, H Ross; Anderson, Laurie M; Andrews, Kathryn G; Atkinson, Charles; Baddour, Larry M; Barker-Collo, Suzanne; Bartels, David H; Bell, Michelle L; Benjamin, Emelia J; Bennett, Derrick; Bhalla, Kavi; Bikbov, Boris; Abdulhak, Aref Bin; Birbeck, Gretchen; Blyth, Fiona; Bolliger, Ian; Boufous, Soufiane et al. (2012). "Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010". *Lancet* **380** (9859): 2095–128

Lo, Y. M. D., Mehal, W. Z., Wordsworth, B. P., Chapman, R. W., Flemmg, K. A., Bell, J. I. , and Wamscoat, J. S. (1991) HLA typing by double ARMS. Lancet 338,65,66

Lo, Y. M. D., Patel, P., Newton, C. R., Markham, A. F., Flemmg, K. A., and Wainscoat, J. S. (1991) Direct haplotype determination by double ARMS: specificity, sensitivity and genetic applications. Nucleic Acids Rex 19, 3561-3567.

Lucarelli G, Giardini C, Angelucci E.(1997), Bone marrow transplantation in thalassemia. In: Winter JN, ed. Blood stem cell transplantation. Boston: Kluwer Academic,:305-15.

Lukopolous, D.; Hadji, A.; Papadakis, M.; Karobaba, P.; Sinopoulo, K. (1990), Prenatal diagnosis of Thalassemia and sickle cell syndromes in Greece. Ann. N.Y. Acad. Science, (612), 226-231.

Maggio, A.; Giambona, A.; Cai, S.P.; Wall, J.; Kan, Y.W.; Chehab, F. F. (1993), Rapid and simultaneous Thalassemia typing of Hemoglobin S, Hemoglobin C, and seven Mediterranean mutations by covalent reverse dot blot analysis: application to prenatal diagnosis in Sicily. Blood, , 81(1), 239-242.

Maggio, Aurelio; d'Amico, Gennaro; Morabito, Alberto; Capra, Marcello; Ciaccio, Calogero; Cianciulli, Paolo; Di Gregorio, Felicia; Garozzo, Giovanni et al. (2002). "Deferiprone versus Deferoxamine in Patients with Thalassemia Major: A Randomized Clinical Trial".*Blood Cells, Molecules, and Diseases* **28** (2): 196.doi:10.1006/bcmd.2002.0510.

Mathias LA, Fisher TC, Zeng L, et al.:(2000), Ineffective erythropoiesis in beta-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. Exp Hematol 28:1343–1353.

Mayoclinichttp://www.mayoclinic.com/health/thalassemia/DS00905/DSECTION=complications |url=missing title (help). Retrieved 20 September 2011.

Meral A, Tuncel P, Surmen-Gur E, et al.:(2000) ,Lipid peroxidation and antioxidant status in beta-thalassemia. Pediatr Hematol Oncol 17:687–693.

Micheli P, Penati P, Momigliano LG. (1935) Ulteriori richereche sulla anemia ipocromica splenomegalica con poichilocitosi. Atti Soc Ital Ematol Haematol (Pavia);16:Suppl 1:10-3.

Mirasena, S.; Shimbhu, D.; Sanguansormsri, M.; Sanguansormsri, T. (2008), Detection of Thalassemia mutations using a multiplex amplification refractory mutation system assay. Hemoglobin, , 32(4), 403-409.

Montalenti, C. and Siniscalco, M.: Frequency of microcythaemia in some Italian districts. Nature 165: 682-683, 1950.

Nathan DG, Gunn RB. Thalassemia, (1966): the consequences of unbalanced hemoglobin synthesis. Am J Med;41:815-30.

Newton, C. R., Graham, A., Heptmstall, L. E., Powell, S. J., Summers, C , Kalsheker, N., Smith, J. C., and Markham, A. F. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 17,2503-2516.

Niederau C, Fischer R, Purschel A, Stremmel W, Haussinger D, Strohmeyer G.(1996), Long-term survival in patients with hereditary hemochromatosis. Gastroenterology 110:1107

Old JM, Varawalla NY, Weatherall DJ. (1990), Rapid detection and prenatal diagnosis of b-thalassemia. Studies in Indian and Cypriotpopulation in the UK. Lancet;336:834–837.

Old, J.M.; Varawalla, N.Y.; Weatherall, D.J. (1990), Rapid detection and prenatal diagnosis of Thalassemia; studies in Indian and Cypriot populations in the UK. Lancet, 336(8719), 834-837.

Old, J.M.; Khan, S.N.; Verma, I.; Fuchareon, S.; Klenthous, M.; Ioannou, P.; Navaratnam, K.; Fisher, C.; Riazuddin, S.; Saxena, R.; Winichagoon, P.; Kyriacou, K.; Ai-Quobaili, F.; and Khan, B (2001),,. A multicenter study in order to further define the molecular basis of Thalassemia in Thailand, Pakistan, Sri Lanka, Mauritius, Syria and India, and to develop a simple molecular diagnostic strategy by Amplification Refractory Mutation System - Polymerase Chain Reaction. Hemoglobin, 25(4), 397-407.

Old JM. Antenatal Diagnosis of Hemoglobinopathies. In: Goulden NJ and Steward CG, (2004), editors. Methods Pediatric Hematology: Methods and Protocols. Volume 91. New Jersey: Humana Press Inc;. p. 33–62.

Old, J.M.; Higgs, D.R. (1982),Gene Analysis. Methods in hematology. In: The Thalassemias., Weatherall, D.J. Ed.; Churchill Livingstone, Edinburgh,; Vol. 6, pp. 74-102.

Old, J.M.; Petrou, M.; Modell, B.; Weatherall, D.J. (1984), Feasibility of antenatal diagnosis by DNA polymorphism in Asian Indian and Cypriot populations. Br. J. Hematol., , 57(2), 255-263.

Olivieri NF, Brittenham GM.(1997), Iron-chelating therapy and the treatment of thalassemia. Blood 89:739-61. [Erratum, Blood;89:2621.]

Olynyk JK, OÕNeill R, Britton RS, Bacon BR.(1994), Determination of hepatic iron concentration in fresh and paraffin-embedded tissue: diagnostic implications. Gastroenterology 06:674-7.

Orkin, Stuart H.; Nathan, David G.; Ginsburg, David; Look, A. Thomas; Fisher, David E.; Lux, Samuel (2009). *Nathan and Oski's Hematology of Infancy and Childhood* (7th ed.). Philadelphia: Saunders.

Orvieto R, Leichter I, Rachmilewitz EA, Margulies JY.(1992), Bone density, mineral content, and cortical index in patients with thalassemia major and the correlation to their bone fractures, blood transfusions, and treatment with desferrioxamine. Calcified Tissue Int ;50:397-9.

Patrinos, G.P.; Giardino, B.; Riemer, C.; Miller, W.; Chui, D.H.K.; Anagnou, N.P.; Wajcman, H.; Hardison, R.C.(2004), Improvements in the Hb Var database of human hemoglobin

variants and Thalassemia mutations for population and sequence studies. Nucleic. Acid Res., (32), 537-541.

Patrinos, G.P.; Kollia, P.; and Papadakis, M.N. (2005), Molecular diagnosis of inherited disorders: Lessons from hemoglobinopathies. Human Mutation, 26(5), 399-412.

Perutz, M.F.; Rossmann, M.G.; Cullis, A.F.; Muirhead, H.; Will, G.; North, A.C.T. (1960). "Structure of H". *Nature* **185** (4711): 416–422.

Petrou, M.; Modell, B.(1995) Prenatal screening for hemoglobin disorders. Prenat. Diagn., 15(13), 1275-1295.

Porter JB.(1997) A risk-benefit assessment of iron-chelation therapy. Drug Saf ;17:407-21.

Recherches stir l'anCmie erythroblastique infantile des peuples de la Mediterranee eorientale; (1938), e'tude anthropologique, etiologique et pathogenique; la transmission hereditair de la maladie. Ann.med. 43:104-125,.

Reed W, Lane PA, Lorey F, et al. (2000), Sickle cell disease not identified by newborn screening because of prior transfusion. J Paediatrics; 136:145–146.

Rietti F. (1925),Ittero emolitico primitivo. Alti Accad Sci Med Nat Ferrara;2:14-9.

Rioja L, Girot R, Garabedian M, Cournot-Witmer G.(1990) ,Bone disease in children with homozygous beta-thalassemia. Bone Miner 8:69-86.

Rosatelli, M.C.; Tuveri, T.; Schelas, M.T. (1992), Molecular screening and fetal diagnosis of beta Thalassemia in Italian population. Hum. Genome, 89(6), 585-589.

Rund D, Oron-Karni V, Filon D, Goldfarb A, Rachmilewitz E, Op-penheim A.(1997), Genetic analysis of b-thalassemia intermedia in Israel: diversity of mechanisms and unpredictability of phenotype. Am J Hematol 5416-22.

Salvasen GS, Dixit VM:(1999), Caspase activation: the induced proximity model.Proc Natl Acad Sci U S A 96:10964–10967.

Samavat A, Modell B (November 2004). "Iranian national thalassaemia screening programme". *BMJ (Clinical Research Ed.)* **329** (7475): 1134–7.

Sangani, B.; Sukumaran, P. K.; Mahadik, C.; Yagnik, H.; Telang, S.; Vaz, F.; Oberroi, R.A.; Modell, B.; Merchant, S.M. (1990) Thalassemia in Bombay: the role of medical genetics. Bull. WHO, , 68(1), 75-81.

Saxena, R.; Jain, P.K.; Thomas, E.; Verma, I.C. (1998), Prenatal diagnosis of Thalassemia ,An experience in a developing country. Prenatal. Diagn.,18(1), 1-7.

Schrier SL. Pathobiology of thalassemic erythrocytes. Curr Opin He-matol 4:75-8.

Shaji, R.V.; Edison, E.S.; Poonkuzhali, B.; Srivastava, A.; Chandy, M. (2003), Rapid detection of b-globin gene mutations and polymorphisms by temporal temperature gradient gel electrophoresis. Clinical. Chem., 49(5), 777-781.

Shalev O, Repka T, Goldfarb A, et al. Deferiprone (L1) (1995),chelates patho-logic iron deposits from membranes of intact thalassemic and sickle RBC both in vitro and in vivo. Blood 86:2008-13.

Shesely EG, Kim H-S, Shehee WR, Papayannopoulou T, Smithies O,Popovich BW. (1991),Correction of a human HbS-globin gene by gene targeting. Proc Natl Acad Sci U S A 88:4294-8

Sheth, J.J.; Sheth, F.J.; Pandya, P.; Priya, R.; Davla, S.; Thakur, C.; Flavian, V. (2008), Beta-thalassemia mutations in western India. Ind. J. Paediatric., 75(6), 567-570.

Shinar E, Rachmilewitz EA.(1993), Haemoglobinopathies and red cell membrane function. Baillieres Clin Hematol; 6:357-69.

Shinar E, Rachmilewitz EA, Lux SE (1989), Differing erythrocyte membrane skeletal protein defects in alpha and beta thalassemia. J Clin Invest 83:404–410

Smith, C. H. (1943): Familial blood studies in cases of Mediterranean (Cooley's) anemia; diagnosis of the trait, or mild form of the disease. Arn.J.Dis. Child. 65:681-701.

Sodani, P; Isgrò, A; Gaziev, J; Paciaroni, K; Marziali, M; Simone, MD; Roveda, A; De Angelis, G et al. (2011). "T cell-depleted hla-haploidentical stem cell transplantation in thalassemia young patients"

Sorensen S, Rubin E, Polster H, Mohandas N, Schrier S. (1990),The role of membrane skeletal-associated alpha-globin in the pathophysiology of beta-thalassemia. Blood 75:1333-6.

Stickel, Felix; Hampe, Jochen (2010). "Dissecting the evolutionary genetics of iron overload in non-alcoholic fatty liver disease". *Journal of Hepatology* **53** (5): 793–4.doi:10.1016/j.jhep.2010.06.010. PMID 20739088.

Sylvester-Jackson DA, Page SL, White JM, et al. (1993), Unbiased analysis of the frequency of b-thalassemia point mutations in a population of African-American newborns. Arch Pathology Lab Med; 117:1110–1114.

Taher AT, musallam Km, El-Beshlawy A, Karimi m, Daar S, Belhoul K, Saned mS, graziadei g, Cappellini mD.(2010), Age-related complications in treatment-naive patients with thalassaemia intermedia. Br J Haematol; 150(4):486-489.

Tan JAMA, Yap SF, Tan KL, Thong MK. (1998),. The use of the Amplification Refractory Mutation System (ARMS) as an effective and economical tool for prenatal diagnosis of β-thalassaemia in Malaysian subjects. Int Med Res J;26(2):65–68.

Tanner, Mark A, J Malcolm Walker, Sunil V Nair, Martina Pibiri, Annalisa Agus, Mark A Westwood, Gillian C Smith, Carlo Dessi, Renzo Galanello, and Dudley J Pennell. "Combined Kaye, Todd B.; Guay, André T.; Simonson, Donald C. (1993). "Non-insulin-dependent diabetes mellitus and elevated serum ferritin level".*Journal of Diabetes and its Complications* **7** (4): 245. doi:10.1016/S0002-9610(05)80252-1.

Tan KL, Tan JAMA, Wong YC, Wee YC, Thong MK, Yap SF. (2001), Combine ARMS: a rapid and cost effective protocol for molecular characterization of b-thalassemia in Malaysia. Genet Test;5:17–22.

Tavazzi D, Duca L, Grazidei G, et al.:(2001) ,Membrane-bound iron contributes to oxidative damage of beta-thalassemia intermedia erythrocytes. Br J Haematol 112:48–50.This study identifies the several forms of membrane-bound iron that could lead togeneration of ROS.

Tebbutt, A. H.: Mediterranean (1950), (Cooley's) anemia in Australia. M.J. Australia 1:50-52,

Terrenato, L; Shrestha, S; Dixit, KA; Luzzatto, L; Modiano, G; Morpurgo, G; Arese, P (1988). "Decreased malaria morbidity in the Tharu people compared to sympatric populations in Nepal.". *Annals of tropical medicine and parasitology* **82** (1): 1–11.

Thakur (Mahadik), C. (2000), Biochemical and Molecular characterization of Thalassemia in Maharashtra. PhD Thesis, University of Mumbai: India, October.

Thakur, C.B.; Vaz, F.; Banerjee, M.; Kapadia, C.; Natrajan, P.G.; Yagnik, H.; and Gangal, S (2000), Prenatal Diagnosis of Thalassemia and other hemoglobinopathies in India. Prenatal. Diagnosis, 20(3), 194-201.

The genetics of human hemoglobin differences: problems and perspectives. (1956), Ann. Human Cenet. 21:1-30.

The thalassemia syndromes. Conference on Hemoglobin(1958), National Academy of Sciences-National Re-search Council. Washington D. C., , pp. 283-286.

Thein SL, Hesketh C, Taylor P, et al.(1990) ,Molecular basis for dominantly inherited inclusion body b thalassemia. Proc Natl Acad Sci U S A 87:3924-8.

Thein, S.L.; Old, J.M.; Wainscoat, J.S.; Petrou, M.; Modell, B.; Weatherall, D.J. (1983), Population and genetic studies suggest a single origin for the Indian deletion β° thalassaemia. Br. J. Hematol., , 57(2), 271-278.

Thong MK, Tan JAMA, Tan KL, Yap SF. (2005), Characterisation of β-globin gene mutations in Malaysian children: A strategy for the control of β-thalassaemia in a developing country. J Trop Pediatr.;51(6):328–333.

Thongnoppakhun, W.; Jiemsup, S.; Yongkiettrakul, S.; Kanjanakorn, C.; Limwongse, C.; Wilairat, P.; Vanasant, A.; Rungroj, N.; Yenchitsomanu, P.N. (2009), Simple, efficient and cost-effective multiplex genotyping with Matrix Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry of Hemoglobin. J. Mol. Diagnosis., 11(4), 334-346.

Valenti, Luca; Canavesi, Elena; Galmozzi, Enrico; Dongiovanni, Paola; Rametta, Raffaela; Maggioni, Paolo; Maggioni, Marco; Fracanzani, Anna Ludovica; Fargion, Silvia (2010) . "Beta-globin mutations are associated with parenchymal siderosis and fibrosis in patients with non-alcoholic fatty liver disease". *Journal of Hepatology***53** (5): 927–33.

Varawalla NY, Old JM, Sarkar R, Venkatesan R, Weatherall DJ. (1991), The spectrum of β-thalassemia mutations on the Indian subcontinent: the basis of prenatal diagnosis. Br J Hematol.;78(2): 242–247.

Varawalla, N.Y.; Old, J.M.; Weatherall, D.J. (1991), Rare beta Thalassemia mutations in Asian Indians. Brit. J. Hematol., 79(4), 640-645.

Vaz, F.E.E.; Thakur, C.B.; Banerjee, M.K.; Gangal, S.G. (2000), Distribution of Thalassemia Mutations in the Indian population Referred to a diagnostic center. Hemoglobin, , 24(3), 181-194.

Verma IM, Somia N.(1997), Gene therapy Ñ promises, problems and prospects. Nature 389:239-42.

Verneris M, Ma L, Bai H, et al.:(1999) The role of FAS and FAS ligand in the accelerated apoptosis of erythroid precursors in Cooley's anemia [abstract]. Blood 94(suppl 1):205a.

Wang, W.; Kham, S.K.Y.; Yeo, Gare-Hoon.; Thuan-Chong Q.; Chong, S.S. (2003), Multiplex minisequencing screening for common Southeast Asian and Indian thalassemia mutations. Clin. Chem., , 49(2), 209-218.

Weatherall DJ, Clegg JB. The thalassaemia syndromes. 4th ed. Oxford, England: Blackwell Scientific (in press).

Weatherall DJ, Clegg JB.(1996), Thalassaemia Ñ a global public health prob-lem. Nat Med 2:847-9.

Weed, Robert I.; Reed, Claude F. and Berg, George (1963). "Is hemoglobin an essential structural component of human erythrocyte membranes?". *J Clin Invest.* **42** (4): 581–8. doi:10.1172/JCI104747

Westgren M, Ringden O, Eik-Nes S, et al(1996), Lack of evidence of permanent engraftment after in utero fetal stem cell transplantation in congenital hemoglobinopathies. Transplantation 61:1176-9.

Whipple GH, Bradford WL. (1936),Mediterranean disease Ñ thalassemia (erythroblastic anemia of Cooley): associated pigment abnormalities simulating hemochromatosis. J Pediatr 9:279-311

Wickramasinghe SN.(1976), The morphology and kinetics of erythropoiesis in homozygous b-thalassaemia. In: Congenital disorders of erythropoiesis. Amsterdam: Elsevier , 221-37.

Wilborn M, Therrell BL, McCabe ERB. (1994), Newborn screening for sickle cell disease in transfused infants. National Newborn Screen ing Meeting, June 7–11, Seattle, Washington,.

Wu, D. Y., Ugozzoh, L., Pal, B. K , and Wallace, R. B. (1989) Allele-specific enzymatic amplification of beta -globin genomic DNA for diagnosis of sickle cell anemia. Proc. Natl. Acad Sci. USA 86,2757-2760

Yuan J, Angelucci E, Lucarelli G, et al. (1993),Accelerated programmed cell death (apoptosis) in erythroid precursors of patients with severe beta-thalassemia. Blood 82:374-7.

Yuan J, Kannan R, Shinar E, et al.:(1992), Isolation, characterization, and immunoprecipitation studies of immune complexes from membranes of β–thalassemic erythrocytes. Blood 79:3007–3013.

Zeng Y, Huang S. The studies of hemoglobinopathies ad thalassemia in China-the experiences in Shanghai Institue of Medical Genetics. Clin Chim Acta 2001; 313: 107-11

Zermati Y, Garrido C, Amsellem S, et al. :(2001), Caspase activation is required for terminal erythroid differentiation. J Exp Med 193:247–254.

Zhang Y-H, McCabe ERB. (1992), RNA analysis from newborn screening dried blood specimens. Hum Genet; 89:311–314