1 Introduction

Globin genes:

Haemoglobin contains two α-like globin chains and two β-like globin chains, each forming a complex with one molecule of haem. The globin chains are encoded by α and β-globin gene clusters located on chromosomes 16 and 11 respectively (Thein and Weatherall 1988).

α-globin gene cluster:

Human α-globin cluster resides near the telomere of the short arm of chromosome 16 and encompasses approximately 30 kb (Fig: 1.1). It includes an embryonic gene (ζ), two adult genes (α1 and α2), three pseudogenes (ψζ1, ψα2, ψα1) and a gene of unknown significance (θ1) (Higgs et al, 1989).

β-globin gene Cluster:

The β-globin gene cluster spans approximately 60 kb on the short arm of chromosome 11 (Fig-1.1). It is composed of an embryonic gene (ε), a duplicated fetal gene (γ), a pseudo β-gene, a minor adult gene (δ) and the adult β-gene (Bunn and Forget 1986).

Structure of globin genes:

Throughout the vertebrates, the basic intron-exon structure of the globin genes remains constant. The globin genes are compact (1 to 2 kb) and have three coding regions called exons that are interrupted by two intervening sequences (IVS) of variable length called introns (Collins and Weisman 1984). The two IVS regions in the α-globin genes are located between codons 31 and 32 and between codons 99 and 100. Whereas, in the β-globin genes the IVS regions interrupt the sequences between codons 30 and 31 and between codons 104 and 105 (Fig: 1.1) (Thein and Weatherall 1988). The IVS-I is shorter than the IVS-II in both α and β-genes whereas the IVS-II in α-genes is considerably shorter than in the β-globin genes.

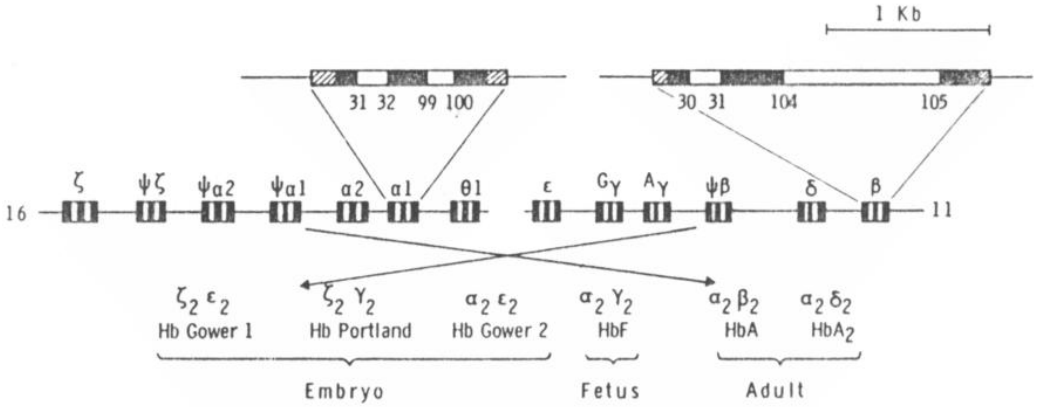

Fig: 1.1. Organization of the human α and β-globin gene clusters on chromosome 16 and 11. The genetic control of various embryonic, fetal and adult haemoglobins is also shown. Hatched boxes in the individual genes represent 3' and 5' untranslated regions, black boxes represent exons and the white boxes represent intervening sequences (IVS) (Thein and Weatherall 1988).

Globin gene expression:

The α- and β-globin loci are activated towards the end of the third week of gestation. There is a transition during development from the production of embryonic haemoglobin (Hb Portland, $\zeta_2\gamma_2$; Hb Gower-1 $\zeta_2\varepsilon_2$; Hb Gower-2 $\alpha_2\varepsilon_2$) to fetal haemoglobin (Hb-F, $\alpha_2\gamma_2$) to adult haemoglobins (Hb-A, $\alpha_2\beta_2$; Hb-A_2, $\alpha_2\delta_2$) (Fig: 1.1) (Peschle et al, 1985). The α-globin gene expression remains constant throughout life. The protein products of α1- and α2-genes are identical, however, the steady state level of α2- mRNA predominates over the α1- mRNA by approximately 3:1 (Liebhaber et al, 1986). On the other hand ζ,-, ε-, and γ-globin gene expression is down-regulated and β-globin gene expression increases with the transition from fetal to adult haematopoiesis. The γ to β-globin switching is normally complete by six months after birth (Cunningham and Jane1996).

Temporal control:

The globin genes are expressed in a developmental sequence. As development proceeds the site of erythropoiesis shifts from the yolk sac in the early embryo, to the liver in fetal life, and finally to the bone marrow in the postnatal life (Weatherall and Clegg 1981). Tuan et al, (1985) observed that *cis*-acting erythroid specific DNAase-I hypersensitive sites are present in a broad

region of more than 20 kb 5' to the ε-globin gene. The DNAase-I hypersensitive region confers a high level, position independent, expression on linked globin genes (Grosveld et al, 1987). The term Locus Control Region (LCR), has been used to describe the cis-acting sequences responsible for this effect (Orkin 1990). It is postulated that temporal regulation of β-like globin genes results from competition between embryonic, fetal and adult globin genes for interaction with a common LCR (Townes and Behringer 1990).

Trans-acting factors like GATA binding proteins, synthesized in the yolk sac, fetal liver and bone marrow, may bind to a DNA sequence motif (T/A)GATA(A/G) present in the ε, γ and β-globin promoters for the ordered expression of the respective genes (Orkin 1990). Two such proteins, GATA-1 and GATA-2 have been shown to be essential for transcriptional control of erythroid specific gene expression (Shivdasani and Orkin 1996).

Existence of an LCR for the α-globin cluster has been suggested in the sequences upstream from the ζ-globin gene (Higgs et al, 1990a).

Promoter function:

The region located upstream to the coding sequence of many structural genes which controls individual gene expression and provides a site for binding of RNA polymerase is called promoter region. At least three preserved definable elements contribute in varying degrees to the promoter function of globin genes (Collins and Weisman 1984). The first preserved 5' untranslated sequence is the "TATA" or "ATA" box located approximately 25-30 bp upstream from the initiation site in both α-like and β-like globin genes (Fig 1.2). The "ATA" box is thought to play an important role in locating the site at which the transcription initiates. The second important component is the "CCAAT" box located approximately 80 bp upstream from the transcription initiation site. Its position is somewhat more variable than the "ATA" box. The third component of the promoters is the "GGGGYG" (Y: a pyrimidine nucleoside) or the inverted type "CRCCC" (R: a purine nucleoside) sequence that is located 90 bp upstream from the initiation site (Collins and Weisman 1984). The normal human δ-globin gene does not have a perfectly conserved "CCAAT" sequence and this, in part, may be responsible for the low normal expression of the gene (Bunn and Forget 1986).

Transcription:

The transcription initiation site for globin genes correspond to the Cap site located 50 bp upstream from the initiation codon (AUG) which also marks the 5' end of the mature cytoplasmic mRNA (Fig: 1.2). The transcription goes on to include the exons introns and to the sequences beyond the highly conserved 3' "AATAAA" polyadenylation site (Collins and Weisman 1984).

Post transcriptional processing:

Following transcription the 5' end of the transcript is capped by addition of a methylated guanylic acid residue. This process is essential to prevent exoneucleolytic degradation of the nascent transcript (Nevins, 1983). The mRNA transcript is also cleaved and subsequently polyadenylated at a site marked by the "AAUAAA" sequence (Collins and Weisman 1984). Another important posttranscriptional event is splicing in which the introns are removed from the transcript. The process begins with cleavage of the 5' junction between exon and the intron. This results in looping back of the 5' end of the intron to form a 5'-2' phosphodiester bond with an “A” residue about 30 nucleotides 5' to the 3' splice junction (Padgett et al, 1984). The resulting structure is called a lariat. The first two nucleotides at both 5' and 3' end of the intron are essential for splicing. Nearly all eukaryotic genes have a highly conserved "GT" sequence at the 5' end and "AG" sequence at the 3' end of every intron. Marked similarities in sequence have been observed at the exon-intron boundaries in all eukaryotic genes. These identified sequences are called consensus sequences. For donor splice sites (5’ consensus sequence) they include the last 3 nucleotides of the exon and the first 6 nucleotides of the intron and for the acceptor splice sites (3’ consensus sequence) they are the last 10 nucleotides of the intron and the first nucleotide of exon (Shapiro and Senapathy, 1987).

CAATTTCTTATTTGTGTAATAAGAAAATTGGGAAAACGATCTTCAATATGCTTACCAAGCTGTGATTCCAAATATTACGTAAATACACTTGCAAAG
GAGGATGTTTTTAGTAGCAATTTGTACTGATGGTATGGGGCCAAGAGATATATCTTAGAGGGAGGGCTGAGGGTTTGAAGTCCAACTCCTAAGCCA
GTGCCAGAAGAGCCAAGGACAGGTACGGCTGTCATCACTTAGACCTCACCCTGTGGAGCCACACCCTAGGGTTGGCCAATCTACTCCCAGGAGCAG
GGAGGGCAGGAGCCAGGGCTGGGCATATAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAA
ACAGACACCATG**GTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTG**
GGCAG*GTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCAC*
*TGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAG***GCTGCTGGTGGTCTACCCTTGGACCGAGAGGTTCTTTGAGTCCTTTGGGGATCTGTC**
CACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCT
CAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGG*GTGAGTCTATGGGACCCTTGATGTTT*
TCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTCATAGGAAGGGGAGAAGTAACAGGGTACAGTTTAGAATGGGAAACAGACGAATGATTGCATC
AGTGTGGAAGTCTCAGGATCGTTTTAGTTTCTTTTATTTGCTGTTCATAACAATTGTTTTCTTTTGTTTAATTCTTGCTTTCTTTTTTTTTCTTCT
CCGCAATTTTTACTATTATACTTAATGCCTTAACATTGTGTATAACAAAAGGAAATATCTCTGAGATACATTAAGTAACTTAAAAAAAAACTTTAC
ACAGTCTGCCTAGTACATTACTATTTGGAATATATGTGTGCTTATTTGCATATTCATAATCTCCCTACTTTATTTTCTTTTATTTTTAATTGATAC
ATAATCATTATACATATTTATGGGTTAAAGTGTAATGTTTTAATATGTGTACACATATTGACCAAATCAGGGTAATTTTGCATTTGTAATTTTAAA
AAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCA
TGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATTTCTGCATATAAATATTTCTGCATATAAATTGT
AACTGATGTAAGAGGTTTCATATTGCTAATAGCAGCTACAATCCAGCTACCATTCTGCTTTTATTTTATGGTTGGGATAAGGCTGGATTATTCTGA
*GTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAG***CTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACT**
TTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCT
TTCTTGCTGTCCAATTTCTATTAAAGCTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCT
GCCTAATAAAAAACATTTATTTTCATTGCAATGATGTATTTAAATTATTTCTGAATATTTTACTAAAAAGGGAATGTGGGAGGTCAGTGCATTTAA

Fig: 1.2. Sequence of the β-globin gene. The sequences important for promoter function, capping, initiation, termination and polyadenylation are underlined. The exons are represented as bold and the introns are shown as italics (After Collins and Weisman 1984).

Translation:

Once processed the mRNA transcript is transported out of the nucleus and acts as a template on which the amino acid residues are sequentially added to form the polypeptide. The process is initiated at the initiation codon (AUG) and is terminated when a stop codon (UAA) is encountered (Bunn and Forget 1986).

Thalassaemia:

Historical background:

(Bunn and Forget 1986):

Thomas Cooley gave the first description of thalassaemia in 1925. He described a syndrome among children of Italian decent characterized by profound anaemia, splenomegaly, and bony abnormalities. The term "thalassaemia" derived from the Greek θαλασσα (the sea) was introduced by Whipple and Bradford in 1932 to show the apparent association between the disease and the Mediterranean. A number of Italian workers, between 1925 and 1935 described the syndrome of mild anaemia and increased osmotic resistance of red cells, who described it as "la malattia di Rietti-Greppi-Micheli". In 1936 Caminopetros in Greece and in 1937 Angelini in Italy concluded that the Cooley's anaemia is a recessively inherited disorder resulting from homozygous inheritance of the Rietti-Greppi-Micheli syndrome.

Definition and classification:

Thalassaemias are a heterogenous group of genetic disorders of haemoglobin synthesis, that result from a reduced rate of production of one or more of the globin chain(s) of haemoglobin (Weatherall 1996). They are divided into the α-, β-, δβ-, or γδβ-thalassaemias according to the type of chain(s) affected (Table: 1.1). The quantitative reduction in globin chain synthesis in thalassaemia syndromes differentiates these from the structural changes seen in abnormal haemoglobins. The structurally abnormal haemoglobins are mostly produced in normal amounts. However, some abnormal haemoglobins, Hb-E and Hb-Knossos for example, are also associated with reduced globin chain production.

Table: 1.1 Classification of thalassaemias (Weatherall et al, 1989; Weatherall 1996).

α-Thalassaemias:

- α^{o} -Thalassaemias
- α^{+} -Thalassaemias
  - Deletion
  - Non-deletion
- With α-chain Hb variants
- With β-chain Hb variants
- With β-Thalassaemia

β-Thalassaemias:

- β^{o} -Thalassaemias
- β^{+} -Thalassaemias
- With β-chain Hb variants
- With α-chain Hb variants
- With α-Thalassaemia

δβ-Thalassaemia:

- $(\delta\beta)^{o}$ -Thalassaemia
- $(^{A}\gamma\delta\beta)^{o}$ -Thalassaemia
- $(\varepsilon\gamma\delta\beta)^{o}$ -Thalassaemia
- δ-Thalassaemia
- γ-Thalassaemia

Hereditary Persistence of Fetal Haemoglobin (HPFH):

- Deletion
  - $(\delta\beta)^{o}$ -HPFH
- Non-deletion
  - Linked to β-globin gene cluster
  - Unlinked to β-globin gene cluster

World wide distribution of thalassaemia:

Disorders of haemoglobin are considered to be the most common genetic disorder world wide. About 250 million people (4% of the world population) carry a gene for an abnormal haemoglobin (WHO 1993). α^{+}-thalassaemia is the commonest globin mutation. Each year about 240,000 infants are born with a major haemoglobin disorder. Sickle cell disease accounts for nearly 78% of the affected births, β-thalassaemia major or Hb-E/β-thalassaemia accounts for about 20%, and about 1.6% is accounted for by Hb-S/β-thalassaemia (WHO 1985).

The disease has a high prevalence in a broad belt including the Mediterranean the Middle

East, Indian subcontinent and the South East Asia (Weatherall and Cleg 1981). The highest concentration of α-thalassaemia is found in the Southeast Asia where up to 17,000 severely affected cases are born every year (WHO 1987).

Population genetics of thalassaemia:

The world distribution of thalassaemia and other major abnormal haemoglobins coincides with that of Plasmodium falciparum malaria. It is thought that heterozygotes are resistant to falciparum malaria and the selective advantage this provides compensates for the continual loss through the death of the homozygotes (Weatherall and Clegg 1981). The high frequency of thalassaemia genes, in populations where these are prevalent, exist in a state of balanced polymorphism due to heterozygote advantage on one hand and continuing loss through homozygotes on the other hand (Modell and Berdoukas 1984).

The heterozygote advantage against falciparum malaria may be the result of an increased susceptibility to red cell damage by oxidative stress in thalassaemia that leads to premature destruction of the parasite (Friedman et al, 1981). A strong correlation between the degree of parasite invasion and the lack of deformability of thalassaemic erythrocytes may also contribute to resistance against invasion by malarial parasite (Weatherall et al, 1989).

Molecular pathology of α-thalassaemia:

Most α-thalassaemias are caused by large gene deletions. Two α-thalassaemia phenotypes are recognized (1) α^{o}-thalassaemia or α-thalassaemia-1 is characterized by complete absence of α-globin chains and (2) α^{+}-thalassaemia or α-thalassaemia-2 in which there is mild reduction in α-globin synthesis. α^{o}-thalassaemia results from deletions that involve two α-globin genes (--/αα), whereas α^{+}-thalassaemia usually results from deletion of one α-globin gene (- α/αα) (Higgs et al, 1990b).

α-thalassaemia-2 is the commonest type of thalassaemia. It has a frequency of 30% in some parts of Africa (Dozy et al, 1979) and 30-50% in some parts of India (Kulozik et al, 1988; Gupta et al, 1991). The commonest α-thalassaemia-2 determinant ($-\alpha^{3.7}$) results from deletion of 3.7kb of DNA extending from IVS-II of α2-gene to the corresponding part of α1-gene. Another α-thalassaemia-2 determinant ($-\alpha^{4.2}$) results from deletion of 4.2kb of DNA

extending from the 3' end of ψα-gene to the 3' end of α2-gene (Embury et al, 1980). Less common deletions causing α-thalassaemia-2 include $-\alpha^{3.5}$ (Kulozik et al, 1988).

α1 and α2-genes are embedded within two highly homologous 4 kb duplicated segments. These regions are divided into homologous sub-segments (X, Y, and Z) by non-homologous elements (I, II, and III). Reciprocal recombination (Fig: 1.3) between Z segments, which are 3.7kb apart, produces chromosomes with only one α-gene ($-\alpha^{3.7}$, rightward deletion) that causes α-thalassaemia (Embury et al, 1980) and others with three α-genes ($\alpha\alpha\alpha^{anti\ 3.7}$) (Goossens et al, 1980). The events can be subdivided, depending on exactly where within the Z box the crossover takes place, into $-\alpha^{3.7I}$, $-\alpha^{3.7II}$, and $-\alpha^{3.7III}$ (Higgs et al, 1984). Recombination between homologous X boxes, which are 4.2kb apart, gives rise to $-\alpha^{4.2}$-thalassaemia (Embury et al, 1980) and $\alpha\alpha\alpha^{anti4.2}$ chromosome (Lie-Injo et al, 1981).

α^{o}-thalassaemia is caused by deletions of both α-globin genes (--/αα). A large number of such deletions have been reported in people from Southeast Asia and Mediterranean (Higgs et al, 1990b). These deletions appear to have resulted from illegitimate recombinations that are poorly understood (Lukens 1993).

Less commonly α-thalassaemia phenotype is caused by mutations affecting the function of intact gene. Non-deletional forms of α-thalassaemia have been reported in the Mediterranean, Southeast Asians, Indians, Blacks and Saudi Arabians (Higgs et al, 1990b; Baysal et al, 1995b). Most of the mutations occur in the dominant α2 gene that produces a more severe phenotype than mutations affecting the α1 gene (Higgs et al, 1989).

Molecular Pathology of β-thalassaemia:

In contrast to α-thalassaemia majority of β-thalassaemias are caused by point mutations affecting gene regulation or expression. The study of globin chain synthesis in thalassaemia homozygotes reveals two major types of β-thalassaemia (1) with some residual β-chain synthesis (β^{+}-thalassaemia) and (2) no β-chain synthesis (β^{o}-thalassaemia) (Lukens 1993).

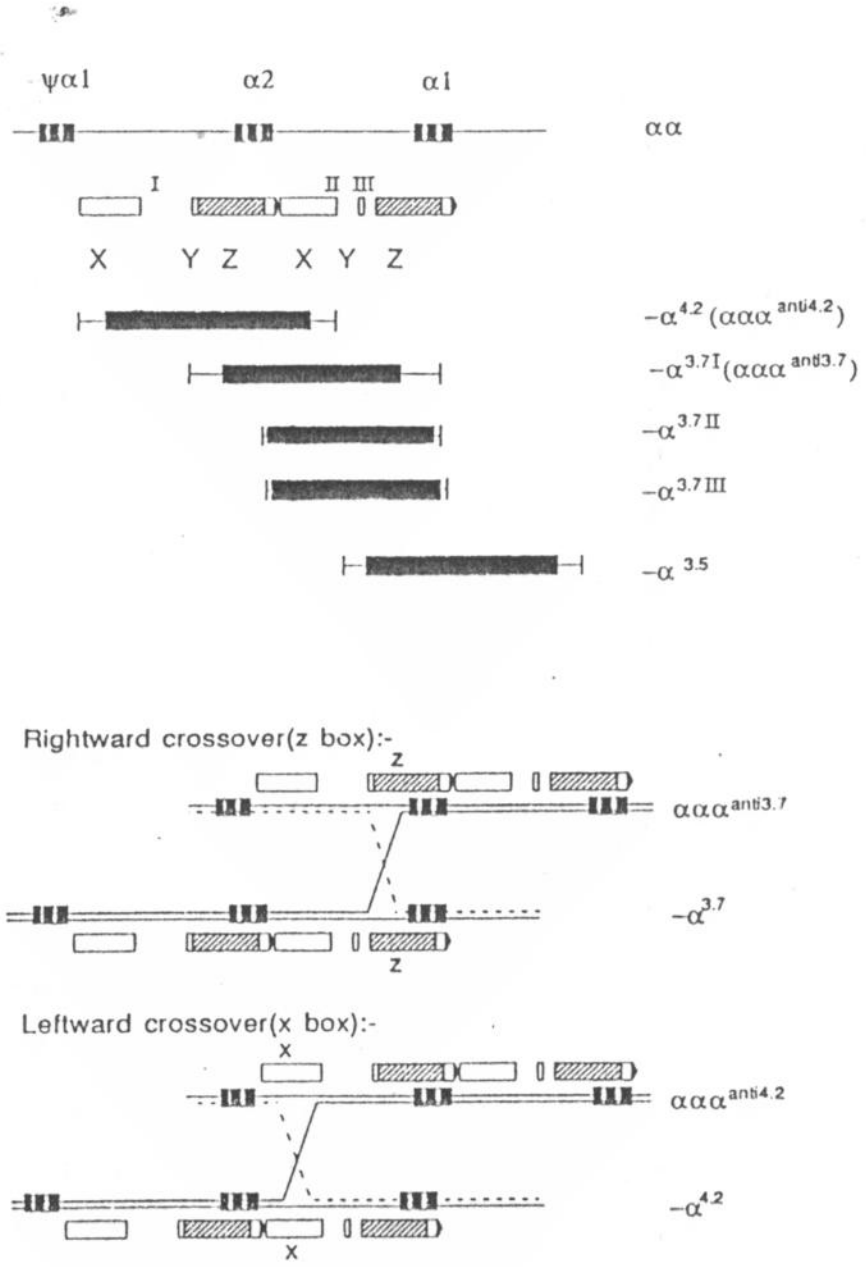

Fig: 1.3. The duplicated XYZ box arrangement containing the α genes. Nonhomologous regions (I, II, and III) are indicated. The extent of each deletion is indicated by the solid blocks and the limits of the breakpoints are represented by solid lines. Misaligned chromosomes crossing over to produce the $-\alpha^{3.7}$, $\alpha\alpha\alpha^{anti3.7}$ and $-\alpha^{4.2}$, $\alpha^{anti\ 4.2}$ haplotypes are also shown (Higgs et al, 1989).

Studies on the effect of thalassaemia mutations on globin gene expression demonstrate abnormal RNA processing (Triesman et al, 1983). Over 150 different mutations have been identified as a cause of β-thalassaemia (Baysal and Carver 1995). Studies on the

molecular genetics of thalassaemia in various ethnic groups have shown that each group tends to have its own set of common mutations (Kazazian et al, 1990). These mutations affect the gene expression by a variety of mechanisms.

Transcriptional promoter mutations:

The mutations resulting in reduced promoter function are mostly concentrated in the "TATA" box (Fig: 1.2) and in the proximal and distal "CACACCC" sequences at -90 and -105 neucleotides upstream of the gene (Kazazian et al, 1990). The promoter mutations tend to reduce the binding of RNA polymerase and lower the level of transcription to 20-30% of normal (Leukens 1993). The mutations in the promotor region of β-globin gene, for example C-T substitution at position -88 relative to Cap site (Orkin et al, 1984) produces a mild phenotype. A mutation at the Cap site of the β-globin gene also affects the transcription and capping with secondary effect on translation (Myers et al, 1986). Several mutations in the promoter region of β-globin gene are known to cause β^+-thalassaemia (Baysal and Carver 1995).

Chain termination mutations:

Premature termination of transcription may be caused by a nonsense mutation. A single nucleotide substitution in a codon, normally coding for an amino acid, can change it to a stop codon. CAG-TAG substitution at codon 39 is a classical example of nonsense codon (Trecartin et al, 1981). Premature chain termination may also be induced when the reading frame is shifted either by an insertion or deletion of nucleotide(s). The four nucleotide deletion (-TTCT) in codons 41 and 42, for example, results in premature introduction of a stop codon in the new codon 59 (Kimura et al, 1983). All chain termination mutants tend to produce β^o-thalassaemia phenotype (Lukens 1993). A large number of nonsense and frame-shift mutations have been documented as a cause of β^o-thalassaemia (Baysal and Carver 1995).

Mutations affecting RNA splicing:

The normal splicing may be disturbed by mutations involving the critical sequences at the exon-intron splice boundaries (Kazazian et al, 1990). Consensus sequence changes have been observed in donor site of IVS-1. G-C substitution at position +5, for example, results in production of normally and abnormally spliced RNA that causes severe β^+-thalassaemia

phenotype (Triesman et al, 1983). T-C substitution at position +6 of the IVSI, however, results in a milder β^+-thalassaemia phenotype (Orkin et al, 1982a). The altered mature RNA resulting from these mutations is ineffective as a messenger for β-globin synthesis. Nucleotide substitutions can lead to appearance of a consensus splice site sequence within an intron or an exon, the new splice site competing with normal splice sequences. G-A substitution at IVSI-110 creates a new acceptor splice site that resembles the normal acceptor splice site. The new site is used preferentially by the normal donor site that results in a β^+-thalassaemia phenotype (Spritz et al, 1981). A cryptic splice site, that mimics the consensus sequence for a splice site but is never used, may become used due to a mutation. A substitution at Codon 26 (Hb-E), for example, leads to use of a cryptic site at a low level of efficiency (Orkin et al, 1982b). At least 38 mutations result in abnormal RNA splicing and cause thalassaemia ranging in severity from mild β^+ to β^o-thalassaemia (Baysal and Carver 1995).

RNA Cleavage and polyadenylation mutants:

Six mutations in the polyadenylation site, all causing β^+-thalassaemia, have been reported (Baysal and Carver 1995). Mutations at the poly-A site, AA<u>T</u>AAA-AA<u>C</u>AAA in American Blacks for example, can result in cleaving of only a small percentage of the RNA transcript (Orkin et al, 1985). It is presumed that deficient β-globin synthesis is secondary to abnormal elongated transcripts (Kazazian et al, 1990).

Deletions as a cause of β-thalassaemia:

Deletions causing β-thalassaemia are rare. However, 13 different deletions of varying lengths have been reported (Baysal and Carver 1995). The commonest deletion removes 619 bp of IVS-II, exon-3 and sequences 3' of the gene (Spritz et al, 1982). Some deletions involving a large portion of the 5' untranslated region of β-globin gene are associated with an unusually high Hb-A_2 (Motum et al, 1992). Craig et al, (1992) have reported a 10.3kb deletion involving the 5’ untranslated region of β-globin gene in an Asian Indian which is associated with an unusually high Hb-A_2 level. These mutations probably remove competition of β-globin gene promoters for the common Locus Control Region.

Dominant β-thalassaemia mutations:

A dominant form of β-thalassaemia, in which heterozygotes also become symptomatic, has been identified. A total of 27 different mutations, mostly involving the 3rd exon, are associated with this uncommon phenotype (Baysal and Carver 1995). At the molecular level they result from different mechanisms including a highly unstable β-variant as a result of single base substitution (Thein et al, 1991) or deletion of intact codons (Park et al, 1991), truncated β-chain due to premature termination, and an elongated β-globin with an altered carboxy terminal end as a result of frame-shift mutation (Thein et al, 1990). The dominant thalassaemia mutations in a heterozygous state form hyper-unstable haemoglobin variant that precipitate in the erythroid cells and lead to a syndrome of thalassaemia intermedia (Thein 1992).

β-thalassaemia due to unknown mutations:

Sometimes cases of typical β-thalassaemia are seen without any detectable mutation in the β-globin gene or its immediate flanking regions (Semenza et al, 1984; Kazazian et al, 1990). It is thought that these result from a mutation in another gene located elsewhere in the genome, which is important in β-globin gene expression (Cao et al, 1994).

World-wide distribution of β-thalassaemia mutations:

The spectrum of β-thalassaemia varies widely in different world populations. Each population has its own set of common mutations that account for most of the mutations. However, a large number of rare alleles are also observed in each ethnic population (Kazazian et al, 1990). Allele frequencies vary from one country to another or even between different regions of the same country (Rosatelli et al, 1992a). Because so many alleles are found in each region, many individuals with β-thalassaemia major carry two different alleles (are compound heterozygotes). However, in populations where the number of alleles is very limited or consanguineous marriage is common the incidence of true homozygotes is increased (Rosatelli et al, 1992b; Varawalla et al, 1991a). The prevalence of common mutations in various world populations is presented in Table: 1.2.

Table: 1.2. β-thalassaemia carrier rate and mutations in various world populations.

Country: (n) **(Reference)**	**Carrier rate: (WHO 1985)**	**Mutations:**	**%**
Italy: (914) (Rosatelli et al, 1992a)	2-15%	Cd 39 (C-T) IVSI-110 (G-A) IVSI-1 (G-A) IVSI-6 Others	40% 23% 10% 10% 17%
Greece: (642) (Loukopoulos 1996)	8%	IVSI-110 (G-A) Cd 39 (C-T) IVSI-1 (G-A) IVSI-6 (T-C) Others	43% 21% 14% 9% 13%
Sardinia: (3000) (Rosatelli et al, 1992b)	13%	Cd 39 (C-T) Fr 6 (-A) Fr 76 (-C) Others	96% 2% 1% 1%
Spain: (58) (Amselem et al, 1988)	<1%	Cd 39 (C-T) IVSI-6 (T-C) IVSI-110 (G-A) Others	64% 15% 9% 12%
Cyprus, (Turks): (232) (Baysal et al, 1992)	16-18%	IVSI-110 (G-A) IVSI-6 (T-C) IVSI-1 (G-A) IVSI-745 (C-G) Others	74% 8% 7% 7% 4%
Cyprus, (Greeks): (705) (Baysal et al, 1992)	16-18%	IVSI-110 (G-A) IVSI-6 (T-C) IVSI-1 (G-A) IVSI-745 (C-G) Others	80% 6% 5% 5% 4%
Turkey: (429) (Basak et al, 1992)	2-4%	IVSI-110 (G-A) IVSI-6 (T-C) Fr 8 (-AA) IVSI-1 G-A) Others	42% 10 5% 5% 38%
Malta: (32) (Scerri et al, 1993)	1.2-3%	IVSI-6 (T-C) Others	78% 22%
Azerbaijan: (135) (Kuliev et al, 1994)	6%	Fr 8 (-AA) IVSII-1 (G-A) IVSI-110 (G-A) Others	33% 21% 13% 33%
Bulgaria: (128) (Huisman 1990a)	3%	IVSI-110 (G-A) Cd 39 (C-T) IVSI-6 (T-C) IVSII-754 (C-G) Others	24% 22% 10% 10% 34%
Israel: (122) (Rund et al, 1990)	1-20%	Fr 44 (-C) IVSI-110 (G-A) -28 (A-C) IVSII-1 (G-A) Others	20% 18% 15% 14% 33%
Egypt: (48) (Novelletto et al, 1990)	2.5%	IVSI-110 (G-A) IVSI-6 (T-C) IVSI-1 (G-A) Others	27% 19% 10% 44%

Table: 1.2. (Contd…) β-thalassaemia carrier rate and mutations in various world populations.

Country: (n) (Reference)	**Carrier rate: (WHO 1985)**	**Mutations:**	**%**
Algeria: (172) (Labie et al, 1990)	5-6%	Cd 39 (C-T) IVSI-110 (G-A) Fr 6 (-A) IVSI-1 (G-A) Others	26% 25% 18% 15% 16%
UAE: (50) Quaife et al, 1994)	3%	IVSI-5 (G-C) Fr 8-9 (+G) IVSI -25 bp Others	66% 8% 8% 18%
Iran: (50) (Taghi personal communication)	2-4%	IVSII-1 (G-A) IVSI-5 (G-C) IVSI-6 (T-C) Others	22% 13% 9% 56%
North West Pakistan: (167) (Varawalla et al, 1991a)	2-6.5%	Fr 8-9 (+G) IVSI-5 (G-C) Fr 41-42 (-TTCT) Cd 15 (G-A) Others	35% 44% 10% 7% 4%
India: (535) (Varawalla et al, 1991a)	1-17%	IVSI-5 (G-C) Del 619 bp IVSI-1 (G-T) Fr 41-42 (-TTCT) Fr 8-9 (+G) Others	39% 25% 13% 10% 8% 5%
Burma: (170) (Brown et al, 1992)	β-Thal: 1-3% Hb-E: 1-25%	Hb-E IVSI-1 (G-T) IVSI-5 (G-C) Fr 41-42 (-TTCT) Others	41% 20% 16% 12% 11%
Thailand: (128) (Winichagoon et al, 1990)	β-Thal: 3-9% Hb-E: 0-40%	Fr 41-42 (-TTCT) Hb-E Cd 17 (A-T) IVSII-654 (C-T) Others	25% 19% 13% 6% 37%
China: (93) (Chan et al, 1987)	2-4%	Fr 41-42 (-TTCT) IVSII-654 (C-T) Cd 17 (A-T) -28 (A-G) Others	48% 22% 10% 8% 12%
Malaysia: (?) (Yang et al, 1989)	β-Thal: 1-10% Hb-E: 0-30%	IVSI-5 (G-C) Cd 19 (A-G) Fr 41-42 (-TTCT) Others	49% 15% 12% 24%
Indonesia: (72) Lie-Injo et al, 1989)	β-Thal: 3% Hb-E: 1-8%	IVSI-5 (G-C) Hb-E IVSII-654 (C-T) Others	44% 18% 10% 28%
Blacks: (38) (Gonzalez-Redondo et al, 1988)	1-3%	-29 (A-G) -88 (C-T) Cd 24 (T-A) Others	55% 21% 8% 16%

Pathophysiology of thalassaemia:

(Weatherall and Clegg 1981)

Quantitative reduction of one or more globin polypeptide chains has two major consequences: decreased haemoglobin synthesis and an imbalance between α and non-α chain production. The former is a major determinant of red cell hypochromia, but is of little clinical significance. Unbalanced globin chain production is the major determinant of the clinical outcome. In the absence of a complementary globin chain the free normal chains aggregate and precipitate within the cytoplasm, damage red cell membranes, and lead to premature destruction of erythroid cells. β-chain tetramers seen in α-thalassaemias (Hb-H) are unstable and lack the physiologically useful properties of normal haemoglobin. They tend to oxidize easily and precipitate within erythroid precursors. However, these tetramers remain soluble for a while and therefore result in a clinical syndrome that is not very severe.

In β-thalassaemia deficiency of β-chains result in accumulation of unpaired α-chains. Because of their great instability free α-chains aggregate to form insoluble inclusions in erythroid precursors. The burden of inclusions is sufficient to cause large scale ineffective erythropoiesis. The spleen traps inclusion bearing red cells that enter the circulation, where many incur irreparable mechanical and metabolic injury. In response to the ineffective erythropoiesis and shortened red cell survival there is an enormous increase in marrow activity. Expansion of the medullary cavities by erythroid tissue occurs at the expense of cortical bone.

Clinical and Laboratory features of α-thalassaemia syndromes:

Carriers of single α-gene deletion (-α/αα) are clinically normal. About half have mild hypochromia and microcytosis. Heterozygotes of α-thalassaemia-1 (--/αα) or homozygotes of α-thalassaemia-2 (-α/-α) usually do not have anaemia. They have distinct microcytosis and hypochromia (Huisman 1996). Hb-H disease (--/-α), on the other hand, has clinical picture of chronic haemolytic anaemia of variable severity. The syndrome of hydrops fetalis results from deletion of all 4 α-genes (--/--). The affected fetus is usually delivered prematurely and is either still born or dies shortly after birth (Lukens 1993). The haematological picture of different types of α-thalassaemias is summarized in Table: 1.3. Carrier detection for α-thalassaemia is difficult by routine haematological methods. Gene

mapping by restriction endonucleases or detection by PCR are reliable for detection of α-thalassaemia carriers (Kulozik et al, 1988; Baysal and Huisman 1994).

Table: 1.3. Summary of haematological findings in individuals with α-thalassaemia (Adapted from Higgs et al, 1989).

Phenotype	**No of functional α-genes**	**Hb-Barts at birth (%)**	**Hb-H %**	**MCV**	**MCH**	**α/β chain synthesis ratio**
Normal	4	0	0	85-100	~30	~1.0
α^{+}-thalassaemia trait	3	0-2	0	75-85	~26	~0.9
α^{o}-thalassaemia trait	2	2-8	0 (occasional)	65-75	~22	~0.7
Hb-H disease	1	10-40	1-40 (many)	60-70	~20	~0.4
Hydrops fetalis	0	~80	+	110-120	low	0.0

Clinical and Laboratory features of β-thalassaemia syndromes:

Heterozygous β-thalassaemia (thalassaemia minor) is mostly asymptomatic but causes prominent abnormalities of red cells. A typical case has haemoglobin in the normal or lower normal range, MCV is $\leq$75 fl, MCH $\leq$25 pg, and Hb-A_2 is $\geq$3.5% (Lukens 1993). The most severe form of homozygous β-thalassaemia is recognized as thalassaemia major. The infant is normal at birth but usually within six months of age progressive pallor develops and the patient fails to thrive. In the absence of transfusion therapy haemoglobin concentration slowly falls to 3-5 g/dl and the characteristic clinical picture emerges. The child has a small stature, relatively large head, and moderate to marked hepato-splenomegaly (Modell and Berdoukas 1984). Table: 1.4 summarizes the clinical and haematological features of β-thalassaemia syndromes.

Thalassaemia intermedia:

The term Thalassaemia Intermedia (TI) is used to describe patients with the clinical picture of thalassaemia, which, although not transfusion dependent, is associated with a more severe degree of anaemia than that found in heterozygous carriers for α- or β-thalassaemia (Weatherall 1996). The intermediate forms of thalassaemia show a markedly heterogenous clinical picture. At the molecular level several factors may be responsible for the mild phenotype of thalassaemia that may exist in each patient either independently or in

combinations. These are summarized in Table: 1.5.

Mild β-thalassaemia mutations, for example C-T substitution at -88 (Orkin et al, 1984), C-T substitution at -101 (Gonzalez-Rodondo et al, 1989), and A-C substitution at the Cap site (Wong et al, 1987), when co-inherited with another severe β-thalassaemia mutation are often associated with a mild (intermedia) phenotype. Many of the promoter region mutations in heterozygous state are associated with minimal or no increase in Hb-A_2 level.

Table: 1.4. Clinical and haematological features of β-thalassaemia syndromes (Modified from Lukens 1993).

	Thalassaemia Major	**Thalassaemia Intermedia**	**Thalassaemia Minor**
Severity	++++	++	+,±
Genetics	Homozygous/double heterozygous	Homozygous/double heterozygous	Heterozygous
Splenomegaly	++++	++,+++	+, 0
Jaundice	+	++	0
Bony changes	++++,++	+, 0	+, 0
Haemoglobin	<7 g/dl	7-10 g/dl	>10 g/dl
Hypochromia	++++	+++	++
Microcytosis	+++	++	+
Target cells	10-35%	++	+
Stippling	++	+	+
Reticulocytes	5-15%	3-10%	2-5%
Nucleated RBC	+++	+, 0	0
Hb-F	20->94%	30-100%	1-2%
Hb-A_2	1-8.7%	<1-10.0%	3.5-8.0%

Table: 1.5. Molecular basis of thalassaemia intermedia (Cao et al, 1994).

1. *Mild defects of β-globin chain production*
    a. Homozygosity for mild β-thalassaemia
    b. Compound heterozygosity for mild and severe β-thalassaemia

2. *Homozygosity or compound heterozygosity for severe β-thalassaemia associated with*
    a. α-thalassaemia
    b. Genetic factors enhansing γ-chain production
        Gγ promotor mutation (homozygous Xmn-I polymorphism)
        Heterocellular HPFH

3. *β-thalassaemia due to large promoter region deletions*
4. *Homozygosity for δβ-thalassaemia or its combination with β-thalassaemia*
5. *Double heterozygosity for β-thalassaemia and triplicated α-globin gene*
6. *Heterozygosity for hyperunstable Hb variants (Dominant thalassaemia)*

Co-inheritance of α-thalassaemia in the form of deletion of two genes (--/αα or -α/-α) may raise the MCV and MCH in a β-thalassaemia heterozygote to a normal range (Melis et al, 1983). The severity of homozygous β-thalassaemia is also reduced by co-inheritance of two gene deletion α-thalassaemia (Cao et al, 1994). Co-inheritance of single α-gene deletion (-α/αα), however, affects the phenotype of only β^+-thalassaemia homozygotes (Gringras et al, 1994). Co-inheritance of triplicated α-genes (αα/ααα), on the other hand, may increase the severity of β-thalassaemia (Galanello et al, 1983).

Some patients of typical β-thalassaemia major produce larger quantities of Hb-F that compensates the globin chain imbalance created by lack of β-chain production. Studies in Sickle cell disease (SS) and β-thalassaemia heterozygotes have shown that A-T polymorphism at position -158 relative to $^{G}\gamma$ Cap site (recognized by Xmn-I) is associated with 3-11 fold increase in production per $^{G}\gamma$-globin gene (Gilman and Huisman 1985). Patients of homozygous β-thalassaemia associated with homozygous Xmn-I polymorphism at position -158 usually have a thalassaemia intermedia phenotype (Thein et al, 1987; Gringras et al, 1994).

Management of thalassaemia:

The mainstay of thalassaemia management is regular blood transfusions, iron chelation therapy and provision of other support facilities. A hyper-transfusion regimen that maintains a mean haemoglobin of 12 g/dl is recommended. Hyper-transfusion allows normal growth and physical activity, reduces marrow hyperplasia and the associated bony abnormalities and prevents splenomegaly and hypersplenism. The main disadvantage of hyper-transfusion is the accumulation of excess iron in the body that damages many organs including heart, liver and endocrine glands (Cao et al, 1992). Iron that accumulate over a period of time due to repeated blood transfusions and increased absorption from the intestine needs to be removed by regular use of iron chelators. Desferrioxamine is the most extensively used iron chelator (Giardina and Grady 1995). Its main disadvantage, other than the high cost, is a continuous subcutaneous route of administration. An oral iron chelator L1 is also available but the associated risk of agranulocytosis has limited its use (Brittenham 1992).

Splenectomy is considered for patients who require more than 200 ml of packed red cells/kg/year (Piomelli 1995). Thalassaemia patients on regular treatment protocols also require hormone replacement therapy (Kattamis and Kattamis 1995). Allogenic bone marrow transplantation is the only curative treatment available at present. Success rate of over 92% in selected cases has been achieved at some centres (Lucarelli et al, 1995). Reactivation of fetal haemoglobin production by 5-azacytidine or hydroxyuria may have some benefit in patients of thalassaemia intermedia (Olivieri 1996). The prospects of gene therapy, for the present, appear to be remote (Beuzard 1996).

The intermediate form of thalassaemia requires careful surveillance. The patients should be watched carefully in early childhood, and if there are signs of growth retardation or increasing bone deformity, they should be put on regular transfusions (Cao et al 1992). Patients of thalassaemia intermedia, who are kept off transfusion, usually develop splenomegaly that becomes a source of pooling of blood. In such patients splenectomy can be beneficial.

Thalassaemic children who are not given any treatment usually die before two years of age. Those who get adequate treatment reach their adult life. The prognosis of children who are inadequately treated is poor.

Prevention of thalassaemia:

Although thalassaemia patients can be managed to a great extent by regular blood transfusions and other supportive measures, the high cost of treatment creates severe health burden in a community where the disorder is common. The burden has gradually increased because of the rise in life expectancy following introduction of modern supportive measures (Angastiniotis et al, 1986). Nowadays, therefore, a fundamental aspect of the management of these disorders is prevention. Several thalassaemia prevention programmes in the Mediterranean have shown that the birth rate of new thalassaemics can be brought down to almost zero (Cao 1987). The cardinal features of a thalassaemia prevention programme include (1) public education, (2) population screening, (3) genetic counselling, and (4) prenatal diagnosis (Fig 1.4).

Prerequisites:

The planning and organization of a comprehensive thalassaemia prevention programme has certain prerequisites. Political will and commitment by the government who is also the public health planner is essential. A basic situation analysis at the National level is necessary to determine priorities. In the absence of reliable epidemiological data preliminary information on trends and hospital statistics may be useful. The funding resources may be made available from governmental as well as non-governmental sources (Alwan and Modell 1997).

Public education:

All programmes for prospective control of thalassaemia in the Mediterranean had an intensive awareness campaign and involvement of population. Public education was achieved by means of mass media, posters, informative booklets, and by special teaching on thalassaemia in schools. More personal approaches through lectures and discussions with the community leaders of the population were very useful. The health care workers and the paramedical staff played a major role in implementation of the preventive programmes (Cao 1987). In addition to the public awareness campaign it is also essential to create an awareness amongst the state authorities about the possibility of an ever increasing expenditure on new cases of thalassaemia if no control measures are taken (Loukopoulos 1996).

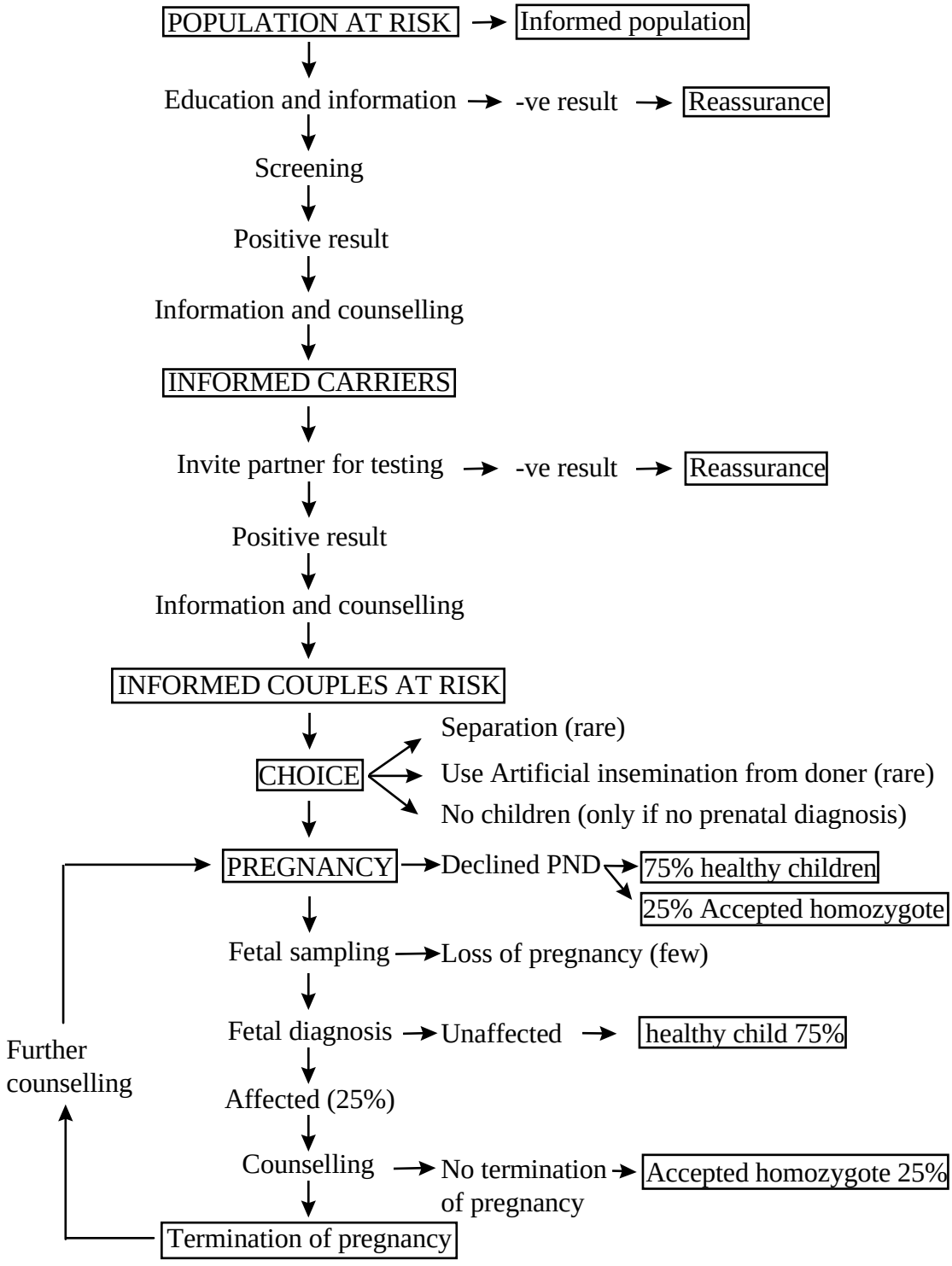

Fig: 1.4. An outline of the basic principles of thalassaemia prevention including public education, carrier screening, genetic counselling, and prenatal diagnosis (Based on Petrou et al, 1990).

Carrier screening:

Programmes designed to reduce the frequency of β-thalassaemia major births include the couples who already have an affected child or who are in their first pregnancy (WHO 1983). Retrospective diagnosis i.e. identification of a couple after the birth of an affected child, although very useful for individual couples, has only a limited effect on the number of affected children in the community (Cao 1987). The retrospective approach, however, may be useful in communities where final family size is large because a cessation of reproduction can reduce the birth rate of new thalassaemics to almost 50% (Alwan and Modell 1997). Initial programmes in the Mediterranean involved parents with an affected child and prospective diagnosis i.e. identification of the couples at risk before giving birth to an affected child, was instituted at a later stage (Cao et al, 1981). The choice of testing varies between premarital or at marriage as in Greece (Fessas 1986), mandatory screening of couples before marriage as in Cyprus (Angastiniotis et al, 1986) screening in the antenatle clinics as in the UK (Petrou 1994), or school leavers as in some areas of Italy (Bianco et al, 1985). An obvious disadvantage of screening during pregnancy is the finding of a couple at risk when the pregnancy is already advanced. Late testing may produce dramatic emotional stress on couples.

Screening the relatives of known carriers can also be an efficient approach for detecting thalassaemia. The approach can identify a large number of carriers (Mouzouras et al, 1980; Cao 1987). A useful and cost effective strategy adopted by several programmes is to test one member of a couple, the other member being tested when the first is found to be a carrier.

The carrier detection methods should be able to pick up β-thalassaemia trait, as well as δβ-thalassaemia, γδβ-thalassaemia, Hb-S, Hb-E, and Hb-C which, when co-inherited with β-thalassaemia, may result in thalassaemia syndromes. The methods should also be able to identify silent β-thalassaemia alleles and co-existing α- and β-thalassaemia trait (Cao et al 1994). A scheme (Fig 1.5), that can identify almost all carriers, should include red cell indices, haemoglobin electrophoresis, Hb-A_2 and Hb-F estimation, and globin chain synthesis studies (Modell and Berdoukas 1984). Molecular genetic studies can supplement the identification of silent β-thalassaemia carriers and those with co-existing α-thalassaemia (Cao et al, 1994).

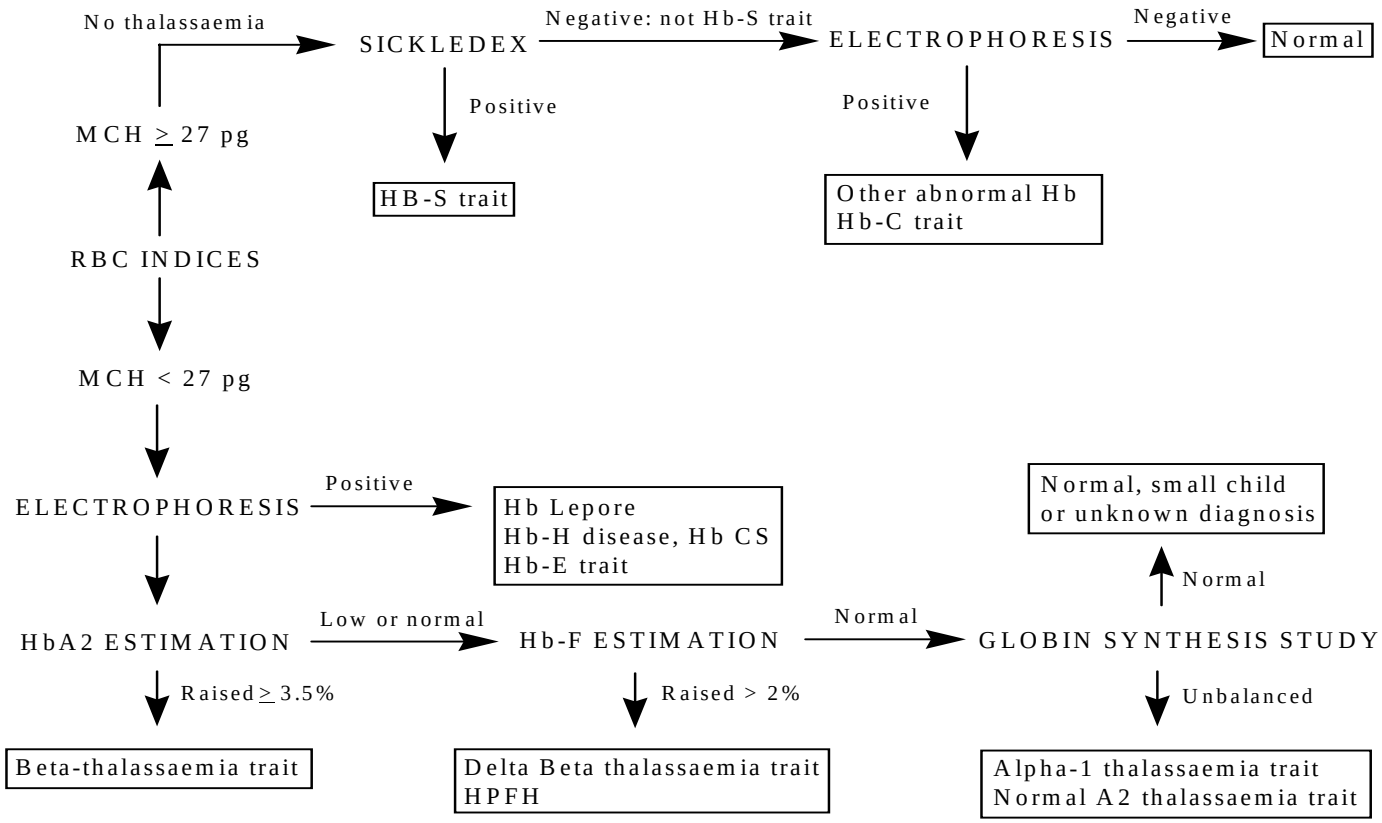

Fig: 1.5. Flow chart for identification of carriers of a haemoglobin disorder (Modell and Berdoukas 1984).

Data from various populations suggest that MCH is a more sensitive indicator than MCV for identifying unusual forms of β-thalassaemia (Modell and Berdoukas 1984). A cut off limit for MCH below which Hb-A_2 estimation should be done to confirm the diagnosis of thalassaemia trait may be 27 pg. An important diagnostic problem, especially in the Asians, arises from the high incidence of iron deficiency that is the commonest cause of severe microcytosis. Hb-A_2 level in β-thalassaemia trait with concomitant iron deficiency, however, remains in the higher range unless severe anaemia is present (Cao et al, 1994).

In large scale population screening for thalassaemia in a developing country red cell indices may be replaced by a cost effective one tube osmotic fragility test as a screening method and confirmation by Hb-A_2 estimation (Kattamis et al, 1981; Bianco and Silvestroni 1983).

Genetic counselling:

Genetic counselling aims at replacing misunderstandings about the cause of genetic disease with correct information and increasing people's control on their own and their family's health by informing them of the resources available for diagnosis, treatment and prevention. The basic principles of genetic counselling include autonomy of the individual or couple, right to complete information and highest standard of confidentiality (Harper 1993). The main components of genetic counselling are correct diagnosis in the presenting family member, explanation of the nature prognosis and treatment of the disorder, estimation of genetic risk for parents and family members, the options for avoiding genetic risks and supporting the individual or couple in making the right decision. The ethical principles governing genetic counselling need to be reviewed in the light of social and religious structures particularly in relation to the issues of prenatal diagnosis, and counselling in relation to customary consanguineous marriage (Alwan and Modell 1997).

Counselling is generally based on a private interview with the individual carrier or the couple. It should be non-directive and any mandatory measures restricting the individual freedom should be avoided because this may have negative effects (Angastiniotis and Hadjiminas 1986). In countries where high-risk ethnic minorities exist, in the UK for example, it may be difficult to provide adequate counselling because of problems of language, culture, and trust (Modell and Berdoukas 1984). In a situation like this the services of a trained counsellor with the same cultural religious and linguistic background

can be of great help (Petrou et al, 1990).

Prenatal diagnosis:

The basic objective of prenatal diagnosis is to give an informed choice to the at risk couple (Fig 1.4) (Petrou et al, 1990). The affected pregnancies are terminated only on the parent's request, the main consideration being for the future and quality of life of the child. The concept of prenatal diagnosis and termination of pregnancy has been accepted only slowly over the past 25 years and there is ongoing debate about its ethical and social implications. The decision whether or not to terminate an affected pregnancy is never taken lightly even in countries where prenatal diagnosis is fully accepted (Alwan and Modell 1997). Thalassaemia preventive programmes carried out before prenatal diagnosis was available and thus based solely on heterozygote detection and genetic counselling have not been effective in reducing the birth incidence of new cases of thalassaemia major (Barrai and Vullo 1980a). The introduction of prenatal diagnosis (Kan et al, 1975) gave a new dimension to thalassaemia prevention.

Prenatal diagnosis was initially done by globin chain synthesis ratio on fetal blood obtained in mid trimester gestation by fetoscopy or placental aspiration (Fairweather et al, 1978) and then by cordocentesis (Cao et al, 1986). With the development of sophisticated ultrasound scanners the practice has now shifted to first trimester fetal sampling by chorionic villus sampling (Ward et al, 1983). The rate of miscarriage with chorionic villus sampling is less than 1% (Centre for Disease Control and Prevention, Atlanta 1995). The laboratory diagnosis is now carried out by DNA analysis. Polymerase Chain Reaction (PCR) has simplified the process of mutation detection in the fetal tissue (Old et al, 1990).

In-vitro fertilization and embryo transfer has also enabled diagnosis to be carried out on just a few cells obtained from the embryo prior to implantation (Monk and Holding 1990). However, the present method of pre-implantation diagnosis are cumbersome, expensive and experimental, but if successful may be made acceptable reliable and simple (Alwan and Modell 1997).

Evaluation of thalassaemia control programmes:

The results of prevention programmes in various Mediterranean populations are

summarized in Fig: 1.6. The best results have been achieved in Cyprus where the incidence has declined by almost 97%. This has been helped by a small size and population of the country, high standard of living, high literacy rate, low birth rate, high standard of health care and helpful attitude of the Church in endorsing premarital screening (Angastiniotis et al, 1986). In the UK, where the programme is active since 1977, the birth rate of thalassaemia major has fallen to just 50% of the expected. In the UK screening and counselling is well delivered to British Cypriots, but very inadequately to British Asians particularly Pakistanis (Modell et al, 1984). An important reason for the low acceptance rate for prenatal diagnosis amongst British Pakistanis and Bangladeshis is that they are referred late in the second trimester and many then refuse prenatal diagnosis. Due to the availability of first trimester diagnosis the acceptance rate of prenatal diagnosis has improved substantially in these communities (Petrou et al, 1990). Almost 80% of Pakistani couples referred in the first trimester now request prenatal diagnosis (Petrou 1994). Unfortunately, many such couples are identified late as the screening is done in antenatal clinics.

Cost effectiveness of thalassaemia prevention:

Cost analysis of prevention versus treatment, done in many Mediterranean countries and the UK, shows that the cost of prevention is equivalent to treating the affected new born for just one year (Ostrowsky et al, 1985; Cao 1987). Table: 1.6 shows six possible policies for thalassaemia (Alwan and Modell 1997). A comparison of the cost of the six policies in a population (Fig 1.7) shows that the policy involving best possible patient care, plus premarital and antenatal "prospective" carrier screening and genetic counselling, with availability of prenatal diagnosis is the most cost effective.

DNA analysis for thalassaemia:

In the mid 1970s, two revolutionary techniques became available that radically simplified the analysis of DNA structure. The first evolved from the discovery of DNA cleaving enzymes called restriction endonucleases which could cut a DNA molecule only at specific sequences (Weinberg 1985). The other technical revolution came with the ability to sequence DNA (Sanger et al, 1977). The discovery of Polymerase Chain Reaction (PCR) in the mid 1980’s has further simplified the analysis of DNA (Mullis and Faloona 1987).

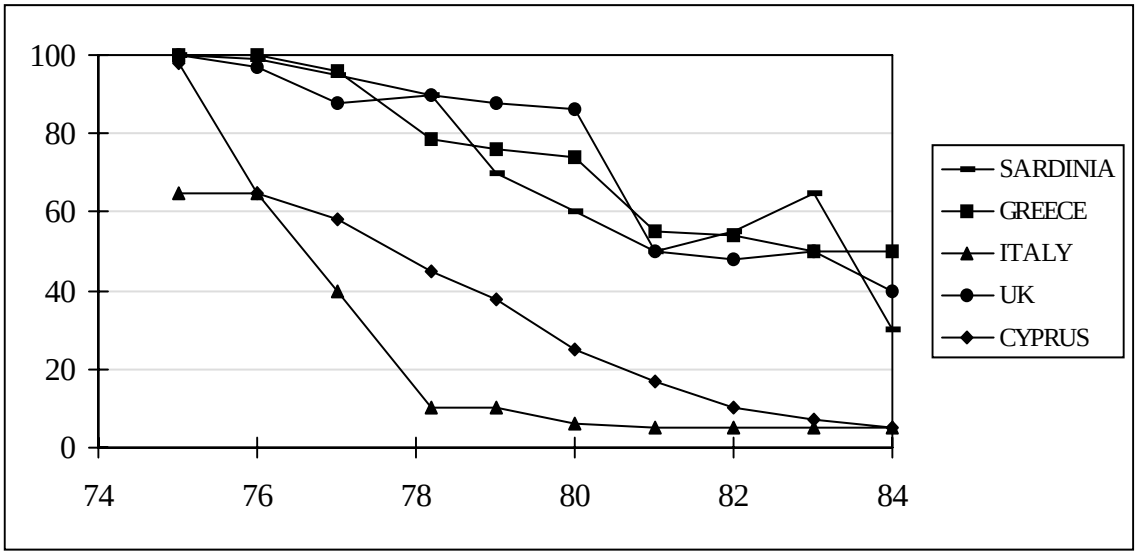

Fig: 1.6. Fall in the birth rate of infants with thalassaemia major associated with four national thalassaemia prevention programmes (Based on Cao 1987).

Table: 1.6. Six possible policies for thalassaemia. Policies 1-5 are currently in existence in different countries of the Eastern Mediterranean Region (EMR) (Alwan and Modell 1997).

Policy 1	A "baseline situation" in which no treatment, counselling or prevention is available. (Until recently this was the situation throughout most of the Middle East, but there has been rapid progression towards “policy 2” in many countries during the last 10 years.)
Policy 2	The best possible patient care, plus "retrospective" genetic counselling after the first affected child is diagnosed. (This is now common in many countries of the Region.)
Policy 3	The best possible patient care, plus "retrospective" genetic counselling, plus the option of prenatal diagnosis in subsequent pregnancies. (Available for a limited number of families in the Region at present.)
Policy 4	The best possible patient care including retrospective genetic counselling, plus “prospective” (premarital) carrier screening and counselling, but no prenatal diagnosis. This is in place in some countries of the EMR.
Policy 5	The best possible patient care, plus premarital and antenatal "prospective" carrier screening and genetic counselling, with availability of prenatal diagnosis. At present, the only country of the Region implementing this policy is Cyprus.
Policy 6	The best possible patient care, plus premarital, and family- and population-based “prospective” carrier screening, plus genetic counselling, but no prenatal diagnosis.

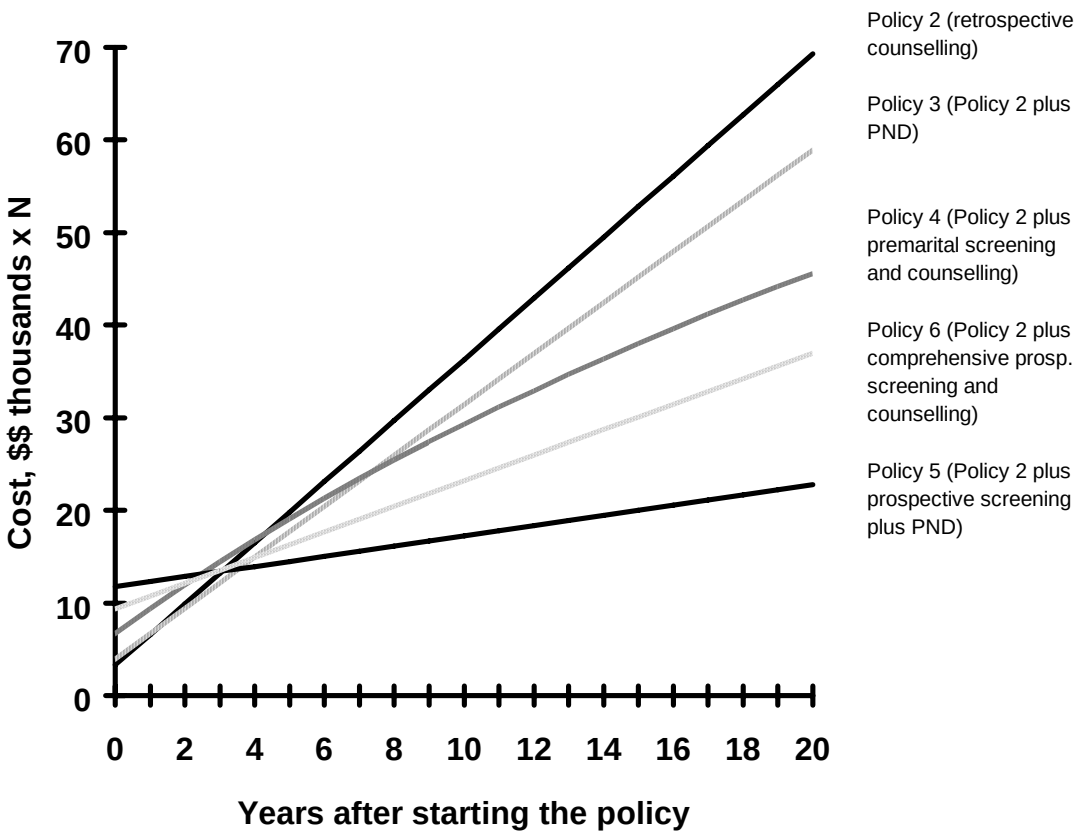

Fig: 1.7 Projected evolution of total costs of treating and preventing thalassaemia over the first 20 years, with the six different policies described in Table: 1.6 (Alwan and Modell 1997).

Southern blotting:

Localization of particular sequences within genomic DNA can be accomplished by the transfer technique of Southern (1975). Genomic DNA is digested with one or more restriction enzymes and the resulting fragments are separated according to size by electrophoresis through an agarose gel. DNA is then denatured in situ, transferred to nitrocellulose filter or nylon membrane and is then hybridized to radiolabelled probe. Southern blotting has been extensively used in the past for analysis of gene defects in thalassaemia (Boehm and Kazazian 1989).

Restriction Fragment Length Polymorphism (RFLP):

The term RFLP denotes a variation in fragment sizes when genomic DNA from different individuals is cleaved with the same restriction enzyme. There are several polymorphic sites

that are closely linked to the β-globin gene (Antonarakis et al, 1985). These polymorphic sites can be recognized by the use of restriction endonucleases (Fig: 1.8) which cleaves double stranded DNA at a specific sequence (Varawalla et al, 1992).

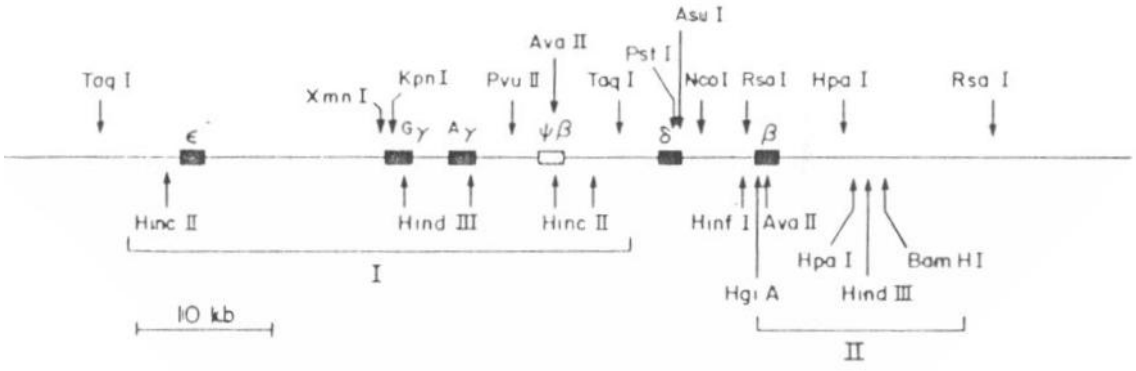

Fig: 1.8 Restriction endonuclease site polymorphisms in human non-α globin gene cluster. The sites shown below the line have been most intensively studied and are commonly used to define chromosomal haplotypes in genetic analyses. Polymorphisms at the sites encompassed in brackets I and II are usually in linkage disequilibrium with one another within each bracketed area, but random recombinations or associations occur between polymorphisms of group I and those of group II (Bunn and Forget 1986).

Many β-thalassaemia mutations can also be identified by restriction enzymes (Thein and Weatherall 1988). RFLP analysis can be used for prenatal diagnosis of thalassaemia by identifying the restriction sites closely linked to the β-globin gene (Old et al, 1986a). A disadvantage of the linkage based diagnosis is that a meiotic recombination between the polymorphic site and the β-globin gene can be a potential source of error (Old et al, 1986b). Requirement of a previously affected or normal child in the family or the grand parents, lack of informativeness of the marker sites in some families (Antonarakis 1989) and the presence of a mutation outside the β-globin gene cluster e.g. in the locus control region (Kazazian et al, 1990) are other disadvantages.

Genomic sequencing:

Sanger et al, (1977) described a method of determining the sequence of unknown DNA. The method is based on the principle that the inhibitory activity of 2',3'-dideoxythymidine triphosphate (ddTTP) on DNA polymerase-I is incorporated into the growing oligonucleotide chain in the place of thymidylic acid (dT). Because the ddT contains no 3'-hydroxyl group, the chain cannot be extended further, so that termination occurs

specifically at positions where dT should be incorporated. If a primer and template are incubated with DNA polymerase in the presence of a mixture of ddTTP and dTTP, as well as the other three deoxyribonucleoside triphosphates, a mixture of fragments, all having the same 5' end with ddT residue at the 3' ends, is obtained. When this mixture is separated by electrophoresis on denaturing polyacrylamide gels the pattern of bands shows the distribution of dTs in the newly synthesized DNA. By using analogous terminators for the other nucleotides in separate incubation reactions and running the samples in parallel on a gel, a pattern of bands is obtained from which the sequence of DNA can be read.

Sequencing of DNA is an extremely useful tool in identification of an unknown gene defect. Currently, sequencing is carried out on fragments of either double stranded or single stranded DNA that are amplified by Polymerase Chain Reaction (Engelke et al, 1988; Rao 1994).

Polymerase chain reaction (PCR):

Polymerase Chain Reaction (PCR) is an in-vitro technique that allows amplification of a specific DNA region (Mullis and Fallona 1987). The amplification of DNA is achieved by using oligonucleotide primers. These are short, single stranded DNA molecules that are complimentary to the ends of a defined sequence of the DNA template. The primers are extended on single stranded denatured DNA (template) by heat stable DNA polymerase in the presence of deoxynucleoside triphosphate (dNTPs) under suitable reaction conditions. This results in the synthesis of a new DNA strand complementary to the template strand. DNA polymerase is used to synthesize new strands of DNA in successive cycles of heat denaturation of the template, annealing of primers by cooling the mixture and primer extension at a temperature suitable for the enzyme. Each cycle of amplification creates new DNA strands that become templates for the next cycles.

PCR products consist of fragment(s) of DNA of size(s) defined by the boundaries of the PCR primers. The simplest and most commonly used method of PCR product analysis is its electrophoresis on agarose or polyacrylamide gels. DNA is a negatively charged molecule and migrates towards the positive electrode when it is placed in an electrical field. This movement is inversely proportional to the molecular weight of DNA. Once separated by electrophoresis, DNA can be visualized by staining with ethidium bromide

or silver nitrate (Newton and Graham 1994).

PCR is an extremely sensitive technique and in about 20 cycles there will be 2^{20} fold amplification. Therefore the commonest source of error in PCR involves contamination at the pre PCR step (Newton and Graham 1994). It is important to include negative controls and reagent blanks with every batch of reactions. Other useful measures to avoid contamination may be the physical isolation of PCR reagents and products, autoclaving solutions, avoiding splashes, use of positive displacement pipettes and aliquoting reagents (Kwok and Higuchi 1989).

The basic technique of PCR has been used in several approaches that identify known and unknown gene defects. These include Amplification Refractory Mutation System (ARMS), dot blot, reverse dot blot, Denaturing Gradient Gel Electrophoresis (DGGE), Single Strand Conformation Polymorphism (SSCP) and chemical cleavage of mismatches (Newton and Graham 1994).

Amplification refractory mutation system (ARMS):

In PCR the yield of reaction is critically dependent on a perfect match between the 3' end of the primer and the template DNA. A primer designed to match the change encountered in a point mutation will only amplify the mutant sequence and similarly the primer for a normal sequence will amplify only the normal sequence and not the mutant sequence (Newton et al, 1989). Kowk et al, (1989) have shown that additional mismatches at position -3 or -4 from the 3' end of the primer can further improve the specificity of the reaction. They also demonstrated that A:G, C:C and to some extent G:G mismatches gave the best results. Studies by Old et al, (1990) and Varawalla et al, (1991) have proven the efficacy of ARMS method in detecting β-thalassaemia mutations.

Denaturing gradient gel electrophoresis (DGGE):

The method is based on the principle that electrophoretic mobility of a double stranded DNA molecule through linearly increasing concentration of denaturing agents is retarded by its denaturation (Myers et al, 1987). As the DNA fragment proceeds through the gradient gel, it will reach a position where the concentration of the denaturing agent equals the melting temperature (T_m) of its lowest melting domain. This results in marked retardation of

its electrophoretic mobility. The T_m of a melting domain is dependent on its nucleotide sequence. Therefore, when DNA fragments, that differ by a single nucleotide change in their lowest melting domain, are electrophoresed through denaturing gradient gels, branching and consequent retardation of their mobility will occur at different positions along the gel. This allows their separation. The DGGE can not resolve DNA fragments differing by base changes located in the highest T_m domains. This problem can be overcome by attaching a G-C rich domain (40-45 bases) into the fragment to be analyzed (Myers et al, 1985). The G-C rich domain has a high T_m and it prevents complete denaturation of the DNA molecule. The PCR-DGGE combination is a very useful tool for detection of single neucleotide changes in DNA (Fodde and Losekoot 1994).

Dot blot and reverse dot blot:

The technique of dot blot involves immobilization and denaturation of PCR products to a membrane, such as nitrocellulose or nylon, followed by hybridization with the allele specific oligonucleotides. Using stringent conditions an oligonucleotide that is fully complementary to one allele will hybridize to that allele only. The other allelic variants will, similarly, be hybridized by its specific oligonucleotide probe (Amselem et al, 1988).

The reverse dot blot is similar to dot blot but the process is reversed i.e. allele specific oligonucleotides are immobilized onto a membrane and hybridization is then carried out using a radioactive or biotinylated PCR product. It offers the advantage of simultaneous analysis of several mutations in a single test (Newton and Graham 1994).