Hemoglobinopathies in Iran : molecular spectrum, prevention and treatment.

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Chapter I

The origin of the Iranian ethnic groups

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The evolution and genetic variability of all life forms is associated with their origin and their habitat. In mankind this is not only evident from the visible ethnical and cultural differences but in particular from the differences and specificity of DNA mutations and polymorphisms as well.
Mutations inducing hemoglobinopathies are one of the classic examples of the association between specific populations and their habitat, the last factor providing the selective pressure (malaria), the first, and the specific mutation. Originating from particular individual and being common in an ethnic group, mutations will eventually migrate and be positively selected in the presence of malaria and ultimately become predominant in the population.
Since the knowledge of the mutation spectrum present in a population is essential for the molecular diagnosis and prevention of hemoglobinopathies, the knowledge of the ethnical background of a particular area is equally important. The more heterogeneous the ethnical background is, the larger the molecular spectrum and the higher the variability in frequency with which mutations occur between sub populations. Therefore the history of hemoglobin mutations and the history related to the formation of the predominant Iranian ethnicities are briefly summarized in this chapter.

The history of hemoglobin mutations

Hemoglobin mutations are in fact as old as the history of evolution. The actual Thalassemia and Sickle Cell Disease (SCD) mutations are supposed to go back for perhaps 10 to 25.000 years. However, the selection of these mutations (including G6PD) by malaria is supposed to start with the beginning of agriculture or at least when the cohabitation between human and anopheles (which transmits the parasite *Plasmodium*) reached a sufficient population density of both the guests and the parasite.

The prehistoric period

Remains of both Homo Sapiens Neanderthalensis and Homo S. Sapiens have been found in coexistence in multiple areas of the Middle East. It is generally assumed that modern man has been evolved in this area since about 100.000 years ago. For millennia multiple migration flows on the way to Europe, Asia and America, have passed through the “Middle East” that would evolve into the birthplace of agriculture and the “cradle of human civilizations”. On the way to the east, ancient populations in Iran would have become influenced by the Neolithic agriculture born in Mesopotamia but very strongly keeping the nomadic pastoral culture coming from central Asia which is still found in the actual tribal populations. Peoples living at a low population density with either agricultural or nomadic cultures must have undergone very little malaria selection. As shown in the Ferrara area (Italy), in densely populated malaria regions the selective pressure may amplify thalassemia carrier frequency from 0.005 to 0.2 in 20 generations. Thus we must assume that malaria selection would have started only when significant population densities would have risen in the first large communities in historic time.

A recent historic summary

Shortly before the 18th century BC, Indo-European pastoral nomadic populations migrated to the Iranian plains. These shepherds called themselves Aryans, a word that in Sanskrit means noble, and gave their name to the new land "land of Aryans" or Iran. Aryans kept coming and spreading in the area until the 10th century BC, contributing to the Mesopotamian cultural mix. Independently from the predominant group they became known by different names such as Medes, Iranians, and Persians. During the rule of Ciro the Great (559-530 BC), Persians reached as far west as Asia Minor, Greece, Egypt, and as Far East as present-day Afghanistan, Turkestan and part of India.
Towards the end of the 4th century BC, this vast empire fell into the hands of Alexander the Great. Alexander's successors, the Seleucid dynasty, lost hold over the eastern part of the empire to the Persian Arsacid dynasty (2nd century BC to 3rd AD). Between the 4th and the 7th century AD Persians were constantly at war with Romans and Byzantines but remained independent under the Sassanid dynasty.
The Arab conquest covered the region in 641. Unlike most other provinces of the Arab Empire, Persians retained their own language, arts and literature. With the fall of the Caliphate of Baghdad in 874 AD, Persia attained virtual independence, first under the descendants of Tahir, the last Arab viceroy, and later under the Seleucidian Turkish and the Persian dynasties.
The Mongol invasion began in 1258 and brought dynastic conflicts making in the end way for the Persian ruler Ismail Shah, whose grandson Abbas I (1587-1629) succeeded in uniting the whole country. He expelled the Turks from the west, the Portuguese from the Hormuz region, and also conquered part of Afghanistan. From that time on a country that in some period extended from Syria to North India was virtually ruled by Iranian dynasties until 1925. The rising of democratic movement conduced to a constitutional monarchy and to the Pahlavi dynasty, which lasted for about 50 years until in 1979 the Islamic Republic was established.

The formation of the present ethnicity in Iran

All major historic events have generated the complex variety of ethnic groups living in various parts of modern Iran. **Persians, Azeri, Gilaki and Mazandarani,** for as far as one can define them as homogeneous groups, they represent 83% of the population. The remaining most important ethnic minorities in Iran can be associated with their specific history, culture, customs, and language. The Kurds, the Baluchis, the Arabs, the Bakhtiyari, the Qashqaie, the Turkmans, Lurs and Assyrians represent these minorities.
The following list is a general outline of the major and minor ethnic groups in modern Iran

The Persians

Persia is derived from the word Pars, or Persis, a word already adopted by the ancient Greeks defining the great Persian Empire as a vast geographical and cultural domain. The term "Persia or Persian" is derived from the major ethnic group still living in central Iran where the city of Shiraz and the province of Fars are. Persians represent about 50 % of the population in the country and can be considered as the most autochthonous population in Iran. Persians are almost exclusively Shia' Muslims.

The Azeri (Turk)

There are two streams of opinion concerning the origin of Iranian Turks. The first maintain that they are the descendants of the Turks who either migrated to Iran in the 7th and 11th centuries (A.D.) or invaded parts of Iran at various stages. The second claims that they are original inhabitants of Iran on whom the invaders have imposed their languages throughout centuries of occupation. The Iranian Azeri (also called Turks) live mainly in the north west of Iran in the Eastern and Western Azerbaijan and Ardebil provinces (capitals Tabriz, Urumiyeh and Ardebil respectively). Other Azeri are mostly scattered throughout many region of Iran such as the Zanjan province up to Qazvin, in and around Hamedan, in Tehran, around Qom and Saveh, Khorasan province. Some of the central and southern ethnic groups, the Qashqaie for example, are Turkish speaking but are not Azeri.

The Turkish language spoken in Iran is associated with the language spoken in the Caucasus, but it has undergone different developments in several regions. The dialect spoken in both the Azerbaijan province in Iran and in the Republic of Azerbaijan is Oghuz, which is the mother tongue of the Iranian Turks. The Oghuz dialect however has two accent groups, the northern spoken in the Azerbaijan Republic and the southern in Iran. The language, culture and customs differences among Azeri are significant. Although the Turks are thought to be the largest non-Farsi speaking ethnic group in Iran (24%), they cannot be considered as a homogeneous entity, however, virtually all Azeri are Shia' Muslims.

The Gilaki and Mazandarani

Gilaki and Mazandarani are two groups of the same population presumed to originate from a mixed ethnicity of Caspian and Kadussi with the Aryan. They differ in name because they live in Gilan and Mazandran, two different provinces on the western and eastern coast of the Caspian Sea respectively. They represent together about 8% of population in Iran. Their predominant religion is Shia' Muslims.

The Kurds

The Kurds are an ethnical entity scattered in different countries. Kurdistan, their original territory, is divided between southeast Turkey, northeast Iraq, northwest Iran, south Russia and part of Syria.

Iranian Kurds mainly reside in Iranian Kurdistan, Kermanshahan, and south of the Western Azerbaijan province. Part of them also lives in the north of Khorasan province. Kurds speak a Northwest Iranian language and have several dialects. By the mid-1970s, fewer than 15 percent of all Kurds were nomadic but migration of rural Kurds to the Kurdish cities as Kermanshah, Sanandaj, and Mahabad, as well as to larger Iranian towns is ongoing. In total about 4.2 million Kurds are living in Iran. After, Persians, Azeri, Gilaki and Mazandarani, Kurds are the fifth most important ethnic group in the country and account for about 7% of the total population. Most Kurds follow the Sunnis Muslim teaching, some of them are Shia's, and some are followers of the Yazidi and Ahle-Haq Muslims sects. Followers of Qaderi and Naqshbandi Muslim Sufism are also common in some parts of the Iranian Kurdistan, particularly in its southern regions. A minority of Kurds are adherents to Judaism.

The Baluchis

Baluchis are nomads of unclear origin that populated Central Iran until in the 11th century A.D. they moved to the South and Southeast region called today Baluchistan.
Baluchis speak their own language, a west Iranian language with two branches of northern (Sorhadi) and southern (Makrani) and live under a tribal system.
Most of the principal Baluchi tribes live at the Iranian border with Pakistan and Afghanistan. They include the Yarahmadzai, the Nauri, the Gomshadzai, the Saravan, the Lashari, and the Barazani tribes. Along the coast of the Gulf of Oman live the important tribes of Sadozai and Taherza. A few tribes in the Sistan area are also regarded as Baluchi, but they speak Sistani an abandoned the Persian dialect.
The Baluchis population was estimated to be 600,000 in Iran in the mid-1980s but according to the recent census, it is estimated that, they are about 1.2 million. They are part of a larger group, living in an area which includes the Baluchestan Province in Pakistan and areas in southern Afghanistan. About half of the Baluchis are semi nomadic or nomadic, the rest are settled farmers or live in towns of their triads. Iranian Baluchis represent about 2% of the Iranian population and are mostly of the Hanafi sect of the Sunni Muslim faith.

The Arabs

Arabian tribes migrated in the early 6^{th} centuries AD, probably moving in from the Arabian Peninsula to Khuzistan, in southwest Iran where they now still live. Arabian tribes are scattered in an areas between the Arvandroud (Shatt al-Arab) and the Persian Gulf, to the west of the Bakhtiyari territory, and some of them have inter-mixed with the Bakhtiyari tribe. Arabs have retained the Arabic language and many of their tribal customs.
The largest Arab tribe in Iran is the Bani-Kaab. Its numerous clans inhabit the Minoo Island, Khorramshahr, and Shadegan on both sides of the Karun River, the city of Ahwaz, west and south of the Dezful River and between the Dezful and Shushtar rivers. Other important tribes are: the Bani-Lam, Bani-Saleh, Bani-Torof, Bani-Tamim, Bani-Marvan, the Al-Khamiss, the Bavi and the Kenane.
In the population census that has been taken before the first Iran-Iraq war (1976) the size of the Arab ethnic groups in Iran population was estimated at about 300,000 individuals but according to the latest population census (2002) 1.8 million Arabs live in the South-West provinces representing about 3% of the Iranian population.

The Bakhtiyari

The general opinion is that the Bakhtiyari are probably of Kurdish origin. The Bakhtiyari tribe is composed of clans living in the mountain regions between the Chaharmahal, Fars, Khuzistan and Lurestan provinces.
The tribe is divided into two separate branches: Haft Gang and Chahar Gang. The former consist of 55 and the latter of 24 clans. Arab and Lur clans (see below) have mixed with the Bakhtiyari tribe. The Bakhtiyari tribes represent less than 1% of the Iranian population and are also called the Great Lur. Most of the Bakhtiyari are Shia' Muslim.

Turkmans

The Turkmans are an ethnic minority who speak the same Turkish-based language spoken in the Republic of Turkmenistan. They live in the Turkmen Sahra and in Gorgan, a fertile plain near the border with the Republic of Turkmenistan.
Iranian Turkmans have been living in Iran since 550 AD, but they started forming tribes from 750 AD. They are the descendants of Central Asian Turks, who retained their ethnic identity during the Mongol invasion.
The most important Iranian Turkmen tribes are Kuklans and Yamotes. The Kuklans have six branches, and live in the central and eastern Turkmen Sahra. The Yamotes have two large clans, the Atabai and Jaafarbai, and live in the west of Turkmen Sahra.
The Turkmen population is estimated to be around one million (less than 2 % of the Iranian population. The highest concentration of Turkmans will be found in the town of Gonbad Kavus, which is the center of Turkmen Sahra, Bandar Turkmen, Aq-Qala, and Gomishan. The largest group of Turkmen Muslims follows the Hanafi sub-stream of the Sunni Muslim, but some Turkmans are followers of the Naqshbandieh Muslim Sufism.

Lures

Lur is the name of an ethnic group living in the mountainous areas of the southwest Iran (Lurestan province). Lures appear to be of the same ethnic origin as the Kurds.
The Luri language is close to Kurdish. There are four main Lur groups: the Bala Garideh, Delfan, Selseleh and Tarhan. The Bala Garideh are the genuine Lur who are divided into important tribes such as Dirakvand, Janaki, Amaleh, Sagvand, etc. Most Lures (about 2% of the Iranian population) are Shia's Muslim.

The Qashqaie

According to the general opinion consider the Qashqaie population is considered as Turkish speaking descendent of the Khalaj clan, who lived between India and the Iranian Sistan, and migrated to central and southern Iran. The actual Qashqaie territory extends from Abadeh and Shahreza in the Isfahan province to the Persian Gulf coast.
The Qashqaie emerged as an ethnic entity in the eighteenth century and became one of the best-organized and most powerful tribal confederations in Iran. At present the Qashqaie population counts about 250,000 people divided in numerous clans. The major ones are the Kashkooli, Sheesh Blocki, Khalaj, Farsi Madan, Safi Khani, Rahimi, Bayat, and the Darreh Shuyee.
The Qashqaie are still nomads moving their herds between summer and winter pastures in the south and the north of Shiraz. Since the mid-1960s, many Qashqaies have settled in villages and towns.
The majority of Qashqaies are following the Shies Muslim faith.

The Assyrians

Assyrians are East Syrian Christian communities in Iran. The ancient name "Assyrian" derived from that of the god Assur, designed the Semitic population of north Mesopotamia and capital city. This Christian group speaks modern dialects of the Assyrian, an Aramaic language that evolved from old Syrian. Language and religion provide a strong cohesive force and give the Assyrians a sense of identity with their coreligionists in Iraq, in other parts of the Middle East, and also in the United States. Less than 100,000 Assyrian live in Iran.

The nomadic life in general

Historic and political factors have been influential in keeping nomadic life patterns in Iran. Political instability, conflicts between local rulers, heavy tax collection campaigns of urban rulers at the time of financial problems, etc. Hence, the nomads earn their living principally by raising livestock taking a safe distance from the administrative control. A census completed in 1987 estimated the total population of nomadic tribes more than 1.5 million in almost 200.000 families. The total number of tribes is 96, but there are an additional 547 independent clans. Some of these do have neither a solid tribal structure nor a large number of households. Many are the remnants of old tribes and clans, which have disintegrated over time or settled in a particular region.
The Kerman and Hormozgan provinces have the highest number of tribes (28), the largest numbers of clans, 295 in total, and migration patterns extending to the Sistan & Baluchestan and in a part of Khorasan provinces. The highest number of nomadic households is found in the Chaharmahal & Bakhtiyari, Khuzistan and Isfahan provinces.

The Languages

About 70 percent of the population speaks Indo-Iranian languages which are however not a homogeneous group but consists of a variety of Indo-Iranian, Semitic, and Turkish languages. Farsi is the official language of the country. Modern Farsi had developed by the 9th century from ancient and "middle" Persian. Farsi has considerable Parthian and middle Persian elements, with additional influences from other Iranian languages. "Perso-Arabic" (an expanded version of the Arabic script), has been the official and cultural written language, which soon developed after the Arab invasion. Its grammar is simpler than that of middle Persian, and it has absorbed a vast Arabic vocabulary. About 28 percent of the population speaks various Turkish dialects, Arabic and Assyrian. The Arabic dialects are spoken in Khuzistan and along the Persian Gulf. As a Semitic language, Arabic is related to Hebrew, Syrian, and Ethiopian. Although Arabic has significantly influenced the Farsi vocabulary, it has no linguistic relationship with Persian.

Modern Iran at a glance

Geography
Area: 1.6 million sq. km. (636, 294 sq. mi.).
Cities: Capital: Tehran. Other main cities: Isfahan, Tabriz, Mashhad, and Shiraz.
Terrain: Plains, desert and mountains.
Climate: Semiarid; subtropical along the Caspian coast, tropical in the south coast.

Population
66 million inhabitants (2002 set)

Ethnic groups
Persians 51%, Turk (Azeri) 24%, Gilaki and Mazandarani 8%, Kurd 7%, Arab 3%, Lur 2%, Baloch 2%, Turkmen 2%, other 1%.

Age structure
0-14 years: 36%
15-64 years: 60%
65 years and over: 4%

Religions
Shi'a Muslim 89% Sunni Muslim 10%; Zoroastrian, Jewish, Christian, and Baha'i 1%

Languages
Persian and Persian dialects 60%, Turkish and Turkish dialects 26%, Kurdish 7%, Luri 2%, Baluchi 2%, Arabic 2%, other 1%.

Health
Infant mortality rate: 28.07 deaths/1,000 live births (2002),
Life expectancy at birth: total population: 70.25 years (2002).
Female: 71.69 years; Male: 68.87 years

Government
Type: Islamic republic.
Constitution: Ratified December 1979, revised 1989.
Executive: "Leader of the Islamic Revolution" (head of state); president and Council of Ministers.
Legislative: 290-member Islamic Consultative Assembly (Majles). Judicial: Supreme Court.

Administration:
28 provinces: Este-Azarbaijan, West-Azerbaijan, Ardebil, Esfahan, Elam, Bushehr, Tehran, Chaharmahal-Bakhtiyari, Khorasan, Khuzestan, Zanjan, Semnan, Sistan&Baluchestan, Fars, Qazvin, Gom, Kurdistan, Kerman, Kerman shah, Kohgiloyeh&Boyerahmad Golestan, Gilan, Lorestan, Mazandaran, Markazi, Hormozgan, Hamedan, Yazd

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Chapter II:

Hemoglobin, globin genes, gene products Malaria and hemoglobinopathies.

Hemoglobin

Hemoglobin (Hb), the major protein in the red cells, is an oxygen transporting tetramer consisting of two dimers of α-like and β-like globin chains. Each chain contains a heme, a porphyrin ring harboring an iron atom. The Hb tetramer has a molecular weight of approximately 65 KD and shows a reversible allosteric behavior. By a shifting of 15° between the α/β dimers gradual oxygenation and de-oxygenation can take place depending from the O_2 tension of the surrounding. O_2 binding will take place without any problem at any pressure of the first 5 km of the earth atmosphere. O_2 release will take place at the low tension of the capillary circulation in our body tissues. The release mechanism being enhanced by 2, 3, diphosphoglycerate, a ligand competitive to oxygen, which will keep the released O_2 out of the heme pocket.
The complex physiokinetics of the Hb tetramer allows a very efficient oxygen delivery to all tissues of our body that are reached by billions of red cells, all filled with the right amount of Hb tetramers. When insufficient Hb reach the tissues and more O_2 is needed, hypoxia-mediated signaling will release the kidney hormone erythropoietin which will stimulate the bone marrow to the production of new red cells, all derived from a limited number of committed red cells progenitors expressing α and β globin. By this regulatory mechanism, new red cells are continuously generated and at the required speed. Red cells will last about 120 days in the peripheral circulation before being sequestrated by the reticuloendothelial tissue in spleen and liver and Iron is completely recycled. At this rate about 3 kg of Hb are synthesized on average each year of our life. To build up such a huge amount of perfectly functioning protein we are provided with a number of highly conserved genes on the α- and β-globin gene clusters.

The history of hemoglobin genes

Globin-like genes in plants have four exons and three introns but code for globins similar to those of the invertebrate [1]. These similarities strongly support the hypothesis that the evolutionary history of proto-globin genes predates the divergence of plants and animals [2].
In mammals, hemoglobin not only transports oxygen and CO_2, but also regulates the nitric oxide level [3]. Since nitric oxide is a major regulator of blood pressure, this indicates that hemoglobin is involved in the control of blood pressure in a way that may facilitate a more efficient delivery of the oxygen load to the tissue [4]. The functions of detoxification of nitric oxide might have an ancestral origin related to the low oxygen concentration in the earth atmosphere of 4 billion years ago [5].
Hemoglobin genes, as we know them today in man, have a long history going back to more than 1 billion years. They all evolved from a proto-myoglobin derived from the ancestral genes of invertebrates and plants [6]. Through evolution, two clusters of globin genes separated in early vertebrates approximately 500 million years ago [7]. In man these two clusters are located on chromosome 11 (β-like) [7], and chromosome 16 (α-like) [8]. The myoglobin gene still exists and functions in man, producing the monomeric protein which takes care of oxygen storage in the muscular tissues.
All human globin chains share with each other a very similar primary, secondary and tertiary structure. The amino acid sequences are very similar forming a series of alpha helices and the heme-binding pocket with two histidines. All chains are able to form functional heterotetramers with the typical quaternary structure characteristic of all members of the globin family coding for oxygen transport proteins [9].

Mutations and evolution

All species slowly evolve due to random mutation events on their genome. Positive or negative selection pressure for new mutation is induced by the specific habitat. DNA mutations are rare random events that take place at a supposed rate of 10^{-9} per base pair per year [10]. Mutation hot spots related to the specific DNA structure and the specific tissue do however exist. Mutations take place in all dividing cells of all tissues and in some more than in others with different consequences. Mutations in somatic cells may lead to cancer but are not passed to the progeny. Mutations in gametic cells can be inherited by the progeny and contribute at every generation to the relentlessly slow mechanism of evolution.

Effect of germline mutations

Germline mutations, if transmitted, change the genome of the progeny. However, changes may alter the DNA structure without interfering with a coding region or may modify a gene without changing protein expression or function. These mutations generate polymorphisms and many polymorphisms are found on the genome of all living species. Polymorphisms of the human genome are used in molecular analysis to establish the specific "haplotype" of the individual in regard to a particular section of the genome.

Mutation mechanisms may imply the duplication of a gene or may generate a new hybrid gene, may change the structure of an encoded protein, or lead to a partial or complete loss of expression of one or more genes.

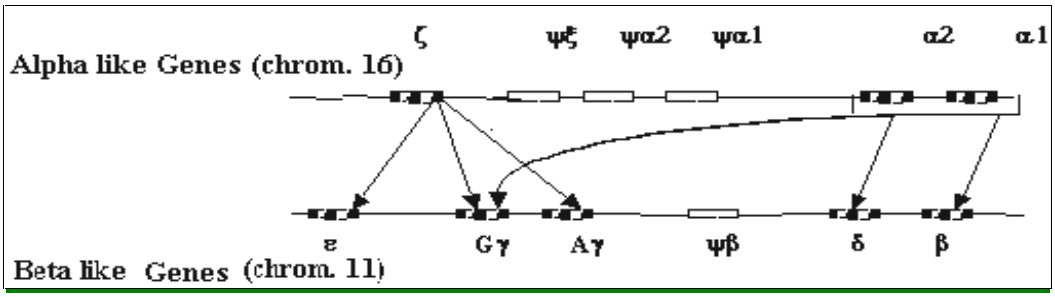
Mutation events are characterized by different mechanisms such as crossover between homologous sequences (generating deletions and triplications of genes). Other mechanisms generate single base substitutions (point mutation) or deletions of a few base pairs changing the reading frame.

Point mutations arise at low frequency owing to chemical instability of purine and pyrimidine bases and to errors during DNA replication. Natural exposure of an organism to certain environmental factors and chemicals also can cause mutation. A common case of spontaneous mutation is the de-amination of cytosine to uracil in the DNA double helix. Subsequent replication leads to a mutant daughter cell in which a T-A base pair replaces C-G base pair. Another cause of spontaneous mutations is copying errors during DNA replication. Although replication generally carries out with high fidelity, errors constantly occur often concentrated in mutation hotspots characterized by semi-palindromic inverted repeats.

The human α and β gene clusters

Through random mutation and selection the primordial myoglobin gene has evolved to a complex gene's family in man. For the last few million years eight specific but very similar genes have been coding for the eight different globins involved in hemoglobin formation in man. Like in primates very similar α and β gene clusters are located on separate chromosomes. The α-genes cluster, about 30 Kb large, is located on the short arm of chromosome 16. It contains three functional genes (ζ, α2, and α1) and three pseudo genes (ψζ, ψα1 and ψα2) with an additional θ gene of uncertain function. The β-genes cluster is located on the short arm of chromosome 11 and is approximately 50 kb large. It contains five active genes (ε, Aγ, Gγ, δ and β) and one pseudo gene (ψβ1). Promoter elements upstream to the initiation codon of each active gene are involved in the initiation of the transcription mechanism. Both clusters contain a regulatory

elements, α-MRE (or HS40) for the α cluster and LCR for the β-cluster, that interact with the specific promoter regions to start erythroid-specific gene expression and to coordinate the developmental regulation of each gene (Fig II-1).



Developmental Period	Embryonic	Fetal	Adult
Hemoglobin type	Hb Gower 1 (ζ2/ε2) Hb Gower 2 (α2/ε2) Hb Portland (ζ2/γ2)	HbF (α2/Gγ2)/(α2/Aγ2)	HbA (α2/β2) 93% HbA_2 (α2/δ2) 2.5% HbF (α2/γ2) <1%

Figure II-1. The figure shows the location of the α- and β- gene clusters activated from 5' to 3' in the embryonic (ζ and ε) fetal (α2, α1, Gγ and Aγ) and postnatal life (α2, α1, δ and β) producing embryonic (Hb Gower1 and 2 and Hb Portland) fetal (HbF) and postnatal tetramers (HbA and HbA2). The expression of fetal hemoglobin drops at birth from 80-90% down to less than 1% in the second year of life leaving HbA as the major Hb in postnatal life.

The hemoglobin switch

As an adaptation to the changing oxygen delivery, different hemoglobins, all composed of two different pairs of globin chains each attached to a heme moiety, are synthesized in the embryo, fetus, and adult (Fig II-2) [11]. The precise mechanisms that control the switch from the production of fetal hemoglobin to that of adult hemoglobin (α2/β2) are still matter of intensive studies [2,11,12]. As a matter of fact severe β-thalassemia (see further in this chapter) is induced by the lack of beta globin expression and becomes manifest with the decline in the synthesis of the well-expressed fetal hemoglobin (α2/γ2) during the first year of life.

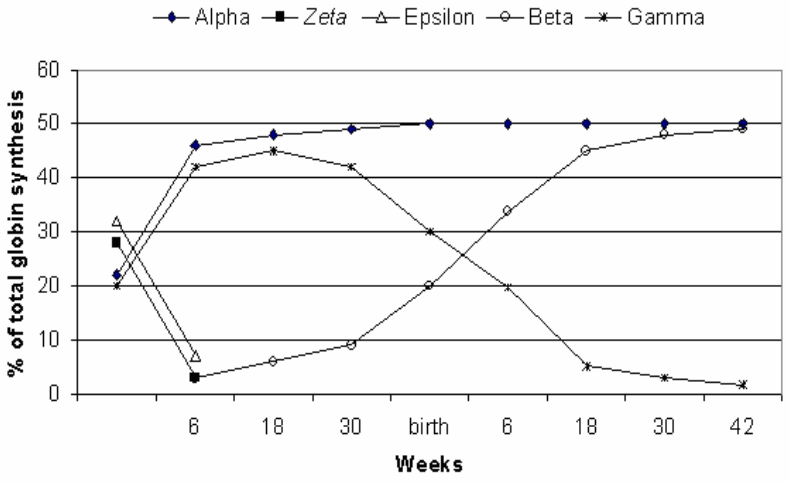


Figure II-2. The switching of γ-gene versus β-gene expression during pre- and perinatal life.

Malaria and Hemoglobinopathies

Many common recessive human diseases, often overwhelming in their effects, are due to mutations in a single gene. These mutations, advantageous in the carrier and frequent in a particular population have been selected by the same evolution mechanism mentioned above. Mutations inducing the frequent forms of hemoglobinopathy are transmitted to the progeny in a Mendelian fashion and have become predominant in particular areas where being a carrier is an advantage.

Haldane hypothesis is correlating the occurrence of α- and β-Thalassemia and sickle cell disease (SCD) to the protecting effect of these traits against *malaria tropica*. This hypothesis has been sustained by many evidences [13] among which the fact that Hemoglobinopathies are the most frequent autosomal recessive disease in mankind, especially frequent in the tropic and sub tropic areas of the old world where *malaria tropica* has been or still is endemic. Different kinds of mutations of the globin genes protect against malaria and the specific mechanism is different but equally effective.

Mutations inducing the change of an amino acid in a globin chain generate a structural defect at the protein level, an **abnormal hemoglobin**. Other mutations disable the expression of the gene inducing an expression defect called **thalassemia**. Both structural and expression defects of the globin genes can be pathogenic in a recessive Mendelian manner and may induce hemoglobinopathy in the progeny of parents who are both healthy carriers of the traits.

The suffix "-pathy" would call up an image of "disease," however most of the hemoglobinopathy defects are recessive traits and not clinically apparent in the carrier. Others produce symptomatic abnormal hematological laboratory findings. A very few produce a significant disease in the carrier and severe pathology is usually only present in the homozygous or the compound heterozygous state. Hemoglobinopathies are discussed in the next chapter.

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Chapter III:

Hemoglobinopathies, the abnormal hemoglobins in general and in the Iranian populations

Hemoglobinopathy classification

A gene mutation may have three effects. It may generate a polymorphism, a modified gene product or it may influence or disrupt the expression of the gene. For the globin genes in particular the first kind of effect, results in structural defect of the hemoglobin molecule **(abnormal hemoglobins).** The second kind, the expression defects, results in **thalassemia** (from the Greek word for sea = thalassa).
Both effects, structural or expressional, may concern either the β- or α-genes. This may result in abnormal hemoglobins induced by amino acid changes on either the β- or the α-chains or in expression defects affecting either the β- or the α-genes inducing β- or α-thalassemia respectively.

Abnormal hemoglobins

The change of an amino acid of a globin chain may have different outcomes on the function of the protein. While some positions on the globin chains tolerate substitutions without compromising the physiologic integrity of the hemoglobin tetramer, other positions are very sensitive to amino acid substitutions. For instance, substitution of a Glutamic acid with a Valine or Lysine at position 6 of the β-globin chain produces the abnormal hemoglobins S (HbS) and C (HbC), respectively. HbS homozygosity and HbS/C double heterozygosity are associated with the well-known phenomenon of intra-erythrocytic polymerization of the protein, causing premature destruction of the red cell (hemolysis) in sickle cell disease (SCD) and HbS/C disease. On the other hand, substitution of Glutamine, Asparagine, and Threonine for Lysine at position 59 of the β-chain produces, respectively, hemoglobins I-High Wycombe, J-Lome, and J-Kaoshiung, all of which are physiologically indistinguishable from normal HbA.

The abnormal globin structure can functionally manifest itself in one or more of the following ways:

- **Altered (increased or decreased) oxygen affinity.**

1. Oxygen affinity tend to alter when mutations affect the portions of the amino acid sequence that compose 1) the regions of contact between α and β chains, 2) the C-terminal regions, and 3) the regions that form the pocket which binds 2,3-DPG.
2. Increased oxygen affinity results into a less efficient oxygen release to the tissue. Decreased oxygen affinity is the opposite phenomenon. These hemoglobins pick up O_2 from the lung less efficiently but deliver more efficiently to the tissue. Depending on the degree of oxygen delivery, chronic hypoxia and polycytemia may arise.
3. A special class of low O_2 affinity hemoglobin variants is characterized by the presence of heme that contains iron in the ferric (Fe^{+++}) oxidation state, rather than the normal ferrous (Fe^{++}) state. These defects are often associated with cyanosis and are usually classified as *methemoglobins* or hemoglobin M (HbM).

- **Altered binding stability between globin chains.**
  Mutations at the contact sites between the globin chains in the Hb tetramer may produce haemoglobins that are intrinsically unstable and precipitate in the red cell. Precipitated tetramers are visible after staining and are called Heinz bodies. These are inclusions attached to the band 3 protein of the red cell membrane and may reduce the deformability of the cell wall, inducing spleen sequestration and "Heinz bodies hemolytic anemia". These defects are usually (semi) dominant and affected heterozygous are treated with splenectomy to reduce chronic hemolysis. Some of these mutations are relatively rare and often de novo. Examples of common unstable hemoglobins characterized by intracellular precipitation are Hb-Köln β98 $^{(Val \rightarrow Met)}$, Hb

Zurich β63$^{(His \rightarrow Arg)}$ and Hb Sabine β91$^{(Leu \rightarrow Pro)}$. Intracellular precipitations also take place when normal β-globin chains form homotetramers in case of HbH disease (β4=HbH) in (--/-α) α-thalassemia (see further in next chapter).

- **Structural defects with thalassemic effect.**
  Some abnormal hemoglobins result clinically in a thalassemia phenotype. This happens mainly when the expression of the mutant is lower than normal or when post-translational events inhibit dimer and tetramer formation. Classic examples are Hb-Constant Spring α142 $^{(+30)}$ and the HbE mutant β26 $^{(Glu \rightarrow Lys)}$. The last being very frequent in Asian populations and causing β-thalassemia major in combination with a variety of Asian β-thalassemia mutations.

- **Altered physical behavior.**
  The classic example of altered physiological behavior is the polymerization of the Hb-tetramers associated with the HbS mutant and with sickle cell disease.
  Not only HbS homozygosity results in the severe SCD phenotype but also various combinations with β-thalassemia mutations and with other structural mutants such as HbC, D, E, O-Arab and other less frequent abnormalities.

Abnormal Hemoglobins worldwide

To date more than 800 abnormal hemoglobins have been described worldwide and new reports keep coming. Mutations are distributed throughout the coding regions and on some codons more than on others (Table III-1).

Table III-1 Summary of abnormal hemoglobins registered up to 2004 and subdivided in cathegories [1]

Total hemoglobin variants	887
Combined thalassemia mutations and hemoglobin variants	44
Mutations involving the α1 gene	222
Mutations involving the α2 gene	265
Mutations involving the β gene	666
Mutations involving the δ gene	69
Mutations involving the Aγ gene	46
Mutations involving the Gγ gene	54
Hemoglobins with altered oxygen affinity	85
Unstable hemoglobins	128
Methemoglobins	9

Abnormal hemoglobins in Iran

The first two surveys for abnormal hemoglobin research were carried out in 1964 and 1965 by the pioneer of hemoglobin investigation in Iran, Samuel Rahbar and colleagues. at Shiraz University hospitals [3]. A third survey was carried out at Tehran University. Eighteen hemoglobin variants, most of them new or rare, were found during this survey and were subsequently reported. Hb J-Iran (1967), HbH (1968); HbL Persian Gulf (1969); HbQ-Iran (1970); HbDaneshgah-Tehran (1973); HbD-Iran (1973); HbArya (1975); Hb Hamadan (1975); Hb Lepore Boston (1975); HbO-Indonesia (1975); HbD-Punjab (1975); Hb Persepolis (1976); HbJ-Kurosh (1976); HbM-Boston (1977); Hb Setif (1977); Hb Osu-Christiansborg (1978); Hb Coventry (1978); Hb Avicenna (1979).
These three surveys were the first initiative that led later on to screening and prevention of hemoglobinopathies in Iran. During the last 10 years a similar population survey has been conducted in southern Iran and in the Hormozgan province (Yavarian M. Unpublished data) focusing on frequent Hb mutations associated with severe pathology. In this way it could be established that HbS, HbE, HbC, and HbO-Arab are frequent mutations in southern Iran to be added to the list of the rare ones previously reported. According to our survey and to the existing literature we can assume that, common and rare mutations together, at least 22 hemoglobin variants occur in all Iran (Table III-2 and III-4). The frequency of the common hemoglobin variants in the Iranian population is however not yet fully defined and needs additional research. The awareness of the clinical relevance and genetic risk associated with these frequent mutations should be taken into consideration by all laboratories in the country dealing with this problem.
The main clinical and laboratory finding associated with the "Iranian" abnormal hemoglobins are summarized in the following paragraphs.

The β-Chain Variants

Only a limited number of recessive or (semi) dominant β-globin chain variants are associated with severe pathology either in the homozygous and/or compound heterozygous, or in the carrier, respectively. Due to the fact that the β globin genes come to expression at a significant rate only after birth, the clinical effect of the potentially pathological β-chains is exclusively postnatal. Moreover, the higher expression of a single mutated β gene (up to almost 50%) compared with the lower expression of a single α gene (25-30% at the most) explain the more significant clinical impact of β chain variants.
In contrast with α chain variants (read further), stable β chain variants always generate a single abnormal tetramer in combination with the normal α globin chains. Therefore, only a single abnormal hemoglobin band or peak is usually observed in addition to the normal pattern in carriers of abnormal β globin chain variants. Due to the high HbF expression (α2/γ2), β chain variants may be not always visible at birth (2-10%) using electrophoresis or HPLC depending from Hb expression, Hb stability and pregnancy duration. An overview of the pathological and non-pathological β globin mutations observed in Iran is summarized in table III-2 and explained in more details in the following paragraphs.

Table III-2: pathological and non-pathological β globin mutations observed in Iran

Variant Hemoglobin	Position and Amino acid change	Cellulose acetate (alkaline pH)
Hb S	Cd6 (A3) Glu→Val	Hb S and Hb A can readily be separated at both alkaline and acidic pH
Hb C	Cd6 (A3) Glu→Lys	Hb X moves to the position of Hb A_2
Hb D-Iran	Cd22(B4) Glu→Gln	Hb X moves about as Hb S
Hb E	Cd26(B8) Glu→Lys	Hb E moves much slower than Hb A, just ahead of Hb A_2 and Hb C
Hb Avicenna	Cd47 (CD6) Asp→Ala	Hb X moves like Hb S at pH 8.5 and 6.2
HbOsu-Christiansborg	Cd52 (D3) Asp→Asn	Hb X in the position of Hb S
Hb Hamadan	Cd56 (D7) Gly→Arg	Hb X occupies the position of Hb S
Hb J-Iran	Cd77 (EF1) His→Asp	Hb X is fast moving
Hb D- Punjab	Cd121 (GH4) Glu→Gln	Hb X moves slower (like Hb S) than HbA
Hb O-Arab	Cd121 (GH4) Glu→Lys	Hb X moves close to HbA (between HbA and Hb S)
Hb Coventry	Cd 141(H19) Leu→0	No separation of Hb X and Hb A

HbS, the sickle hemoglobin

A transition of GAG to GTG at codon 6 of the β-globin gene is results in the HbS mutation. HbS is the most studied Hb variant and thorough information can be found in all textbooks and in many monographies [4, 5, 6]. HbS tetramers polymerize when deoxygenated into long conformations causing the classical deformity of the red cell, which then assume the classical "sickle" shape. The rigid anomalous sickle cells cause reological problems in all tissues where small post-capillary venules may get transiently or permanently infarcted. As mentioned in chapter I, it is assumed that the HbS mutation has reached high frequencies in many populations because of the protection heterozygous individuals have against the severe clinical consequences of *malaria tropica.* Therefore more HbA/S heterozygous than wild type HbA/A are prone to reach the reproductive age in areas where *malaria tropica* has been (or still is) endemic in the history of mankind. In Iran malaria and HbS trait shows parallel frequencies on the shorelines of the Caspian Sea and along the north and south Persian Gulf.

The HbS mutation has independently occurred at least 5 times and is found on five prevalent 5' haplotypes (7). The most important difference between haplotypes is the variable capacity to express HbF in postnatal life associated with presence or absence of the so called Xmn1 polymorphism on the promoter of the Gγ gene [8]. Therefore we may roughly subdivide the HbS haplotypes in two groups, one with the polymorphism including the relatively less severe HbS defects (Senegal, Indian-Saudi), the other without including the more severe type (Cameroon Benin and Bantu).

In the Hormozgan province the average carrier frequency is 0.028 with a peak of 0.051 in the eastern part (Minab&Rodan) and a gene frequency of around 0.01 in the Fars Province at the northwestern border of Hormozgan [9] see also table IV-2.

As mentioned before, the characterization of the HbS mutations occurring in Iran is not fully investigated. However, the many cases with high fetal hemoglobin levels in south Iran [10] indicate that the mutations are likely to be on Asian-Indian or Senegal haplotypes. The cultural background and the connection with the Arabian Peninsula (see

chapter VII) provide the historical support. In Oman has been shown that the frequency of the Benin and Arab-Indian haplotype are 52.1% and 26.7% respectively and that most sickle cell patients of Iranian origin had the Arab-Indian haplotype [11]. The HbS on the Arab-Indian haplotype is a relatively young event which arose presumably 4.000-5.000 years ago, and most probably in the Hindu valley area [12].
Heterozygote HbA/S individuals (sickle cell trait = SCT) have essentially a normal clinical presentation while the homozygous HbS/S (Sickle cell disease = SCD) or the double heterozygous HbS/β^{thal} or HbS/HbD or other more uncommon SCD combinations, present with many severe symptoms. Briefly summarized the symptoms are hemolytic anemia, acute spleen sequestration (ASS), jaundice, painful crises, leg ulcers, acute chest syndrome (ACS), stroke (CVA), congestive heart failure, meningitis, and have lowered resistance to salmonella and pneumococcus organisms in general.
Due to the high frequency of α-thalassemia (see publication 2 and 3) sickle cell trait (HbA/S) may frequently come with α-thalassemia. In these cases lower amount of HbS and some microcytosis and hypochromia is present. It has been suggested that hypochromia (low MCH) might be beneficial in decreasing the vaso-occlusive events in SCD. Similarly, the higher expression of fetal hemoglobin associated with the Indian haplotype is a modulating factor that may, to some extend, reduce the severe symptoms of the classic SCD phenotype.
As discussed in chapter IV, the effect of high fetal hemoglobin (HbF) on the phenotype of β-thalassemia and SCD is very important as a modulating factor as well as a therapeutical intervention. Combinations of HbS with hereditary persistence of fetal hemoglobin (HPFH) and with β-thalassemia defect associated with high HbF expression, induce such a dilution of the HbS tetramers with the high O_2 affinity HbF tetramer that HbS polymerization is strongly reduced leading to milder pathology or even asymptomatic phenotypes.

Hemoglobin C

Hemoglobin C, first described by Itano and Neel [13] in 1950 is generated by a substitution of GAG by AAG at the same codon 6 of the β-globin also mutated in HbS. The deriving amino acid substitution (Glu → Lys) eliminates two negative charges and therefore on alkaline electrophoresis this mutant migrates like HbA2, HbE and HbO-Arab. This variant is one of the most prevalent abnormal hemoglobin and has, like the other probably spread in West Africa because of the advantage in the presence of *malaria tropica* [14]. However a case of HbC in Oman, a country in the proximity of the Hormozgan province, was reported on a different haplotype showing that these common mutations may have occurred several times in different populations [15]. Carriers of HbC show very mild microcytosis and target cells but have no anemia. Homozygotes have mild to moderate splenomegaly mild hemolytic anemia with numerous target cells. Like in HbS heterozygosity, coinheritance of the HbC trait with α-thalassemia reduces the concentration of abnormal hemoglobin because of a preferential binding of the normal α-chains to the wild type β-chains. Combinations of HbC with β-thalassemia are, like Hb S/β-thalassemia quite common in Africans, but unlike the last one, are mild conditions comparable with β-thalassemia trait. Double heterozygosity HbS/C is common in Africans resulting in a less severe SCD disease phenotype in which the typical SCD symptoms are either mild and/or delayed in time. [16,17]. Combinations HbC/E is rare but may occur in mixed African and Asian population. The outcome is mild.
HbC is found in Iran in individuals from Larestan and Bashkerd, at the northwest and east of the Hormozgan province respectively.

Hemoglobin E

HbE was first reported by Itano et al. in 1954 and described later by Hunt and Ingram [18]. This mutation competes with HbS the title for being the most frequent worldwide. It is certainly the most prevalent abnormal hemoglobin in Southeast Asia and the Bodo-Kachari people in northern India have the highest gene frequency in the world (0.50) [19].

HbE is induced by the GAG→AAG mutation at codon 26 causing a Lysine → Glutamic acid substitution induces a β-thalassemia phenotype resulting partially from a lower expression of the mutant as a consequence of the alternative splice site created by the mutation [18] and partially by the instability of the HbE tetramer.

HbE heterozygotes have microcytic hypochromic parameters and the amount of HbE in the heterozygous is about 30% or less. Homozygous patients may present with mild to intermediate hemolytic anemia. Combinations of HbE and β-thalassemia usually result in β-thalassemia major phenotypes [20]

Also the β^E mutation is known to occur on chromosomes with different haplotypes. At least three have been observed among the Southeast Asian populations and two in European families [21, 22] (see Table III-3).

Table III- 3: The HbE haplotypes

Origin	HincII	XmnI	HindIII	HindIII	HincII	HincII	AvaII	AvaII
Southeast Asian	-	+	+	-	+	+	+	-
Southeast Asian	+	-	-	-	-	-	+	-
Southeast Asian	-	+	+	-	+	+	-	+
Czech	+	-	+	+	-	+	+	-
European	+	N.D.	-	-	-	-	+	+

N.D. = not determined. Adapted from [1].

Hemoglobin D-Punjab

This abnormal hemoglobin was first reported in 1951 [23] and in other occasions as HbD-Los Angeles, HbD-Chicago, HbD-North Carolina, HbD-Portugal, and Hb-Oak Ridges. All of them were induced by the same GAA → CAA substitution at codon 121 of the β-globin gene. HbD-Punjab is prevalent in the north Indian state bearing the same name and is regularly found in all populations that have ever lived along the old Silk Road from China to the Mediterranean [24]. HbD-Punjab is also regularly found in the white European population especially in the U.K. Haplotype studies indicate that HbD-Punjab could have a unique evolutionary origin [25, 26, and 27].

HbD-Punjab is the fourth most frequently occurring Hb variant world wide [28] and the third most common hemoglobin variant in Hormozgan Province (0.014), mainly in Bandar Abbas and Geshm Island but also in other Iranian provinces [29].

HbD-Punjab is a stable variant, which is expressed at about 40% in heterozygotes who do not have any clinical abnormalities. However, coinheritances of HbD-Punjab with HbS facilitate polymerization inducing the severe phenotype of sickle cell disease with a severe clinical course [30]. Therefore is the identification of this mutant important. HbD-Punjab migrates on alkaline electrophoresis just like HbS but does not polymerize with the "sickle test" as HbS does. Detection of this mutation at the molecular level might be facilitated by the disruption of the EcoRI site normally located at codon 121 of the β-gene (see chapter VI).

Hemoglobin O-Arab

Firstly described in Jewish Arabs is also known as Hb-Egypt and HbO-Thrace [31,32]. The GAA → AAA mutation at cd121 of the β-globin gene induces a Glu →Lys amino acid substitution. Herewith, the loss of a (-) for a (+) charge makes the mutated Hb migrate at alkaline pH on the HbA2 position.
A high prevalence was observed in Bulgaria and it has been postulated that HbO-Arab perhaps originated from this area and was carried to the northern parts of East and West Africa during the Ottoman Empire [33]. It seems more likely that the mutation had an older African origin and migrated with the Arab invasions. The variant is mainly reported in Arabs, Afro-Americans, Gypsies, Pomaks (population groups from the Balkan countries) and in African Egyptians [34,35,36]. The Heterozygotes presents with 30-40% of HbO-Arab and is not anemic. The Homozygotes patients present with microcytosis and mild anemia. Compound heterozygosity of HbO-Arab and HbS causes severe sickle cell anemia.

More than one structural mutation on the same β-globin gene

At least 19 abnormal hemoglobins with double mutation have been described. These double mutation may occur on 5 alleles, which are considered frequent in Iran. Six double mutations have been reported on the HbS allele, two on the HbC, two on the HbE, one on the HbD and one on the Hb Hamadan allele. Although none of these double mutations have been reported in Iran so far it is likely that, because of the ethnic relation between the two areas, at least the double mutant HbS-Oman could be expected in the country. Hb Oman is the product of the HbO-Arab and the HbS mutation combined on the same β-globin gene. HbS-Oman is expressed at a rate of 14.5- 20 % and it moves slower than HbC at alkaline pH. The double mutation induces a moderately severe hemolytic anemia in the carrier and severe SCD in combination with the regular HbS allele [37].

HbD Iran

This rare hemoglobin was found during a survey in a family from central Iran [38]. The mutation on codon 22 of the β-globin gene involves a transversion of GAA to CAA. On electrophoresis at alkaline pH the mutation moves like HbS and represents 36 - 45 % of the total Hb. At acid pH HbD-Iran do not separate from HbA. Carriers of HbD-Iran present no clinical abnormality [38]. Double heterozygotes with HbD Iran and β-thalassemia have been observed and have the clinical phenotypes of a β-thalassemia carrier [39, 40].

Hb Avicenna

Again a rare mutation found in 1979 [41] during a survey among voluntary blood donors in Iran. The propositus was a young male with no clinical symptoms and normal hematological parameters. Hb-Avicenna migrates like HbS at alkaline pH. This stable abnormal Hb is expressed at a rate of 40% and is caused by a GAT → GCT mutation at codon 47 of the β-globin gene inducing an Asp→Ala amino acid substitution. The ethnic origin of the Iranian patient was not reported.

Hb Osu-Christiansborg

Hb Osu-Christiansborg was firstly reported in 1977 [42] and later by Rahbar et al. in an Iranian family [43], also described in Accra, Ghana, and in Blacks in the USA.
The GAT → AAT mutation at codon 52 of the β-gene induces an Asp →Asn amino acid substitution and abnormal hemoglobin that migrate like HbS at alkaline pH.
Heterozygous individuals present with 42-45 % abnormal fraction, no clinical manifestations and can be mistaken for HbS carriers. Compound heterozygosity Hb Osu-Christiansborg and HbS has been reported [42].

Hb Hamadan

The Hb Hamadan mutation was found in an Iranian family during a random screening. The carrier present with 40% abnormal hemoglobin and no clinical complains [44].
Induced by a GGC → CGC mutation at codon 56 of the β-gene, changing the Gly residue to Arg. This hemoglobin elutes like HbA2 on HPLC (figure III-1) and its abnormal band moves like HbS on alkaline electrophoresis (figure III-2). This mutation is not clinically important but it is easily mistake for HbS because it migrates, like HbD Iran, Hb Avicenna and Hb Osu-Christianborg identical to HbS and could lead to wrong diagnoses.
Hb Hamadan was also found in members of a French Caucasian family, in a family of Turkish descent, and in several Japanese families [45, 1]

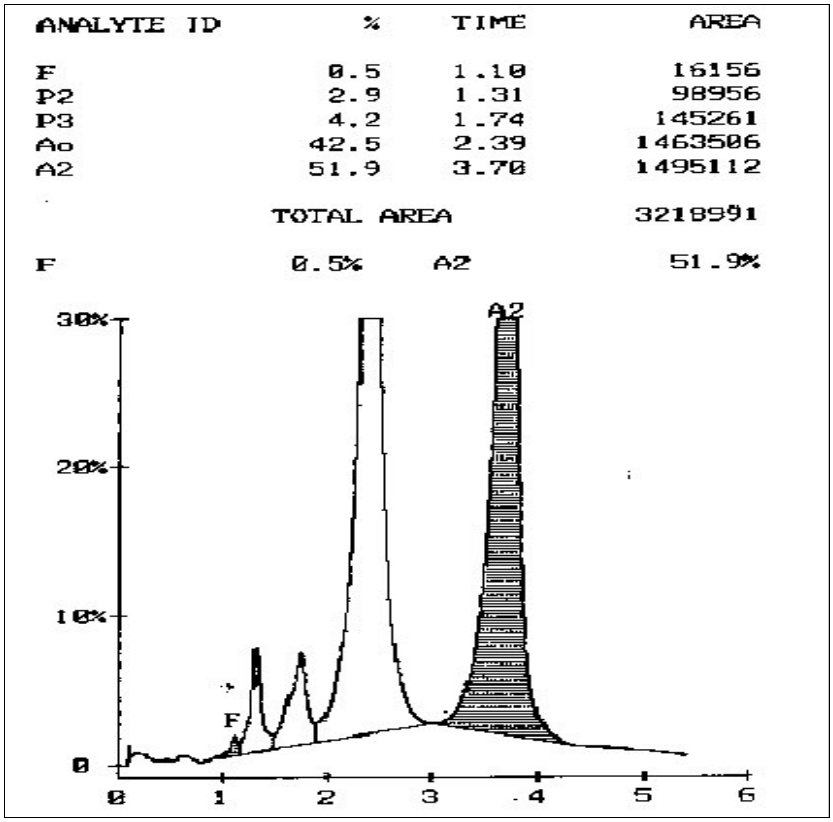

Figure III-1. HPLC shows that Hb Hamadan co-eluting with HbA2.

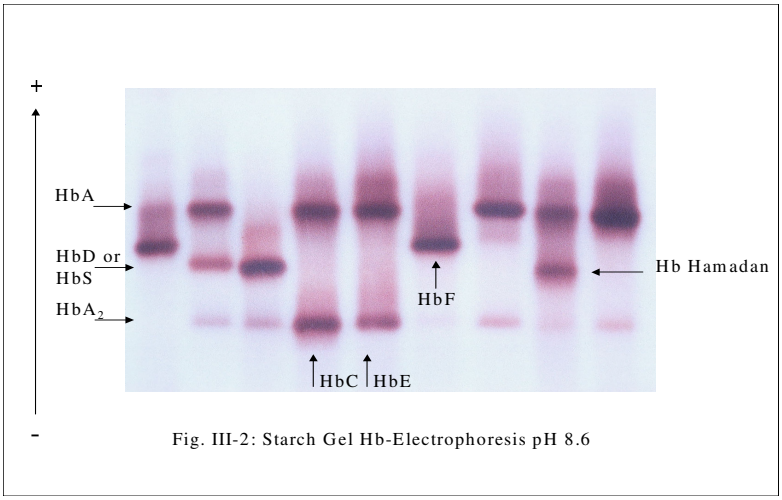

Fig. III-2: Starch Gel Hb-Electrophoresis pH 8.6

HbJ Iran

J-Iran was first seen in an Iranian family in 1967 [46]. The transversion CAC → GAC at codon 77 of the β-globin gene induces the His → Asp amino acid substitution in this mutant. The carrier shows no hematological abnormalities and presents with about 45% abnormal fraction migrating faster than HbA on alkaline electrophoresis.

Hb Coventry – Hb Atlanta

In 1978, Nozari et al. reported Hb Coventry in an Iranian family [47]. This unstable hemoglobin, probably induced by partial oxidation of the Leu residue at position β141 to hydroxyleucine [48], always occurs in association with another unstable Hb (Hb Sydney, Hb Atlanta) [49,50]. Heterozygous individuals are essentially normal. On alkaline electrophoresis Hb Coventry is not separated from HbA.

The α-Chain Variants

The expression of a structural mutation on one of the four α genes rarely exceeds 25% and is compensated by the expression of the three normal α-genes left. Therefore, the clinical consequences of potentially pathological α globin chain variants are generally less significant that those of the β globin chain variants. However, due to the pre-natal expression of the α-genes they may induce pre- and postnatal symptoms.
A common characteristic of structural and stable α gene defects is the presence of two variant tetramers. A major one resulting from the formation of mutated HbA ($\beta 2/\alpha 2^X$), a minor one formed in combination with the δ chains resulting in a double HbA2 fraction and often in a reduced HbA_2 estimation. Due to the high expression of HbF ($\alpha 2/\gamma 2$) at birth, complex electrophoresis patterns may be observed due to the association of the mutated α chains with the normal γ chains. An overview of the α globin mutations observed in Iran is summarized in table III-4 and is explained in more detail in the following paragraphs.

Table III-4: overview of the α globin mutations observed in Iran

Variant Hemoglobin	Position and Amino acid change	Cellulose acetate (alkaline pH)
Hb J-Kurosh	α2 or α1- Cd 19(AB1) Ala→Asp	To the position of Hb J
Hb Arya	α2 or α1- Cd 47(CE5) Asp→Asn	With Hb S
Hb L-Persian Gulf	α2 or α1- Cd 57(E6) Gly→Arg	Between Hb S and Hb G
Hb M-Boston	α2 or α1- Cd 58(E7) His→Tyr	As Hb A
Hb Persepolis	α2 or α1- Cd 64(E13) Asp→Tyr	Slightly faster than Hb D
Hb Daneshgah-Tehran	α2 or α1- Cd 72(EF1) His→Arg	To the position of Hb S
Hb Q-Iran	α2 or α1- Cd 75(EF4) Asp→His	To the position of Hb S
Hb Setif	α2- Cd 94(G1) Asp→Tyr	To the position of Hb S
Hb O-Indonesia	α1- Cd 116(GH4) Glu→Lys	Slightly slower than Hb S

HbJ-Kurosh

Found in an Iranian male in 1976 during a screening among normal blood donors in Tehran [51]. Not characterized at the DNA level at that time it is most likely induced by a GCG→GCC or GAC mutation at codon 19 of the α2 globin gene causing an Ala → Asp amino acid substitution. The major mutant fraction migrates as a faster band at position J on alkaline electrophoresis and the minor fraction associated with the δ chain is visible as a faster portion of the HbA2 fraction. Expressed at 25% in heterozygotes it is not associated with clinical hematological abnormalities.

Hb Arya

Hb Arya is a mildly unstable hemoglobin described in a randomly screened 52 years old Iranian female [52]. A GAC → AAC mutation at cd47 causes an Asp→Asn amino acid substitution in this mutant. The expression of 22% suggests a mutation on the α2 genes. The mutant migrates like HbS on starch gel electrophoresis at alkaline pH. Being mildly unstable only minor hematological abnormalities are present in the carrier.

HbL Persian Gulf

This hemoglobin, reported in 1969 in a woman from the Persian Gulf region [53], is caused by a GGC→CGC mutation at cd 57 inducing a Gly→Arg amino acid substitution. The expression rate of 18% indicates that the mutation is probably on the α1 gene.
The carrier presented without any hematological abnormality. At an alkaline pH the anomalous band migrates between HbS and HbG and shows a second fraction in combination with the δ chain.

HbM-Boston

Hemoglobin M-Boston was described in a 19-year-old Iranian male, his father and three out of his five brothers and sisters, who had a cyanotic appearance from birth on and normal hematological parameters [54].
HbM Boston is a relatively common mutant with decrease O_2 affinity and is described in many ethnic groups under different names (HbM-Gothenburg, HbM-Kiskunhalas, HbM-Norin and HbM-Osaka) [55, 56]. This mutant is caused by a CAC→TAC mutation at codon 58 of presumably the α2 gene and is a methemoglobin. These types of abnormal hemoglobins are characterized by mutations changing the His residue

involved in heme contact (in this case His→Tyr [57,58]. Carrier presents with variable degree of cyanosis due to a decreased O_2 affinity and the expression rate of the mutant. HbM Boston expresses at a 20-30% rate and migrates on acid electrophoresis between HbS and HbC. Hemizygosity of the HbM variants in combination with β-thalassemia may result in a marked cyanosis with significant psychological but however limited clinical complaints.

Hb Persepolis

Hb Persepolis was found in an Iranian male of Indian Sikh origin presenting with normal hematological parameters [51]. The mutant is caused by a GAC→ TAC transition at codon 64 of the α2 (or α1) gene resulting in an Asp→Tyr amino acid substitution.
It moves on alkaline electrophoresis slightly faster than HbD and it can therefore be confused with HbD Punjab. The expression rate (20%) is compatible with a α2-gene defect and the double HbA2 fraction resulting in a low HbA2 estimation is typical for a stable globin chain mutant.

Hb Daneshgah-Tehran

Hb Daneshgah-Tehran was observed in an Iranian blood donor living in Tehran [59]. This variant is generated by a CAC→CGC mutation at cd 72 of the α2 (or α1) gene resulting in a His→Arg amino acid substitution. The variant moves like HbS on alkaline electrophoresis and it can therefore be confused with HbS. The expression rate (25%) is compatible with an α2-gene defect. The carrier had no hematological complains. Hb Daneshgah-Tehran has also been found in an Argentinian family [60].

HbQ-Iran

Hemoglobin Q-Iran was found in an Iranian Family in 1970 [61] and also in Turkish, and Pakistani families [62]. Coinheritance of α-thalassemia and Hb Q-Iran were also reported [62]. HbQ-Iran is generated by a GAC→CAC mutation at cd 75 of the α1 (or α2) gene resulting in an Asp→His amino acid substitution. Also this variant moves like HbS on alkaline electrophoresis and it can be confused with HbS. The expression rate (17-19%) in the carrier is compatible with a α1-gene defect or with a slightly unstable α2-gene variant. The carrier had no hematological complains therefore a stable α1 mutation seems more likely.

Hb Setif

Nozari and et al (1977) reported nine independent cases of this hemoglobin in the Iranian population [62] previously reported in Algerian, Lebanees, and Saudi [64].
Hb Setif is generated by a GAC→TAC mutation at cd 94 of the α2 gene resulting in a Asp→Tyr amino acid substitution. Hb Setif moves like HbS on alkaline electrophoresis and it can be confused with HbS. Although negative on the sickle test Hb Setif seems to have a sickle-like behavior due to intracellular crystallization [65]. Hb Setif is considered to be unstable because the Asp→Tyr substitution at codon 94 is involved in the α1/β2 contact [66]. The expression rate (12-15%) in the carrier is compatible with an unstable α2 gene variant. The carrier had no significant hematological complains.

HbO-Indonesia

Rahbar et al. reported this variant in an Iranian family in combination with Hb D-Punjab [67]. Hb O-Indonesia induced by GAG→AAG mutation at cd 116 of the α1 gene resulting in a Glu→Lys amino acid substitution. This mutant migrates slightly slower than HbS on alkaline electrophoresis and it can be confused with HbS and HbD.
HbO-Indonesia is considered as a slightly unstable variant; however heterozygous individuals have no clinical symptom. An expression rate of 21% was measured in combination with β-thalassemia. The original report was in populations of the Indonesian archipelago described by Lie-Injo in 1957 [68]. The mutant is also reported in Makassar, South Africa, Italy and China [69].

Hb Lepore Boston

Rahbar and et al [70] reported a case of HbLepore-Boston in 1975, in a patient from the central part of Iran. Two additional cases have been observed in the Fars Province (unpublished data) in patients affected by thalassemia major due to combinations of Hb Lepore Boston and β-thalassemia.
Hemoglobins Lepore are a group of gene products resulting from hybrid chains formed by unequal cross over between the δ- and β-globin chains. The abnormal gene product consists partially of δ- and partially of β-chain. Different hybrids can be generated by this mechanism depending from the points of cross over. In Hb Lepore-Boston the point of cross over is between residue 87 of the δ-globin chain and residue 116 of the β-globin chain [71].
Heterozygous individuals have a β-thalassemia minor phenotype with microcytosis, hypochromia and 7 – 13 % of HbLepore-Boston migrating on alkaline electrophoresis on the position of Hb S.
Hb Lepore-Boston-Washington is the most common Hb Lepore type. It is found mainly in Italians and formal Yugoslavians but has also been observed in Rumania, Turkey, Cyprus, Jamaica, Cuba, Greece, England, Australia, Mexico, etc.
Double heterozygous for Hb Lepore-Boston and HbS has results in a relatively mild sickle cell disease [72].

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Chapter IV

Hemoglobinopathies, thalassemia in general and in the Iranian populations

Hemoglobinopathy classification

As explained in the previous chapter a mutation on the globin genes can have two effects. It can generate a different gene product (**abnormal hemoglobins),** disrupt the expression of the gene **(Thalassemia)** or have both effects (i.e. HbE). Structural or expressional mutations may involve either the β- or α-genes. Expression defects of either the β- or α-genes induce β- or α-Thalassemia respectively. Mutations in the globin genes may also be neutral and generate polymorphisms without pathological consequences. Frequent polymorphisms may contribute to the determination of the "gene frame" or gene's cluster "haplotype" and be used for molecular analysis [1].

Some history

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 [2]. Because all early cases were reported in children of Mediterranean origin, the disease was later termed thalassemia, from the Greek word for sea, thalassa [3]. 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 human [4].

The β-thalassemia are widespread throughout the Mediterranean region, Africa, the Middle East, the Indian subcontinent and Burma, Southeast Asia including southern China, the Malay Peninsula, and Indonesia. Estimates of gene frequencies range from 3 to 10 percent in some areas [5]. Within each population at risk for β -thalassemia a limited number of common mutations are found. However, depending from the ethnic heterogeneity of the population, a large spectrum of less frequent mutations is often present. Each mutation is in strong linkage disequilibrium with specific haplotypes which are determined by molecular analysis of polymorphic sites usually by restriction-fragment–length polymorphisms. Theoretically, the 5 polymorphic sites present in the 5' haplotypes sequence of the β-globin gene cluster would allow the formation of 32 (2^5) possible haplotype combinations. But in practice only 19 different β-globin gene haplotype combinations are more or less frequently observed in the general population [6]. Only few of those haplotypes associate specifically with β-thalassemia mutations, helping to demonstrate the independent origin of β-thalassemia mutations in different populations. As said in the previous chapter (II) there is a strong evidence that the high frequency of β-thalassemia throughout the tropics reflects an advantage of heterozygotes against *Plasmodium falciparum* malaria, [7] as it has been reported for α -thalassemia [8].

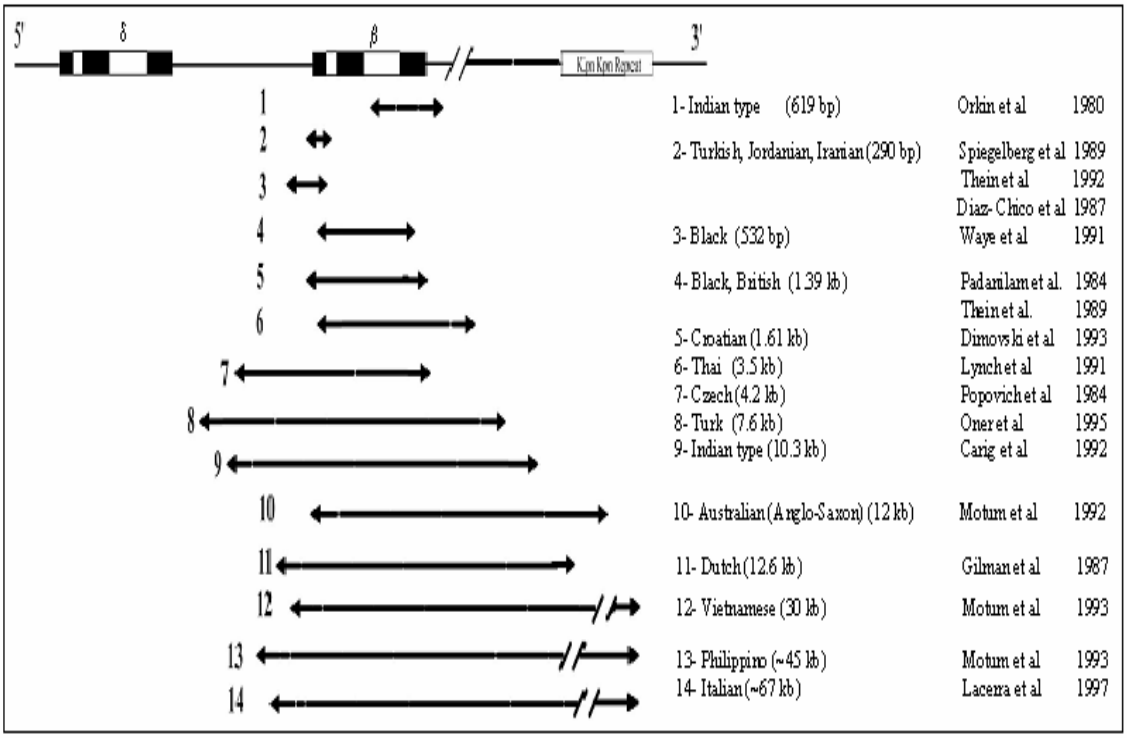
Mutations causing β-Thalassemia

Nearly 200 different mutations have been described in patients with β-thalassemia and related disorders such as the hyper unstable structural mutations with thalassemic phenotype. Although most β-thalassemia mutations concern changes (substitutions, deletions or insertions) involving either a single or a few nucleotide (Table IV-1), large deletions taking away part, the entire or multiple genes also occur (Fig. IV-1) [4]. Roughly large deletions in the β globin gene cluster could be subdivided into two types.

One type involving the 5' upstream sequences (e.i. the Hispanic type) and the other involving the 3' down stream region (i.e. the 290 bp deletion) [9].

All pathological mutations result either in absence (β°-thalassemia) or in reduction of β-globin chains synthesis (β^+-thalassemia). Mutations in or close to the conserved promoter sequences and in the 5' untranslated region that down-regulate transcription, usually results in mild β^+-thalassemia. Deletions 5' to the gene involving the CAP site region also completely inactivate transcription and result in β°-thalassemia. Mutations disrupting the splicing mechanism may block or strongly reduce the precursor messenger RNA (mRNA) resulting in non-functional or unstable mRNA and consequently β°- or β^+-thalassemia phenotypes. Mutations involving the invariant dinucleotide at intron/exon junctions are critical for exon splicing and production of functional mRNA, usually result in β°-thalassemia. The ones in proximity of these conserved nucleotide sequences or those generating "cryptic splice sites", may result in both severe β°- as well as mild β^+-thalassemia. Substitutions or small deletions affecting the conserved AATAAA sequences (PolyA signal) in the 3' untranslated regions result in ineffective cleavage of the mRNA transcript and cause mild β^+-thalassemia phenotypes. Mutations that generate stop codons that interfere with initiation, or termination of globin-chain production usually results in β°-thalassemia. Approximately half of all β-thalassemia mutations interfere with translation. A group of mutations involving the third exon, result in the production of globin chains of considerable lengths that are unsuitable for dimer formation and are too big for rapid proteolytic disposal. This, together with a relative excess of α-globin chains, causes precipitation in the red-cell precursors of both free α and ineffective β chains leading to ineffective erythropoiesis, even in the heterozygous state. This phenomenon is responsible for the so-called Heinz Bodies anemia or dominantly inherited β- thalassemia [10].

Fig IV-1 Deletion involving only the structural β-globin gene. The Kpn repeat box are 6.4 kb length at the 3'end of β-globin gene



*The authors mentioned in this table are not registered in the references of this thesis.

Table IV-1: Beta thalassemia point mutations updated January 2004(Courtesy of P.C. Giordano)

Position	Mutation	Phenotype	Ethnicity	Authors
Transcription defects				
Promoter mutations				
-101	(C→T)	β^+	Turkish/Bulgarian/Italian	Gonzalez-Redondo et al. 1989
-92	(C→T)	β^+	Mediterranean	Kazazian 1990
-90	(C→T)	β^+	Portugees	Faustino et al. 1992
-88	(C→T)	β^+	Balck/Asiatic	Orkin et al. 1984
-88	(C→A)	β^+	Kurdish	Rund et al. 1990
-87	(C→G)	β^+	Mediterranean	Orkin et al. 1982
-87	(C→T)	β^+	German/Italian	Kulozik et al. 1991
-87	(C→A)	β^+	Jugoslavian/Black	Dimovski et al. 1991
-86	(C→G)	β^+	Libanees	Kazazian 1990
-86	(C→A)	β^+	Italian	Meloni et al. 1992
-32	(C→A)	β^+	Taiwanees	Lin et al. 1992
-31	(A→G)	β^+	Japanees	Takihara et al. 1986
-31	(A→C)	β^+	Italian	Cao 1994
-30	(T→A)	β^+	Turkish/ Bulgarian	Fei et al. 1988
-30	(T→C)	β^+	Chinees	Cai et al. 1989
-29	(A→G)	β^+	Am. Black/Chinees	Antonarakis et al. 1984
-28	(A→C)	β^+	Kurdish	Poncz et al. 1983
-28	(A→G)	β^+	Chinees	Orkin et al. 1983
-27	(A→T)	β^+	Corsican	Badens et al 1987
Cap site mutations				
+1	(A→C)	β^+	Asian Indian	Wong et al. 1987
+8	(C→T)	β^+	Chinese	Ma et al 1999
+10	(-T)	β^+	Greek	Athanassiadou et al. 1982
+20	(C→T)	?	Bulgarian	Gonzalez et al 1989
+22	(G→A)	β^+	Turkish/Bulgarian/Italian	Öner et al. 1991
+33	(C→G)	β^+	Greek Cypriot	Ho et al, 1996
+43/+40	(-AAAC)	β^+	Chinees	Hunag et al. 1991
Splicing mutations				
IVS-I,(-3)	(C→T) (cd29)	?	Libanees	Chehab et al. 1987
IVS-I,(-2)	(A→G)(cd30)	β^o	Seph. Jewish	Waye et al. 1997
IVS-I,(-1)	(G→C) (cd30)	β^+	Tunesian/Black	Chibani et al. 1988
IVS-I,(-1)	(G→A)	?	Bulgarian	Kalaydjieva et al. 1989
IVS-I-1	(G→A)	β^o	Mediterranean	Orkin et al. 1982.
IVS-I-1	(G→T)	β^o	Asia/Ind./Chinees	Kazazian et al. 1984
IVS-I-2	(T→G)	β^o	Tunesian	Chibani et al. 1988
IVS-I-2	(T→C)	β^o	Black	Gonzales-Redondo et al. 1989
IVS-I-2	(T→A)	β^o	Algerian	Bouhass et al. 1990
IVS-I,	17 nt del (3' end)	β^o	Kuwaiti	Kazazian & Boehm 1988
IVS-I,	25 nt del. (3' end)	β^o	Asia/Indian	Orkin et al. 1983
IVS-I,	44 nt del. (3' end)	β^o	Mediterranean	Gonzalez et al. 1989
IVS-I-5	(G→C)	β^+	Asian/Melanesian	Treisman et al. 1983
IVS-I-5	(G→T)	β^+	Mediterranen/Black	Atweh et al. 1987
IVS-I-5	(G→A)	β^+	Mediteraanean/Algerian	Lapoumeroulie et al. 1986
IVS-I-6	(T→C)	β^+	Mediterranean	Orkin et al. 1982
IVS-I-110	(G→A)	β^+	Mediterranean	Sprits et al. 1981
IVS-I-116	(T→G)	β^0	Mediterranean	Waetherall et al. 1986
IVS-I-128	(T→G)	β^+	Saudi/Arabian	Wong et al. 1989
IVS-I-129	(A→G)	?	German	Vetter et al. 1997
IVS-I-129	(A→C)	β^0	Sri Lankan	Old et al. 1989
IVS-I-130	(G→C)	β^0	Turkish/ Japanese	Öner et al. 1990
IVS-I-130	(G→A)	β^0	Egyptian	Deidda et al. 1990

Position	Mutation	Phenotype	Ethnicity	Authors
IVS-I-130(+1)	(G→C)	β^0	Middle East	El-Kalla et al. 1997
IVS-II-1	(G→A)	β^0	Mediterranena/Tunesian	Chibani et al. 1988
IVS-II-1	(G→C)	β^o	Iranian	Nozari et al. 1995
IVS-II-2	(-T)	β^o	Chinese	Ma et al. 1999
IVS-II-2/3	(-2+11)	β^o	Iranian	Kaeda 1992 (abs.)
IVS-II-4,5	(-AG)	β^+	Portugees	Faustino et al. 1992
IVS-II-5	(G→C)	β^+	Chinees	Jiang et al. 1993
IVS-II-654	(C→T)	β^0	Chinees	Cheng et al. 1984
IVS-II-705	(T→C)	β^+	Mediterranean	Dobkin et al. 1983
IVS-II-745	(C→G)	β^+	Mediterranean	Orkin et al. 1982a
IVS-II-837	(T→G)	?	Asian/Indian	Varawalla et al. 1991
IVS-II-843	(T→G)	β^+	Algerian	Beldjord et al. 1988
IVS-II-844	(C→G)	β^+	Italian	Murru et al. 1991
IVS-II-848	(C→A)	β^+	Black/Egypt./Iranian	Gonzalez-Redondo et al. 1988
IVS-II-848	(C→G)	β^+	Japanees	Hattori et al. 1992
IVS-II-849	(A→G)	β^o	Black	Antonarakis et al. 1984
IVS-II-849	(A→C)	β^o	Black	Padanilam & Huisman 1986
IVS-II-850	(G→C)	β^o	Joegoslavian	Jankovic et al. 1992
IVS-II-850	(-G)	β^o	Italian	Rosatelli et al. 1992
IVS-II-850	(G→A)	β^o	English/ Scottish	Çürük et al. 1995
IVS-II-850	(G→T)	β^o	Japanees	Ohba et al. 1997
Changes in the coding sequences				
cd 10	(GCC→GCA)	β^+	Asian/ Indian	Pawar et al. 1997
cd 19	(AAC→AGC) Asn→Ser (Hb-Malay)	β^+	SE Asian (Malay)	Yang et al. 1989
cd 24	(GGT→GGA)	β^+	Balck/ Japanees	Goldsmith et al. 1983
cd 26	(GAG→AAG) Glu→Lys (Hb-E)	β^+	SE Asian	Orkin et al. 1982
cd 27	(GCC→TCC) Ala→Ser (Hb-Knossos)	β^+	Mediterranean	Orkin et al. 1984
RNA Cleavage en Polyadenation				
poly a	AA<u>T</u>AAA→AA<u>C</u>AAA	β^+	Black	Orkin et al. 1985
poly a	AATAA<u>A</u>→AATAA<u>G</u>	β^+	Kurdish	Kazazian & Boehm 1988
poly a	AAT<u>A</u>AA→AAT<u>G</u>AA	β^+	Mediterranean	Jankovic et al. 1990
poly a	AATA<u>A</u>A→AATA<u>G</u>A	β^+	Malesian	Jankovic et al. 1990
poly a	AATAAA→(-AATAA)	β^+	Arabian	Rund et al. 1991
poly a	AATAAA→(AA--AA)	β^+	Franch	Ghanem et al. 1992
poly a	AATAAA→AATATA	β^+	Black	Giordano et al. (Per. com. '98)
poly a	AATAAA→(A-----)	β^+	Kurd / UAE	Rund et al. 1992
3' UTR				
+1480	(C→G)	β^+	Greek	Jankovich et al. 1991
+1565-67	(-GCATCTGGATTCT)	β^+	Turkish	Ba_ak et al. 1993
+1570	(T→C)	β^+	Irish	Cai et al. 1992

RNA Translation and post-translation defects

Initiation codon mutations

A<u>T</u>G→A<u>A</u>G	β^o	N-European	Waye et al. 1997
A<u>T</u>G→A<u>C</u>G	β^o	Jugoslavian	Jankovic et al. 1990
A<u>T</u>G→A<u>G</u>G	β^o	Chinees; Korean	Kazazian et al. 1990
<u>A</u>TG→<u>G</u>TG	β^o	Japanees	Hattori et al. 1991
AT<u>G</u>→AT<u>C</u>	β^o	Japanees	Hattori (per. com. 1995)
AT<u>G</u>→AT<u>A</u>	β^o	Italian	Saba et al. 1992
A<u>T</u>G→AT<u>T</u>	β^o	Iranian	Nozari et al. 1995

Position	Mutation	Phenotype	Ethnicity	Authors
Stop/Nonsens codon by base substitution				
cd 4/5/6	(T→A/C→T/G→T)	β^o	N-Indian	Agarwal et al. 1997
cd 7	(GAG→TAG)	β^o	Asian/Indian	Kazazian et al. 1984
cd 15	(TGG→TAG)	β^o	Asian/Indian	Kazazian et al. 1984
cd 15	(TGG→TGA)	β^o	Portugees	Ribeiro et al. 1992
cd 17	(A→T)	β^o	Chinees	Chang & Kan. 1980
cd 22	(G→T)	β^o	Reunion Eilands	Ghanem et al. 1992
cd 26	(G→T)	β^o	Thai	Fucharoen et al. 1990
cd 35	(C→A)	β^o	Thai	Fucharoen et al. 1989
cd 37	(G→A)	β^o	Saudi-Arabian	Boehm et al. 1986
cd 39	(C→T)	β^o	Mediterranean	Trecartin et al. 1981
cd 43	(G→T)	β^o	Chinees	Atweh et al. 1988
cd 43	(GAG→TGA)	β^o	Japanees	Oshima et al, 1996
cd 61	(A→T)	β^o	Black	Gonzalez-Rodondo et al. 1988
cd 90	(G→T)	β^o	Japanees	Fucharoen et al. 1990
cd 112	(T→A)	β^o	Slovanian	Divaky et al. 1992
cd 121	(G→T)	β^o	Polisch/Fr./Zw./Jap./Eng.	Kazazian et al. 1986
cd 127	(C→T)	β^o	English	Hall et all. 1991
Stop/Nonsens codon by frameshift				
cd 1	(-G)	β^o	Mediterranean	Rostatelli et al. 1992
cd 2/3/4	(-9+31)	β^o	N-African	Badens et al. 1996
cd 5	(-CT)	β^o	Mediterranean	Kollias et al. 1989
cd 6	(-A)	β^o	Mediterranean/Black	Kazazian et al. 1980
cd 8	(-AA)	β^o	Mediterranean	Orkin & Goff. 1981
cd 7/8	(+G)	β^o	Slovanean	Indrak (pers. comm. 1998)
cd 8/9	(+G)	β^o	Asian /Indian	Kazazian et al. 1984
cd 9/10	(+C)	β^o	Turkish	Aulehla-Scholts et al. 1990
cd 9/10	(+T)	β^o	Greek	Waye et al. 1994
cd 11	(-T)	β^o	Mexican	Economou et al. 1991
cd 14/15	(+G)	β^o	Chinees	Chan et al. 1988
cd 15	(-T)	β^o	Asian/Indian	Baysal et al. 1984
cd 16	(-C)	β^o	Asian/Indian	Kazazian et al. 1984
cd 22	(-AAGTTGG)	β^o	Turkish	Özçelik et al. 1992
cd 24	(-G,+CAC)	β^o	Egyptian	Deidda et al. 1991
cd 25/26	(+T)	β^o	Tunesian	Fattoum et al. 1991
cd 26	(+T)	β^o	Japanees	Hattori et al. 1997
cd 27/28	(+C)	β^o	Chinees	Lin et al. 1991
cd 28	(-C)	β^o	N-African	El Ashemite et al. 1997
cd 28/29	(-G)	β^o	Japanees	Hattori (pers.comm. 1995)
cd 31	(-C)	β^o	Chinees	Ko et al. 1997
cd 35	(-C)	β^o	Malesian	Yang et al. 1989
cd 36/37	(-T)	β^o	Iraanian/Kurdish	Rund et al. 1990
cd 38/39	(-CC)	β^o	Belgian	Heusterspreute et al, 1996
cd 37-39	(-GACCCAG)	β^o	Turkish	Schee et al. 1989
cd 38/39	(-C)	β^o	Tsjechisch	Indrak et al. 1991
cd 40	(-G)	β^o	Japanees	Hattori (pers. comm. 1995)
cd 40/41	(+T)	β^o	Chinees	Ko et al. 1997
cd 41	(-C)	β^o	Thais	Fucharoen et al. 1991
cd 41/42	(-TTCT)	β^o	Chinees	Kimura et al. 1983
cd 42/43	(+G)	β^o	Japaanees	Ohba et al. 1997
cd 42/43	(+T)	β^o	Japaanees	Oshima et al. 1996
cd 44	(-C)	β^o	Kurdish	Kinniburgh et al. 1982
cd 45	(-T)	β^o	Pakistani	El Khalla et al. 1997
cd 47	(+A)	β^o	Black/ Surinamese	Codrington et al. 1990
cd 47/48	(+ATCT)	β^o	Punjaabi	Garewal et al. 1994

Position	Mutation	Phenotype	Ethnicity	Authors
cd 51	(-C)	β^o	Hungarian	Ringelhann et al. 1993
cd 53/54	(+G)	β^o	Japanees	Fucharoen et al. 1990
cd 54	(-T)	β^o	Algerian	Goossens (pers. comm. 1995)
cd 54/55	(+A)	β^o	Asian/Indian	Garewal et al. 1994
cd 56-60	(+14)	β^o	?	Ghaffari et al. (abs. 1997)
cd 57/58	(+C)	β^o	Punjabi	El-Kalla & Mathews 1995
cd 59	(-A)	β^o	Italian	Meloni et al. 1994
cd 64	(-G)	β^o	Zwiss	Chehab et al. 1989
cd 67	(-TG	β^o	Filippinian	Eng et al. 1993
cd 71/72	(+T)	β^o	Chinees	Chan et al. 1989
cd 71/72	(+A)	β^o	Chinees	Cheng et al. 1984
cd 72/73	(-AGTGA, +T)	β^o	English	Waye et al. 1997
cd 74/75	(-C)	β^o	Turkish	Ba_ak et al. 1992
cd 76	(-C)	β^o	Italian	Maggio et al. 1988
cd 77/78	(-C)	β^o	W-African	Pacheco et al. 1998
cd 82/83	(-G)	β^o	Azerbaijani/ Tsjechisch	Schwartz et al. 1989
cd 84/85	(+C)	β^o	Japanees	Hattori (pers. comm. 1995)
cd 84/86	(+T)	β^o	Japanees	Hattori (pers. comm. 1995)
cd 88	(+T)	β^o	Aziatisch Indiaas	Varawalla et al. 1992
cd 95	(+A)	β^o	Thai	Fucharoen et al. 1994
cd 89/90	(-GT)	β^o	Korean	Ohba et al. 1997
cd 106/107	(+G)	β^o	Black	Wong et al. 1987
Termination codon mutations				
cd 144/ 145	(+CT)	Hb-Cranston	Italian	Bunn et al 1975
cd 147	(+AC)	Hb-Tak	SE Asian	Flatz et al 1971

*The authors mentioned in this table are not registered in the references of this thesis.

Dominantly inherited β-thalassemia phenotypes and Hyperunstable Hb's

Position	Mutation	Phenotype		Ethnicity	Authors
cd 24/25	(-GGT)	β^o		Japanees	Hattori (pers. comm. 1995)
cd 28	(C<u>T</u>G→C<u>G</u>G)	β^o	Hb-Chersterfield	English	Thein et al. 1991
cd 30	(G→C)	β^o	Hb-Monroe	N-African	Gonzales-Redondo et al.1989
cd 31/32	(+CGG)	β^o		Spanish	Negri-Arjona et al. 1996
cd 32	(T→A)	β^o	Hb-Medicine Lake		Coleman et al. 1995
cd 33/34	(-GTC)	β^o	Hb-Korea	Korean	Park et al. 1991
cd 60	(G<u>T</u>G→G<u>A</u>G)	β^+	Hb-Cagliari	Italian	Podda et al. 1991
cd 94	(+TG)		Hb-Agnana	S-Italian	Ristaldi et al. 1989
cd 100	(-CTT+11)	β^o		S-African	Williamson et al. 1997
cd 108	(-G)	β^o	Hb-Manhattan	Ashkenazi J.	Kazazian 1990
cd 109	(-ACGTGCTGGTCT)	β^o		Sweedish	Landin et al. 1996
cd 110	(T→C)		Hb-Showa	Japanees	Kobayashi et al. 1987
cd 114	(-CT,+G)	β^o	Hb-Geneva	Franch/Swiss	Beris et al. 1988
cd 114	(T→C)		Hb-Durham	Irish	de Castro et al. 1992
cd 115	(C→A)	β^o	Hb-Hradec	Tsjechisch	Divoky et al. 1983
cd 120	(+A)	β^o		Filipin	Hopmeier et al. 1996
cd 121	(G→T)	β^o		N-European	Kazazian et al. 1986
cd 123	(-A)	β^o	Hb-Makabe	Japanees	Fucharoen et al. 1990
cd 123	(-ACCCCACC)	β^o		Thai	Fucharoen et al. 1994
cd 124	(-A)	β^o		Russian	Çürük et al. 1994
cd 125	(-A)	β^o		Japanees	Hattori (pers. comm. 1995)
cd 124	(+CCA)	β^o		Russian	Çürük et al. 1994
cd 126	(-T)	β^o	Hb-Vercelli	N-Italian	Murru et al. 1991
cd 126	(-17)	β^o		Pakistani	Ahmed et al. 1996
cd 126	(G<u>T</u>G→G<u>G</u>G)	β^o	Hb-Neapolis	Italian	Pagano et al. 1991
cd 127	(CAG→CCG)	β^o	Hb-Houston	N-Italian	Kazazian 1990
cd 127	(C<u>A</u>G→C<u>G</u>G)	β^o		French	Goossens (pers. comm. 1991)
cd 127	(CAG→TAG)	β^o		English	Hall et al. 1991
cd 127	(-AGG)	β^o		Japanees	Fucharoen et al. 1990
cd 128-9	(-4, -GCTG; +5, +CCACA)	β^o	Hb-Gunma		Hattori et al. 1989
cd 132-5	(-11, -AAAGTGGTGGC)	β^o		Irish	Thein et al. 1990
cd 134-7	(-(G)TGGCTGGTGT(G) and +(G)GCAG(G)	β^o		Portugees	Öner et al. 1991

*The authors mentioned in this table are not registered in the references of this thesis.

β-Thalassemia in Iran

As in other part of the world β-thalassemia is the most common genetic disorder in Iran. Figures on the occurrence of the disease have been provided in the last decade by different sources.
According to the 1992 (1372 AH[1]) report made by the "Iranian Thalassemia Association" the financial requirement for a suitable management of the many thalassemia patients in Iran was at that time 220 million US$. To date, considering the significantly increased survival rate of well-treated patients (see publication 4 and 5 in this thesis) and in price of blood products as well as chelation drugs, this figure has raised as much as 10 times. The same 1992 report estimated that 4 babies affected with β-thalassemia major were born each day in Iran bringing the total number of new cases per year to 1.500 [10].
In 1998, the "Charity Foundation for Special Disease" has provided a formal patient registration. According to this foundation at least 20,000 patients are living in Iran. On the other hand the "Iranian Thalassemia Association" reports that at least 26,000 patients are present in the country in 2002 (12). The first figure, which seems to be more realistic than the second, is shared by the official authorities the "Ministry of Health and Medical Education".
Incidence calculations can be made on the base of the known parameters such as carrier frequency, birth and consanguinity rates. On a total population of 66 million with a birth rate of almost 2.000.000 newborn each year and an incidence based on an average carrier frequency of 5%, the incidence will be comprised between a lower figure of 0.6:1000 in the cases of no consanguinity and an upper figure of 2.2: 1000 in case of a high consanguinity rate. Considering an average consanguinity rate between 30 and 40% as usual in other Islamic societies this calculation would take us to a presumed average incidence of about 0.8:1.000 and to a total of 1500 affected children per year in agreement with the direct observations made in 1992. Obviously, these figures underline the impact of these diseases on the individuals, the society and the public health management and economy of the country.

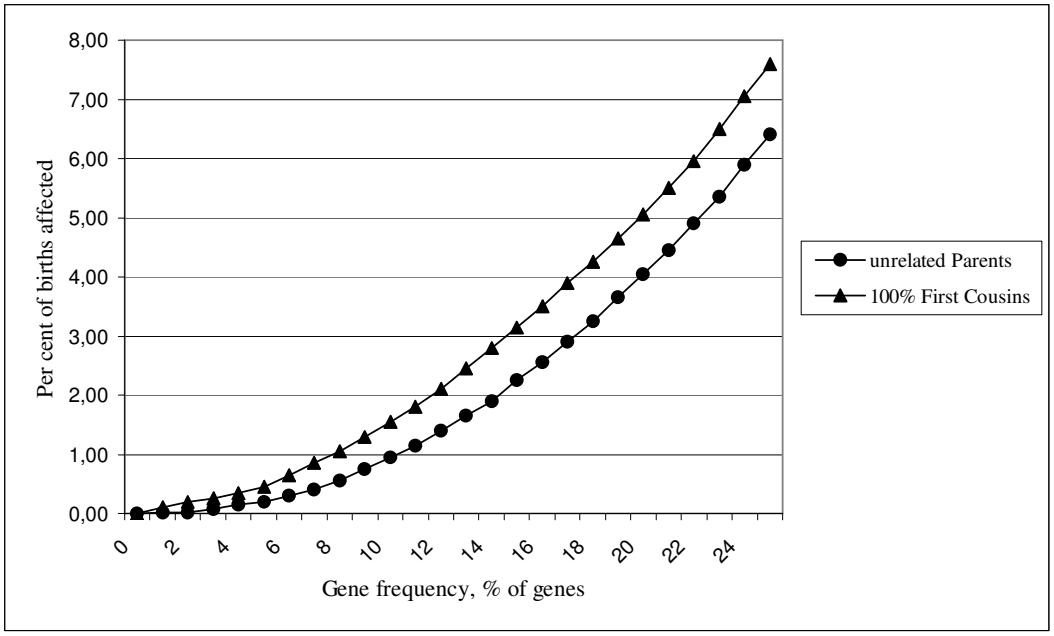
Regional, social and customary differences

Certain regional, cultural and geographical considerations markedly affect the prevalence and management of genetic disorders. Consanguinity is common in most regions of the country. Depending on the area cousin marriage vary from about 10% in the large cities to as much as 70% in the countryside [13]. In addition, populations especially around the south and eastern part of Iran are often small and isolated, favouring a high consanguinity rate. Consanguinity approximately doubles the risk for homozygosity for frequent autosomal recessive conditions such as thalassemia (in general from 2-3:1.000 to about 5:1.000). However, consanguinity may increase as much as 7 times the risk for homozygosity for very rare recessive traits (Fig. IV-2). Although increased risk due to consanguinity may be considered modest in general it can be responsible for high morbidity when β-thalassemia trait is already present in a family and a consanguineous partner is chosen.
The highest rate of thalassemia major is measured around the Caspian Sea in the north of the country and in the Persian Gulf in the Southern area (table IV-2). Expressed in patients: 10,000 individuals, the following figures are measured in the province of Hormozgan (13.5), Mazandaran (13.5), Kohgeloye-va-Boyerahmad (8.0), Bushehr (6.3), Sistan-va-Baluchestan (5.9), Eilam (5.6), Fars (5.4), Kerman (5.3), Gilan (5.1),

1 Solar Iranian calander

Khuzestan (3.9), and Chaharmahal-Bakhtyari (3.5). West-Azerbaijan, with an incidence of 0.2: 10,000 and the lowest carrier frequency (1.4%) is the province with the lowest number of patients.
Hormozgan and Mazandaran, with 9.7% carrier frequency is the top of prevalent regions**.** According to calculations made in the UK [14] each patient needs at least 100,000 US$ for treatment annually. As mentioned in the previous paragraph, considering the prolongation of life expectancies and the ever rising price of cure also in Iran, approximately 2 billions US$ will be needed for an appropriate management of thalassemia patients each year in Iran.

Fig IV-2 Relationship between gene frequency and homozygote births, parents unrelated or 100% first cousins. Adapted from "Prevention of Thalassemias and other Hemoglobin Disorders" [42].



The molecular background of β-thalassemia in Iran

Recent studies on the Iranian β-thalassemia genes have revealed the presence of more than 43 different β-globin gene mutations. Such a great mutational diversity reflects the multiethnicity of the population and poses problems in designing prevention programs (see publication 1 in this thesis).
Although to date, more than 200 β-thalassemia mutations have been described worldwide, a limited number of alleles accounts for the inactivation of most β-globin genes in each specific population or ethnic group [15]. While it is estimated that about 3% of the world population is carrier of a β-thalassemia gene [16], in Iran, about 3.5% of the total population is a healthy carrier but with peaks up to 7% in high prevalence regions (Table IV-2.).
According to the published reports [17-26] and personal unpublished data, collectively 2.247 β-thalassemia alleles have been studied up to now in different populations of different Iranian provinces (data are summarized in table IV-3).
Although, from these data it appears that each province has its own specific mutation spectrum reflecting the ancient and recent ethnic background of the particular geographic area, the prevalence of some mutations can be considered common to the whole country.

Table IV-2. Population, number of registered patients, prevalence of β-Thalassemia major and estimated carrier frequency in 28 Iranian provinces.

Province	*Population* [a]	*Thalassemia patients* [b]	*Patients (Per 10,000)* [c]	*Tthaassemia. carriers (interval of confidence 95% CI)*	
Este-Azarbaijan	3,325,540	115	0.3	38,498	39,266
West-Azerbaijan	2,496,320	55	0.2	23,027	23,623
Ardebil	1,168,011	85	0.7	19,485	20,031
Esfahan	3,923,255	1,173	3.0	132,626	134,033
Elam	487,886	275	5.6	22,328	22,904
Bushehr	743,675	470	6.3	36,086	36,816
Tehran	10,343,965	2,263	2.2	300,410	302,530
Chaharmahal-Bakhtiyari	761,168	266	3.5	27,605	28,248
Khorasan	6,047,661	499	0.8	108,230	109,512
Khuzestan	3,746,772	1,460	3.9	144,271	145,735
Zanjan	900,890	50	0.6	4,021	4,268
Semnan	501,447	84	1.7	12,593	13,031
Sistan&Baluchestan	1,722,579	1,023	5.9	81,364	82,459
Fars	3,817,036	2,048	5.4	171,939	173,531
Qazvin	968,257	106	1.1	19,775	20,324
Gom	853,044	140	1.6	21,292	21,861
Kurdistan	1,346,383	124	0.9	25,283	25,905
Kerman	2,004,328	1,054	5.3	89,243	90,391
Kerman shah	1,778,596	271	1.5	42,964	43,770
Kohgiloyeh-Boyerahmad	544,356	438	8.0	29,676	30,336
Golestan	1,426,288	224	1.6	34,937	35,664
Gilan	2,241,896	1,142	5.1	98,311	99,516
Lorestan	1,584,434	183	1.2	33,334	34,046
Mazandaran	2,602,008	3,506	13.5	183,203	184,824
Markazi	1,228,812	120	1.0	23,745	24,347
Hormozgan	1,062,155	1,436	13.5	74,719	75,755
Hamedan	1,677,957	120	0.7	27,814	28,466
Yazd	750,769	83	1.1	15,379	15,864
Total population	60,055,488	18,813	3.1	2,076,174	2,081,727

[a] = According to the 1996 population census (source: Iran Statistics Centre.
[b] = According to registration report of "*CHARITY FOUNDATION FOR SPECIAL DISEASES*".
[c] = Estimated by Hardy-Weinberg Equilibrium for 2002.

The IVS-II-1 (G→A) mutation

This mutation represents the most common β-thalassemia defect in Iran. The splice junction mutation IVS-II-1 (G→A), firstly reported in an Iranian family in 1995, produces a β°-thalassemia phenotype. The 5' β-cluster haplotype analysis has shown that this mutation may occur on 5 different haplotypes (personal unpublished data). When compared to Mediterranean areas and other regions where this mutation occurs, the IVS-II-1 (G→A) has the highest frequency in Iran (30.8%) with peaks in the northern and central areas (Tehran) of 49.4% and 42.5% respectively [28, 18]. The frequency gradually declines in the south (12%) and the west (6.2%). In the United Arab Emirates (UAE) the frequency is 6% [29]. The high frequency and multiple haplotypes indicate that the IVS-II-1 (G→A) mutation is a native and one of the oldest β-thalassemia defects in Iran.

Table IV-3. Frequency of β-thalassemia mutations described in 2,247 chromosomes in the Iranian population (classified according to Thein SL. [9]).

Defect	Type of Mutation	Frequency (%)
Transcriptional Mutants		
- 88(C-A)	β+	0.30
- 30 (T-A)	β+	0.15
- 28 (A-C)	β+	0.15
-101 (C-T)	β+	0.11
- 87 (C-G)	β+	0.08
5' UTR +22 (G-A)	β+	0.04
RNA processing		
➢ **Splice Junction site**		
IVS-II-1 (G-A)	β°	30.62
IVS-I-1 (G-A)	β°	6.54
IVS-I, 3' end del 25 nt	β°	3.58
IVS-I-130 (G-A)	β°	0.42
IVS-I-130 (G-C)	β°	0.41
IVS-II-850 (G-T)	β°	0.08
IVS-I-2 (T-C)	β°	0.04
➢ **Consensus Sequence**		
IVS-I-5 (G-C)	β+	22.19
IVS-I-6 (T-C)	β+	2.95
IVS-I (-1) (G-C)	β°	1.49
IVS-I-128 (T-G)	β+	0.15
➢ **Cryptic splice sites in introns**		
IVS-I-110 (G-A)	β+	7.60
IVS-II-745 (C-G)	β+	1.81
IVS-II-2, 3(+11/-2)		0.08
IVS-II-654 (C-T)	β+	0.08
IVS-II-1 (G-C)	β°	0.07
Non-functional mRNA		
➢ **Nonsense**		
cd 39 (C-T)	β°	1.61
cd 15 (TGG-TGA)	β°	0.11
cd 15 (TGG-TAG)	β°	0.15
cd 95 (AAG-TAG)	β°	0.04
➢ **Frameshift**		
cd 8 (-AA)	β°	7.77
cd 8/9 (+G)	β°	2.80
cd 36/37 (-T)	β°	1.52
cd 22–24 (-AAGTTGG)	β°	1.45
cd 44 (-C)	β°	1,44
cd 5 (-CT)	β°	1.33
cd 16(-C)	β°	0.45
cd 41/42(-TTCT)	β°	0.44
cd 82/83 (-G)	β°	0.34
cd 15(-T)	β°	0.08
cd 25/26(+T)	β°	0.08
cd 24/25(-GGT)	β°	0.04
cd 42/43 (+G)	β°	0.04
Initiator codon		
Init codon (ATG-ACG)	β°	0.26
Init codon (ATG-ATT)	β°	0,04
RNA cleavage and polyadenylation		
AATAAA-AATAAG	β+	0.23
Cap site mutants		
Cap+1 (A-C)	β+	0,04

The other frequent mutations

The data reported in Table IV-3, shows however that β-thalassemia in Iran can be induced by at least 43 different mutations. The predominant IVS-II-1 (G→A) mutation is followed in decreasing order of frequency by the IVS-I-5 (G→C), the frame shift codon 8 (-AA), the IVS-I-110 (G→A), the IVS-I-1 (G→A), the 3'del 25 nt, the IVS-I-6 (T→C), the codon 5 (-CT), and codon 39(C→T) mutations. This group of mutations covers more than 85 % of the total β-thalassemia defects present in the country.

On average 10.3% of β-thalassemia alleles is still not characterized so far. In the same table the mutations, reported with their allele frequencies, are subdivided into five categories according to the defect type. From the 10 most common alleles six of them are β° type with total loss of expression. The remaining four are of the β^{+} type. As it is observed in most populations the common mutations are usually located around exon 1, IVS-I, and exon II of the β-globin gene.

The IVS-I-5 (G→C) substitution, a severe β^{+} mutation, is the most common allele in the south of the country. This mutation, defined as "Asian/Indian" defect, is very common in a belt comprising the regions of the Indian subcontinent, the UAE (Emirates), Oman and the south of Iran. This reflects the ancient trade between India and the Arabic peninsula and the influence of the Arabic culture. While in Hormozgan and Sistan-va-Balochestan this mutation is presented at very high frequencies (69%, 68% respectively), it decreases in western Iran (26.4–26.6%) and almost desappears in central and northern Iran (<1%). Interestingly, the frequency of this mutation is about 1% in east and southeast Turkey, close to Iran and Iraq [26].

The dinucleotide deletion at codon 8 (-AA), originally described in a Turkish patient [29], occurs mostly in the southwest (17.3% in Khuzestan) [17] and the west part of Iran. This defect, causing a framshift and a premature termination at codon 21 (TGA), has been reported at high frequencies in the neighboring Republic of Azerbaijan [30] at the northwest borders.

The IVS-I-110 (G→A) mutation, the defect occurring of the highest frequency in Cyprus (79.7%) [32], is equally common in most regions of the country (7.3%) but is more represented in the West. This might reflect gene flow from Greece (42.4%) [33] and Turkey (39.3%) and the area of the actual Republic of Azerbaijan (20.2%) and may represent the influence of a century of Persian domination in Anatolia and the development of trade routes between these areas [33]. The higher frequency of this mutation in the southwest (23%) and northwest (18.1%) [18] may reflect a Balkan origin and a gradual gene flow dating from the invasion Alexander and the Seleucid dynasty 312-247 B.C.

The IVS-I, 3'end (-25nt) is also one of the prevalent mutations in the southwest of the country (7.7%). The frequency for this mutation slowly declines from the southwest (Khuzestan) towards the southeast (Hormozgan 1%). A similar pattern is observed for the IVS-I-6 (T→C) mutation (10.5% in the southwest, 6.2% in Boushehr, and 0.4% in Hormozgan [17, 25].

The severe β^{+} mutation at IVS-II-745 (C→G) is a classical Mediterranean mutation which remains confined to the South of Iran. The presence of this mutation may be associated with Jewish migrations to Iran during the Khosro 1st Empire.

Similarly the Mediterranean cd39 (C→T) mutation [38] is mainly found in north and central Iran but is less frequent elsewhere. Possibly originating from the Balkan countries, (Greece, Bulgaria, Romania, and former Yugoslavia), it is almost solely found on the island of Queshem in the south of Iran where founder effect and isolation may have led to the unicity of this defect.

Another interesting observation is the significant concentration of the rare Indian Asian β° cd 8/9 (+G) promoter mutation. While the overall frequency is less than 1% in Iran, two peaks are observed in two particular areas, one in the northeast (Khorasan) and the other in the center of the country (Kherman and Yazd) (62.5%, 11.7% respectively) [18]. This concentration might reflect two circumstances; one connected to the secular trade along the great silk road which extends from Xian in China through the Indian subcontinent to Iran and the East Mediterranean; the other to the invasion of the Mongols (1220 AD) and the Tatars (1380-87 AD)**.**

A comparative distribution of β-thalassemia mutations between Iran and other cultural-related areas is summarized in table IV-4.

Table IV-4. The prevalence (%) of the most common β-thalassemia mutations compared among several populations of the eastern Mediterranean, and Middle-East areas

Mutation	**Cyprus**	**Greece**	**Turkey**	**Bulgaria**	**Lebanon**	**Azerbaijan**	**Iran**
IVS-I-110 (G→A)	79.7	42.4	39.3	24.2	48.5	20.2	**7.3**
IVS-I-6 (T→C)	6.2	7.2	10.1	10.2	6.9	7.1	**3.2**
cd 8 (-AA)	0.2	0.6	5.5	5.5	3.4	21.2	**8.4**
IVS-I-1 (G→A)	5.9	13.2	5.0	3.1	9.8	2.0	**7.2**
IVS-II-745 (C→G)	5.5	6.9	5.0	10.2	3.4	3.1	**1.4**
cd 39 (C→T)	2.4	17.0	3.8	21.9	1.5	2.0	**1.6**
cd 8/9 (+G)	—	0.3	1.3	5.5	—	2.0	**0.5**
Others	0.1	12.4	30.0	19.4	26.5	42.4	**67.1**
References	[33]	[16]	[27]	[28]	[30-32]	[18]	**This study**

Conclusion

A comparison between different regions of Iran shows that the distribution of β-thalassemia mutations differs strongly in type and frequency. When the regional mutation spectra are compared with the overall distribution in the country it can be noticed that the complex multietnicity is causing significant variation. The western and southern parts of Iran, respectively inhabited by Kurdish and Arab populations have a characteristic distribution. Likewise the northern and eastern part shows different population-specific profiles. The limited spread of mutations reported for the northern and eastern regions seems to indicate a more ancestral profile than the western and southern coastal parts of the country, which display a greater heterogeneity because of the recent Arab influence generating new populations of mixed origin. However, due to the different technical approach used by different investigators in determining the regional mutation spectra (reverse dot blot versus DGGE or sequencing) it is difficult to establish the occurrence and the real distribution for the less common mutations.

α-Thalassemia

The α-thalassemias are considered to be the most common hemoglobin defects worldwide. Due to the presence of two genes on each cluster three α-Thalassemia phenotypes can be expected.

1. The mild phenotype, which is induced by three different molecular conditions.
   - The lack of expression of a single gene on one cluster, which is called α^+-thalassemia heterozygosity (-α/αα).
   - The homozygosity for the α^+ condition (-α/-α)
   - The lack of expression of both α-genes on one cluster which is called α°-thalassemia heterozygosity (--/αα)
2. The intermediate phenotype, which is induced by the following condition:
   - Double heterozygosity for the α+ and the α° thalassemia genotype (--/-α) also called HbH disease because of the formation of HbH β4 homotetramers during postnatal life.
3. The severe phenotype
   - Induced by homozygosity for the α°-thalassemia cluster (--/--), also called HbBart's hydrops fetalis syndrome because of the formation of HbBart's γ4 homotetramers in intrauterine life, is associated with perinatal death.

The genotype / phenotype correlation is summarized in table IV-5.

Table IV-5. The correlation between α-globin genotype and phenotype

Genotype	Phenotype
Normal (αα/αα)	No anemia
α^+ Thalassemia heterozygous (-α/αα)	Milde microcytic hypochromic parameters
α^+ Thalassemia homozygous (-α/-α) or α°-Thalassemia heterozygous (--/αα)	Milde microcytic hypochromic anemia
α^+/α° combination (--/-α)	HbH disease, intermediate hemolytic anemia
α° Thalassemia homozygous (--/--)	Hb Bart's, hydrops fetalis severe prenatal hemolytic anemia

The last phenotype is the most severe form of hemoglobinopathy producing a fatal hemolytic anemia and hydrops fetalis resulting in perinatal death.

HbH disease may present with variable phenotypes depending from the type of mutations.

Although α^+ and α°-thalassemia are usually the result of deletion defects, about 10-20% of the cases may be induced by point mutation (40). Combinations of point mutation defects such as those at the polyadenylation site may, produce, in combination with α°-thalassemia deletional alleles, severe or intermediate HbH diseases requiring transfusion therapy [42].

Unlike β-thalassemia, the severe α-thalassemia pathology is present at and before birth. This is because α-chains are essential to the formation of almost all hemoglobins during ontogeny including the embryonic tetramer HbGower 2. Thus, fetuses affected with the --/-- genotype can only produce the early embryonic HbGower1 (ζ2/ε2), Hb-Portland (γ2/ζ2) and the non-functional HbBart's (γ4) while the normal and essential HbF (α2/γ2) and HbA (α2/β2) cannot be formed.

The molecular background of α-thalassemia

The α-globin gene cluster comprises a DNA segment of about 30 kb in length on the short arm of chromosome 16 (16p 13.11-13.33). The two active α genes are embedded within two duplicated sequences of 4 kb in length. The region is divided into highly homologous segments named X, Y, and Z, alternated by three non-homologous segments (I, II, and III). Unequal crossing over between X or Z homologous segments give rise to deletions generating α^{+}-thalassemia alleles. Most common are the $-\alpha^{3.7}$ (Rightward) and the $-\alpha^{4.2}$ (Leftward) deletions which are selected by malaria.
Both deletion fragments still contain a functional α-gene with different expression. (Fig. IV-3_{a-b}). Their counterpart, the $\alpha\alpha\alpha^{anti\ 3.7}$ and the $\alpha\alpha\alpha^{anti\ 4.2}$ triplications may aggravate the otherwise mild heterozygous β-thalassemia state due to the increase in free α globin in the red cell and red cell precursors.

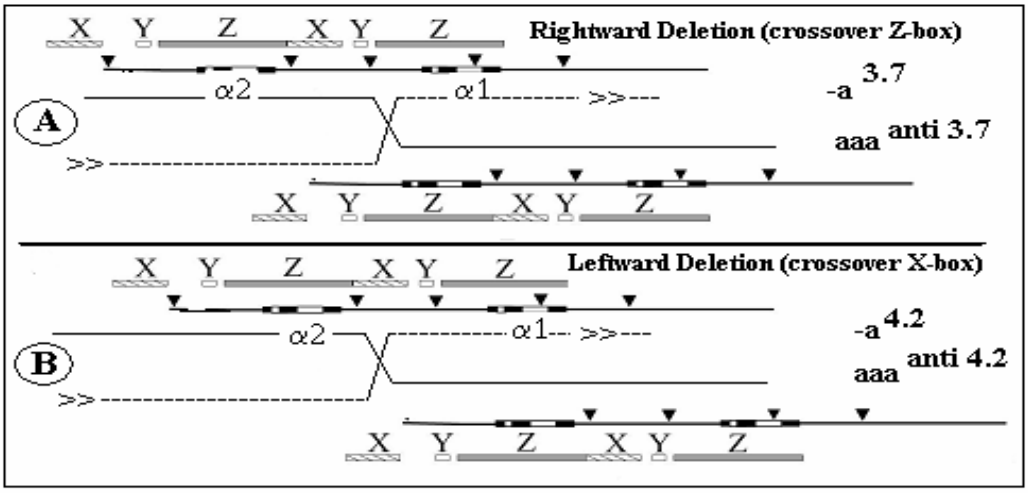


Fig. IV-3a-b. Schematic representation of homologous recombination events between the duplicated α-globin genes. The ApaI restriction sites (▼) are indicated along the fragment. (A) The rightward deletion ($-\alpha^{3.7}$) and ($\alpha\alpha\alpha^{anti3.7}$) resulting from unequal crossing over between misaligned Z boxes. (B). The leftward deletion ($-\alpha^{4.2}$) and ($\alpha\alpha\alpha^{anti4.2}$) resulting from unequal crossing over between misaligned X boxes.

Larger deletions may arise by different mechanisms taking away both α genes of one allele generating various α°-thalassemia genotypes such as the common Southeast Asia (SEA) Mediterranean (MED I and II) and Philippino (Phil) deletions and many others [38,39].
As mentioned before, although most α-thalassemia defects are due to large deletions, single point mutations or small deletions and insertions are not infrequent. [39]. Such defects as for instance the polyadenylation site mutations mentioned above are regularely observed in microcytic hypochromic carriers and produce an $\alpha^{o/+}$-thalassemia allele. These α-thalassemia point mutations are generally indicated as α^{T}. Other frequently occurring non-deletion α- thalassemia defects are mutation on the termination codon generating elongated α chains such as Hb-Constant Spring (Hb^{CS}) and Hb-Icaria. Hb^{CS} is a well-studied variant, usually detectable as a discrete band migrating slower than HbA2 at alkaline pH electrophoresis. Hb^{CS} is regularly found in Southeast Asian populations and less frequently in Mediterranean. The Hb^{CS} mutations found in the two populations originate from independent events [40-41].

α-Thalassemia in Iran

Although HbH disease and Hb Bart's Hydrops Foetalis do regularly occur in Iran, the epidemiology of α-thalassemia defects and their impact on public health are not fully documented. Moreover, α-thalassemia is one of the most significant modulating factors in β-thalassemia major. Therefore a screening of cord blood samples in the Hormozgan region has been conducted to assess the molecular spectrum of these defects in the areas of interest. By monitoring the presence of HbBart's at birth in 660 cord bloods a pre-selection of potential α-thalassemia carriers was done and the molecular background of the disease was estimated. Similarly, by characterizing 13 cases of HbH disease the occurrence of α° thalassemia defects was studied. The molecular spectrum of α-thalassemia is described in publication 2 and 3 in this thesis.

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Chapter V

Hemoglobinopathies and therapy

Hemoglobinopathies and therapy

The homozygous state for β-thalassemia (Cooley's anemia, β-thalassemia major) is a disease in which the total absence of β-globin expression leads to severe anemia. As a recessive trait protecting against malaria, β-thalassemia minor (the heterozygous state) is very frequent in many populations. World wide this leads to about 300,000 newborn each year affected by the major forms of the disease.
The absence of β-globin expression causes severe chronic dyserythropoeisis, hemolysis and hypoxia leading to profound anemia and organ damage. Untreated children usually die between the ages of 4 to 7 years.

The supportive "state of the art" treatment

The current supportive treatment of the severe conditions consists of suppressing the authologous and ineffective erythropoiesis by frequent blood transfusions keeping the Hb levels at near normal values. Transfusion therapy and splenectomy reduce hemolysis and hypoxia to a level at which severe complication usually do not occur before the age of 20. Although this treatment eliminates the severe early pathology it induces progressive iron overload leading to organ damage, hormonal dysbalance and, in the end, to severe cardiac insufficiency. Therefore additional chelating therapy by subcutaneal infusions of Desferal© and or by oral chelating agents Ferriprox© is essential for the "state of the art" treatment of β-thalassemia major. Unfortunately, supportive blood transfusions and chelation therapy are exceedingly expensive in many developing countries. In addition, primary prevention being not available, affected children are born and die at young age.

Bone marrow transplantation

Strenuous and continuous researches have been ongoing in the last decades in search of a cure for β-thalassemia major and sickle cell disease. Many efforts have focussed on improving bone marrow transplantation (BMT) or stem cell transplantation (SCT) [1]. Also the development of technology in stem cell gene therapy and the search for new drugs have been ongoing with variable success. [2-4] Bone marrow transplantation has brought the most significant improvement and has cured many patients transplanted with HLA identical sibs. Unfortunately this option is not available to the majority of the patients and the risk of failure using non-HLA identical donors is still too high to be an acceptable alternative too transfusion / chelation therapy.

The modulating factors

Many factors may modify the phenotype of a patient influencing the pathology expected according to the genotype. Most of these modulating factors are determined by concomitant molecular elements. Some of these are in linkage to the β-gene cluster (*cis*), others are not (*trans*), some are α-gene related, others are related to different molecular factors located on elsewhere in the genome, not directly associated with globin genes expression, but influencing expression, hemolysis proteolysis or other known or unknown factors.
Common polymorphisms, inducing a higher postnatal HbF expression, will be discussed in more detail further in this chapter. Among the most common non β-cluster related modifier factors are the coexisting α-thalassemia defects and the α-gene

triplications. The α-thalassemia defects may reduce the pathology of the severe β-homozygous or compound heterozygous genotype due to the lower amount of free α-chains in the red cells and consequently a milder hemolysis. The α-triplications may, for the opposite reason, aggravate the pathology in β-thalassemia traits that should not suffer of hemolysis at all. Therefore, many efforts have been dedicated in this thesis not only to characterize the molecular spectrum of β-thalassemia but also to estimate the frequency and genotype of α-thalassemia in large areas of the country (publications 2 and 3 in this thesis).

The most significant non-globin gene related factors are the G6PD deficiency (discussed in publication 7) and the effect of Hereditary Hemochromatosis (discussed in publication 6). Another important non-globin gene related factor is the red cell membrane defects. We are intending to examine these factors later on due to the critical element of screening on extremely fresh blood sample and the very extensive molecular diagnostics needed to characterize these diseases.

The most significant β-cluster-related modulating factor remains the γ-gene expression control as a compensatory element of the shortage of β-globin expression in β-thalassemia major and intermedia. Therefore, beside BMT many efforts have concentrated on the development of an effective therapy in this direction to increase the hemoglobin levels in β-thalassemia major patients without blood transfusion (publication 4 of this thesis). Such an option would allow normal growth and development and eliminate transfusional iron overload, which remains the major cause of reduced life expectancy and morbidity.

HbF therapy

Patients affected by β-thalassemia major are born as healthy babies due to the regular expression of their γ-genes providing plenty of perfectly functioning fetal hemoglobin (HbF = γ2/α2). Unfortunately the physiological switch after birth shifting the fetal γ genes expression to the (affected) β gene marks the beginning of the disease. A lot of research efforts have been focused on the switch from fetal to adult genes expression trying to understand and, in the end, to regulate the mechanism. In spite of the fact that the switch mechanism has been fairly explained [5-11], little or no success has been booked in influencing this mechanism [12-15] at the gene control level. However, a number of drugs capable of inducing a temporary increase in γ-gene expression during postnatal life have been studied. These chemotherapeutic agents include hydroxyurea, butyrate analogs, 5-azacytidine, recombinant human erythropoietin, and combination of the various agents. Only the first two have been applied with good results. HU in sickle cell disease and gradually also in β-thalassemia (publication 4 in this thesis) is today an accepted therapy. Due to several adverse practical factors (high dosage and toxicity) butyrate remained in an experimental phase. Both drugs are briefly described in the next two paragraphs.

Hydroxyurea

At first, hydroxyurea (HU) is synthesized by the German chemists W.F.C Dresler and R. Stein, in 1869 [16], a rather simple compound ($CH_4N_2O_2$) (Fig. V-1). Its therapeutic effect was not recognized until the early 1960's. Currently HU is used to treat a variety of malignancies, as well as inhibitor for viral replication in patients affected by human immunodeficiency virus type 1 (HIV-1). In treatment of hemoglobinopathy HU is used to reduce crises in sickle cell disease and to decrease the need for blood transfusions in thalassemia intermedia patients.

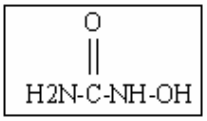

Fig. V-1: The formula of hydroxyurea

During cell division and DNA replication the enzyme ribonucleoside diphosphate reductase (RDR) converts some of the cell ribonucleotides supplies into deoxyribonucleotides. At this point DNA synthesis can begin. The primary effect of HU consists of inhibition of RDR as a "free radical quencher", destroying a tyrosyl free radical that is formed in the catalytic center of the enzyme [17]. Consequently, RDR catalysies the reductive conversion of ribonucleotides to deoxyribonucleotides, components of DNA. This conversion is the rate-limiting step in DNA synthesis. Although HU inhibits DNA synthesis, it has been shown that it has no effect on transcription or translation. HU is specific only for cells in S phase of the cell cycle and cell cultures treated with HU arrest at G1-S interface [18]. Hydroxyurea binds neither chemically nor physically to DNA.

Some cells develop the ability to resist HU's effect and several hypotheses are postulated on this matter. Two explanations appear to be the most accepted: 1) Loss of affinity between HU and RDR and loss of enzyme inhibition [19]. 2) Some cells produce more RDR due to adaptation and amplification of gene expression [18].

Oncogenic risk eventually related to HU treatment was tested in mice by intraperitoneal administration beginning at two days of age. No increase in incidence of tumors was reported. HU has also been tested in combination with other chemical carcinogens to assess the effect of inhibition of DNA synthesis on carcinogenesis. In one study of patients treated with hydroxyurea for essential thrombocytemia who developed leukemia, a statistically non-significant association was found with a 17p chromosomal deletion in leukemic cells. Hydroxyurea does not induce gene mutations in bacteria and does not cause mutations at the HPRT locus in mammalian cells. HU causes chromosomal mutations has a mutagenic effect at the Tk locus in mouse lymphoma cells and is an effective recombinogenic in yeast and induces sister chromatid exchange in mammalian cells. It also induces amplification of gene expression in mammalian cells and may lead to transformation of some but not all cell lines. Although it has been reported to be ineffective in causing germ-cell mutations, it has not been extensively tested for this last point. [20].

In 1984, [21] Platt et al did the first γ-globin genes stimulation trial by Hydroxyurea on sickle cell disease patients. In 1997 Arruda VR et al. reported their trials on β-thalassemia major patients [22]. These first data were followed by a long list of trials usually reporting no long-term major side effects [23]. However, in a study on Polycytemia Vera, 20% patients of the patients under HU treatment developed leukemia after average treatment duration of 8.4 years [24]. Again in Polycythemia Vera patient's chromosomal abnormalities were reported after HU treatment and radiation therapy with P^{32} [25, 26].

In a French study among pregnant SCD patients, HU treated during the first, second and third trimester, no malformations or malfunctions were reported [27].
Others studies [28,29] also concluded that potentially adverse effects on the fetus are not very high and probably overestimated. Like all drugs however, the benefit of HU do not come without side effects. Occasionally, nausea, vomiting, mouth sores and ulcers, diarrhea and skin discoloration and some degree of leukopenia were reported after high dosage but usually these are transient effects [30].

Butyrate compounds

In 1985 Bard H et al. demonstrated that the newborn infants of diabetic mothers are synthesizing significantly more fetal hemoglobin than is expected for their stage of development. They suggested that the in uterus-environment of the fetus of the diabetic mother causes an increase in fetal hemoglobin synthesis [31, 32]. While sodium phenyl butyrate was used for many years as a treatment for urea cycle disorders, an experiment on human K562 cell lines, showed an increase in fetal hemoglobin expression [33]. These finding led to clinical trial of this compound in patients with sickle cell disease [34,35] and thalassemia [36]. Butyrate and its analogues are derivatives of natural fatty acids. There is considerable evidence that butyrate analogues induce erythroid differentiation and stimulate HbF production in human erythroid progenitors in vitro. This effect has also been shown in vivo in different animal models, inducing reactivation of embryonic globin production in chicken [37], delaying the switch from fetal to adult globin in ovine fetuses [38], and increasing HbF production in adult primates [39]. In humans, several fatty acids including α amino-butyric acid [8], arginine butyrate [33], isobutyramide [40], sodium phenylbutyrate [41], propionic acid [35], and 2-propylpentanoic (dipropylacetic) acid [36], have now been shown to stimulate HbF production, indicating a potential therapeutic role in the treatment of the β-globin disorders.
Butyrate is presumed to stimulate HbF expression by acting at sequences near the transcriptional start site of the γ-globin gene promoter [36,42]. Clinical trials with intravenous arginine butyrate have shown an increase in Hb F production, but inconsistent responses in increasing the total Hb level [34, 35]. Sodium phenylbutyrate (SPB), an oral drug derivative of butyric acid, has also been tested on patients with sickle cell disease and β-thalassemia [35,36]. In β-thalassemia, the response was limited to patients who were not transfusion-dependent (thalassemia intermedia) and the results were not predictable [36].
Therapeutical interventions with sodium phenylbutyrate (SPB) are difficult. Adult patients must ingest 35–40 tablets per day. These are therapeutical dosage very near to the toxic limit, resulting in significant dyspepsia, epigastric discomfort and problematic body odor. These discouraging results may limit the practical use of this drug, however, other doses or schedules of administration may improve the applicability of butyrates [43].

The genetic influence on postnatal HbF and F-cell concentration

The switch from fetal to adult hemoglobin synthesis briefly mentioned in chapter I, occurs just before birth and does not lead to a total extinction of fetal hemoglobin (HbF) expression in adult life [44]. A small amount of HbF containing red cells persists in postnatal life [45] and generally there is a correlation between the HbF concentration in postnatal life (0.5-1%) and the number of HbF cells (FC) in healthy adults [5]. Although, HbF and FC values may vary considerably also in abnormal conditions [5,8,46, 47], significant high values are found in conditions referred to as hereditary persistence of fetal hemoglobin (HPFH). Conversely, limited increases and heterocellular distribution of HbF are measured in the so-called “Swiss type” HPFH.

This name is used to classify a heterogeneous group of multifactorial quantitative traits by the similar phenotype, quite distinct from the pan cellular HPFH defects that are caused by deletions of the β-globin gene complex or defined point mutations in the promoters of the γ-globin genes [44]. Although family studies have shown that high levels of HbF and FC are usually inherited, the number of genetic factors involved in the effective expression might be complex and multifactorial.
Several factors have been shown to influence HbF and FC levels in healthy adults, including age [46], sex [5, 46] and genetic variants linked [8] and unlinked to the β-globin locus on chromosome 11p [11, 51]. Two trans-acting quantitative trait loci (QTLs) for FC variability have been mapped, one on chromosome 6q in an extensive kindred with heterocellular HPFH and β-thalassemia [49] and the other on Xp in families with sickle cell disease [12]. Recently, studies in a large English family indicated the presence of at least one other *trans*-acting QTL associated with Hb F and FC variability [42]. It is clear from the isolated family studies that there are several such QTLs for HbF and FC, but their frequency in the general population and contribution to the FC variance are not clear.
Genetic association has been found between *Cis*-acting elements and the HbF and FC expression. Those elements are the $(AT)_x$ Ny $(AT)z$ motifs within the 5'hypersensitive site 2 (HS2) of the β –globin locus control region (βLCR) [12]. In addition the $(TG)_n$ $(CG)_m$ conformation on Aγ- and Gγ-gene IVS-II [13], the –158 polymorphism 5' of the Gγ gene [13] and the $(AT)_x$ T_y repeat conformation in the 5' promoter region of β-gene [14] .

Gene therapy and the Locus Control Region (LCR)

Activation of HbF expression during post-natal life would be the ultimate gene therapy solution for patients affected by thalassemia major and sickle cell disease. Therefore a great deal of studies dedicated to the HbF expression has been conducted during the past years and are still ongoing. In relation to the therapeutic possibilities available at present and therapeutic trials conducted in this thesis (publication 4), a brief summary of these studies mainly focused on the function of the locus control region (LCR) and the 5 sub-elements (HS1 – HS5) comprised in it, are summarized in the following lines.
DNase I hypersensitive site mapping revealed five major hypersensitive sites (HS) all located in the locus control region, from 6 to 22 kb 5' to the human ε-globin gene [51]. The LCR is required to "open" the β-globin locus, after which direct interactions between LCR, erythroid specific factors and individual globin gene promoters confer the stage-specific expression of the β-globin genes [15]. A summary is given in Table V-1.

HS1: This prominent HS sequence, located about 6 kb 5' to the ε-globin gene, has been shown to confer position-independent expression on a linked human β-globin gene in transgenic mice, but it does not increase the level of expression of the β-gene [52]. HS1 has a weak effect at embryonic, fetal and adult stages [53].

HS2: A DNA fragment containing HS2 confers position independent, high expression levels of globin genes in transgenic mice [54]. In transgenic mice, HS2 is equally effective on γ-genes and β-globin gene in both fetal and adult stages [53] and its deletion shows a moderate reduction in expression of all β-like globin genes [55]. Proteins such as GATA1 and/or GATA2 are bound to two consensus-binding sites [56]. Mutations on these consensus sequences cause a reduction in HS2 binding and gene expression [57]. Protein binding sites for AP1 like [58], NFE2 and GATA1 are important for the core function of the HS2 [59]. The HS2 motifs consist of two highly polymorphic short tandems AT repeats interrupted by a short sequence of DNA that can

also be variable [$(AT)_x$ N_y $(AT)_z$]. To date, about 10 different HS2 polymorphisms are known to occur in the normal population [60]. $(AT)_x$ can occur in 8, 9 or 10 copies, N_y in 12, or 14, and $(AT)_z$ in 7, 10, 11, 12 and 13 repeats. One combination of polymorphisms $(AT)_8$ N_{12}GT $(AT)_7$ was found in association with a $^{G}\gamma$ *Xmn*I (C→T) HbS chromosome [8]. The $(AT)_9$ N_{12} $(AT)_{10}$ polymorphism combination was described as the most prevalent one in normal adult individuals with slightly higher HbF (>0.8%) than the average range (0.35 +/- 0.26) [60].

HS3: Four GATA sites and one CAC box are located on the HS3 core [62]. A binding site for AP1-like protein, including NFE2 is located about 30 bp 5'to the HS3 core [63]. It has been shown that DNA fragments containing HS3 are more effective than those containing other single HS's in generating high level expression of the human β-globin gene in transgenic mice [52]. However, the effect of HS3 seems prominent on human γ-globin gene both in embryonic and fetal stages of transgenic mice [53]. Deletion of HS3 results in a significant decrease of ε-globin gene expression and an increase of γ-globin gene expression in transgenic mice embryonic cells [6]. This activity correlates with the presence of the HS3 AP1 motif upstream to the HS3' core sequence [61]. Another erythroid specific transcription factor (YY1) specifically binds to several HS3 motifs including regions 3' to the HS3 core [64].

HS4: The HS4 does not seem to have an enhancer function by it self but is probably required during development to achieve efficient domain opening [62]. 5'HS4 is a highly acetylated fragment of 1.2 kb with the properties of an insulator keeping chromatin in open state [65]. This effect has been described on human β-globin genes in adult transgenic mice while little effect was reported on the same model in embryonic or fetal stages [53].

Table V-1: Transcription factors involved in LCR mediated globin gene regulation

Protein	Consensus binding site	Class	Relatives
NFE2 [70]	YGCTGASTCAY	Basic leucine zipper	Ap1 (Jun+Fos)
LCRF1/Nrf1 [71]	YGCTGASTCAY	Basic leucine zipper	Ap1, Nrf2
GATA1 [72]	WGATAR	GATA zinc finger	GATA2,3,4,5,6
EKLF [73]	CCNCNCCCN	Kruppel zinc finger	Sp1, TEF2
BKLF/TEF2 [74]	CCNCNCCCN	Kruppel zinc finger	Sp1, EKLF
Sp1 [75]	CCCGCCC (GC box)	Kruppel zinc finger	EKLF, TEF2
YY1 [77]	a-VDCCATNWY, b-GACATNTT	Kruppel zinc finger	Cp2
SSP	GGGGCCGGCGGCTG-GCTAGGG		NFE4
USF [77]	CACGTG (E box)	bHLH+ ZIPPER	
TAL1/SCL [76]	AACAGATGGT	bHLH	LYL1, MYOD

NFE2: Nuclear Factor (erythroid-derived 2), LCRF1: Locus Control Region Factor 1, Nrf1: Nuclear respiratory factor-1, GATA1: GATA binding protein 1, EKLF: Erythroid Krupple-Like factor, BKLF: basic Kruppel-like factor, TEF2: Translation Elongation Factor-2, Sp1: trans-acting transcription factor 1, YY1: Ying-Yang-1, SSP: Stage Specific Protein, USF: upstream stimulatory factor, TAL1: T-cell lymphoblastic leukemia factor, SCL: Stem cell leukemia factor, Ap1: Adaptor protein complex, bHLH: basic helix-loop-helix.

HS5: Was reported in 1990 as a hypersensitive site 14.8 kb upstream to the ε-globin gene involved in globin gene transcription [66]. It was suggested that the function of 5'HS5 is that of a chromatin insulator [67]. Recently it has been shown that a conserved

CTCF binding site is present in human 5'HS5 and has an insulating activity in an enhancer-blocking assay [68]. A possible association between HbF levels and polymorphism of the HS2 region has been suggested based on the observation of high levels of expression in Sickle cell patients [69].

The Gγ- and Aγ-genes and their polymorphisms

Polymorphisms upstream to the γ-genes, like the –158 Gγ (XmnI) (C→T) polymorphism, the 4-bp deletion at positions -225 of the Aγ gene, the –202 (C→G) polymorphisms and many others are associated with elevation of the HbF level.
Promoter mutations on the γ genes associated with HPFH are summarized in Table V-2.

Table V-2 – A summary of the γ gene polymorphisms associated with HPFH.

Mutation	Gene	Ethnicity	Gγ (%)	Aγ (%)	Distribution of Hb	HbF%	Authors
del –225 to –222	Aγ	African	66	-		6-7	Gilman JG et al
del –225 to –222	$A\gamma^T$	Sardinian					Manca L.
del –203 to –200 (+CCCC)	Gγ	Tunisian					Pissard S.
-202 C→G	Gγ	African	100	-	pancellular	15-20	Colin FS.
-202 C→T	Aγ	African	10	90		3	Gilman JG.
-198 T→C	Aγ	English	10	90	heterocellular	4 -12	Tate VE.
-196 C→T	Aγ	Italian	5	95	pancellular	10 -15	Gelinas R.
-195 C→G	Aγ	Brizilan	14	86	pancellular	4 -5	Costa FF.
-175 T→C	Aγ	African	-	100		17-38	Stoming TA.
-175 T→C	Gγ	African	100	-	pancellular	28 -29	Craig JE.
-161 C→G	Gγ	African	100	-	pancellular	1-2	Gilman JG.
-158 C→G	Gγ	African	100	-	heterocellular		Durrent LG.
-158 C→G	Gγ	Afro-American	100	-	heterocellular	2 -5	Efremov DG
-117 G→A	Aγ	Italian	10	90	pancellular	8 -10	Colin FS.
-114 C→G	Gγ	Australian	-	-	-	8.6	Motum PI
-114 C→A	Gγ	Algerian	100	-	-	11-14	Zertal-Zidani S.
-114 C→T	Gγ	African	-	-	-	11-14	Fucharoen S.
-114 C→T	Aγ	African	100	-	pancellular	3 –8	Oner R.
-114 to –102 13 bp del. (CAATAGCCTTGAC)	Aγ	African	10	90	-	30	Gilman JG
-110 A→C	Gγ	Czechian	95	5	pancellular	15 -20	Gilman JG
78847 nts del. (GenBank U01317: 40454 to 119300)		Chinese	-	100%	-	9.3 -15.7	Jones RW et al.
35811 nts del. (GenBank U01317: 40681 to 76491)		Black (SE USA)	100	-	-	4 - 16.5	Henthorn PS
113629 nts del (GenBank U01317: 43122 to 156750)		Japanise	61	39	-	100	Matsunaga E
36211 nts del (GenBank U01317: 37057 to 73267)		Turkish	95	5		10-13	Henthorn PS
79208 nts del (GenBank U01317: 37242 to 116449)		Thai	100	-	pancellular	17.2 -20	Fucharoen S.

Adapted from "LE Thalassemie" by Ida Bianco-Silvestroni, Istituto Italiano de Medicina Sociale Editore- Roma 1998.

Transcription factors

Several erythroid specific transcription factors interacting between the locus control region and the promoter of the β-globin genes cluster have been described [70-77].
The mechanism of these factors is partially still unclear. Known transcription factors associated with the expression of the β-gene cluster are summarized in table V-1.

In conclusion

Expression studies indicate that many elements are involved in gene expression in complex and interactive mechanisms. It is difficult at this stage of knowledge to speculate on the value of the many factors involved in gene expression to develop practical therapeutical applications.
Reactivating HbF synthesis in erythroid cells in which the expression of the γ genes is silenced is a major challenge. Many investigations trying to develop pharmacological agents that could reverse the switch from γ- to β-globin chain synthesis were done. These studies were based on the general idea that reactivation of HbF synthesis could be achieved by:

- Methylation and demethylation to induce gene activation,
- Histone acetylation by inhibition of deacetylase enzyme,
- Selection of HbF-producing erythroid progenitors

Buytrates: Hypomethylation is a general feature of the housekeeping genes that are usually expressed in all cells while tissue specific genes, for example, β-like globin genes, are variably methylated [78]. Cytosine methylation and demethylation at CpG dinucleotides may influence transcription. Actively transcribed chromatin is characterized by both hypomethylated DNA and acetylated histones. Butyrate compounds hinder cell growth by inhibiting histone deacetylase, cyclin D1 and D2 [79].
Hydroxyurea: It has been demonstrated that higher levels of HbF are found in erythroid cultures derived from adult erythroid burst forming units (BFU-E) [80]. Blau C. A. et al suggests that the maturation kinetics rather than a specific high HbF reticulocyte response form the basis for preferential F reticulocyte induction. This supports the hypothesis that high HbF reticulocytes arise from acute erythropoietic stimulation through a relatively rapid pathway of maturation [81]. Erythroid specific factor FKLF-2 activates the gamma promoter predominantly by interacting with the gamma CACCC box, and to a lesser degree through interaction with the TATA box or its surrounding DNA sequences [82]. FKLF may favor in an earlier stage γ-globin gene expression in progenitor cells. Hydroxyurea as a cytotoxic agent has damaging effect on the slow maturing erythroid progenitor rather than fast one. Therefore, the selection of fast maturing erythroid lines with relatively higher HbF expression could explain the effect of most cytotoxic drugs increasing HbF.
To reduce the need for transfusion treatment and herewith the complications of iron overload, the effect of alternative Hydroxyurea treatment has been studied on a large number of patients affected with thalassemia intermedia. The results of this study are described in publication 2 in this thesis

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Chapter VI:

Prevention in Iran
and
the Hormozgan experience

The burden of thalassemia in the Iranian population

According to the 2001 census, Iran has a population of over 66 million and about 60 % of the population is between 15 to 64 years of age [1]. More than 1,3 million newborn are expected annually at a birth rate of 20.71 newborn per 1,000 individuals [1]. As mentioned in chapter IV, the average thalassemia carrier frequency in Iran can be assessed at about 5% (1.7% - 9.5%). Therefore, without any prevention program, the expected incidence for β-thalassemia major will be near 1.500 affected newborn per year of which about 500 can be expected from the 65% low consanguinity couples and about 1.000 from the 35% cousin couples.

Such a huge public health problem comprising β-thalassemia and other hemoglobinopathies receives a lot of attention from the public authorities. However, a lot still needs to be done to reduce the social, economical and emotional burden associated with these diseases. The number of thalassemia patients living in Iran is estimated by "Charity Foundation for Special Disease" to be 20,000 individuals.

The second most prevalent hemoglobinopathy in Iran is Sickle cell disease (HbS/S homozygosity). Precise data on the incidence of this disease in the country are not yet available. However, an unpublished survey made by this author in the Hormozgan province shows a carrier frequency of 2.8%. Therefore, considering a birth rate of at least 200.000 newborn a year and a higher consanguinity rate than the country average, the cumulative incidence for HbS/S homozygosity in this region only will be about 300 newborn a year. This figure is an obvious underestimation because of the high frequency of β-thalassemia generating HbS/β-thalassemia combinations, an equally severe form of sickle cell disease.

As mentioned in chapter IV no precise data on the costs of treatment for hemoglobinopathies in Iran are available. However, considering the increase in life expectations and in the costs of blood and drugs, the cumulative public health burden could easily rise to a figure around 2 billions US $ in the near future.

Primary prevention in Iran at present

The "Ministry of Health and Medical Education" has developed a protocol to support a pre-marital screening program. The network includes four levels of expertise:

1) Laboratory. 2) Counseling. 3) Education. 4) Surveillance.

The active prevention protocol starts when a couple decides to be married and asks for the necessary documents. At this point both partners are registered as a future couple and they will be visited by a health care functionary who will give them basic information and will send them to the local screening laboratory for carrier analysis. If they appear to be a couple at risk the first advise will be to renounce marriage. If the couple decides to go on and marry anyway they will be asked to sign a document where they declare to have been informed and to be aware of the risk.

Laboratory. Laboratories are usually in the large cities and are provided with a standard diagnostic protocol for the detection of carriers at the basic hematological and biochemical level according to the internationally approved protocols. The lab personnel have been trained to meet the necessary requirements for specific analysis and quality control. In addition, specialized reference laboratories (4 at the moment) are dedicated to specific genetic analysis and prenatal diagnosis. Each genetic lab must get an approval from the "Genetic Council" and from the central reference lab in Teheran that supervises the quality and capacity of all genetic labs.

Counseling. Counseling centers are found in each large city and are taking care of genetic information and advise for potential couples at risk. The personnel of these centers consist of physicians trained in medical genetics and psychology.

Information: Accessible educational programs for the public are provided through mass media such as newspapers, radio and television, posters in public places.
Education. Virtually all health workers involved in "primary health care" have received professional post-academic education and are kept updated on recent scientific and therapeutic developments by seminars or short-term courses each year. The Deans of all medical faculties are charged with and responsible for the quality of this special education process.
Surveillance. Trained social workers will follow monthly carrier couples that decide to marry and have children. One of the official genetic labs takes responsibility for prenatal diagnosis. After adequate information is given by genetic counselors the parents will take the final decision about the affected pregnancy. If the parents decide to keep an affected fetus they are asked to sign a paper which relief the counsellor from every responsibility.

The choice of the screening stage

"State of the art" treatment for thalassemia and sickle cell disease, not only represents a heavy economical load for public health but also a physical and psychological burden to the patient, the family and the community. In order to prevent new cases in couples at risk screening for thalassemia has started as a pilot study in 4 provinces in 1992 (1371 AH). The initiative was based on the classic elements and on the experience obtained in Italy and Cyprus [4, 5]. The elements being information, carrier diagnostics, counselling, and the intervention of choice in the premarital stage (see Fig. VI-1). Screening at the pre-marital stage was shown to be beneficial, accepted, efficient and economically more advantageous than screening of youngsters during school age or ethnic groups with high incidence.
After this pilot study a pre-marital screening was introduced at the national level in 1996 (1375 AH). Like in Cyprus, in order to be able to register for their official marriage all Iranian couples need to have a certificate attesting that they have been screened for thalassemia at the local health center. However, this measure is less efficient in case of tribal, nomadic or other less educated groups. In these population the marriage proceeds according to the local tribal traditions and falls outside the official channels. Also the nomadic lifestile makes it more difficalt to be followed by the official authorities.

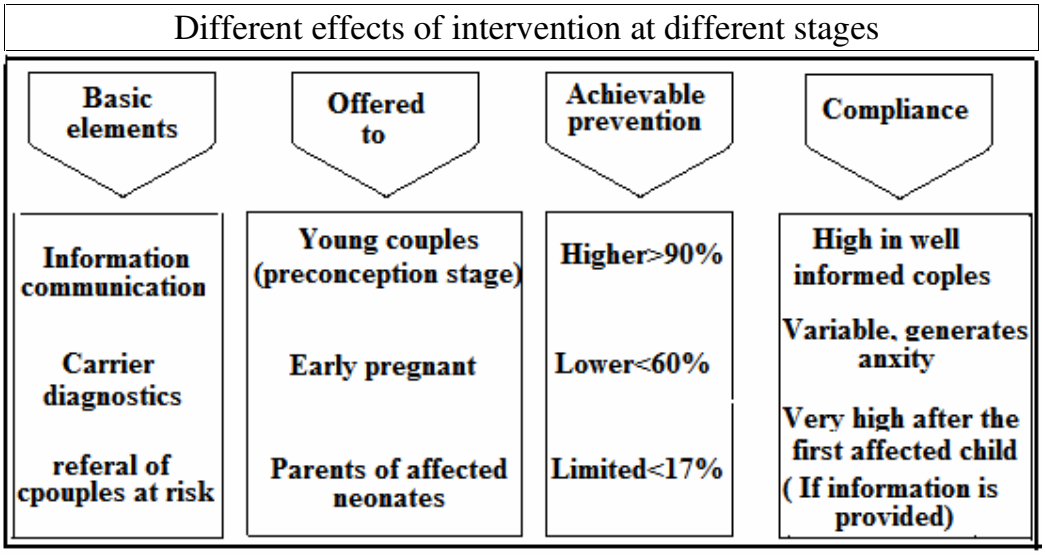

Fig. VI-1: The elements of information, carrier diagnostics and counselling offered at different stages of life and their experimented value and achievable result (courtesy of P.C. Giordano).

Religious elements

Like in conservative Catholics teaching, in conservative interpretations of the Islam it is assumed that any form of interference with the natural course of pregnancy should not be allowed. Other interpreters of the Koran consider life to begin either at the third or even the fourth month of pregnancy and allow the interruption of pregnancy for severe medical reason before these dates. In Iran reformist Islamic spiritual leaders (She, at) have agreed in 1999 upon selective abortion for the prevention of thalassemia major. Less than 10 % of the Iranian population is following the Sunni stream and lives near the Western, Southern, and Eastern borders of Iran. They are following the Saudi or Egyptian Islamic streams and also in those countries the religious leadership approved (fatwa) that thalassemia-affected fetuses could be selectively aborted up to 17 weeks of gestation. Such a religious statement has a powerful influence but no full legal status until the parliament has made a legal decision. To date, two initiatives for a legal agreement are ongoing in Iran, one from a group of members of parliament, the other from the government itself. The discussion is focused on procedural and formal matters, putting the gestation age limit at 17 weeks and giving a legal status to the fatwa. Until then, some doctors in state hospitals may refuse to carry out abortion because the legal state has not yet been reached. However, there are at least three medical centers in the capital city of Tehran practicing prenatal diagnosis for β-thalassemia major.

Consanguinity

Consanguineous marriage increases the chance of homozygosity for recessive traits. The degree of increase is dependent from the prevalence of the trait. The rarer is the trait, the higher is the relative increase. For common traits such as thalassemia in Iran it can be estimated that marriage between 1st cousins will increase the incidence by a factor 2. For the region of Hormozgan, considering a carrier frequency of almost 10%, the incidence would than be 2.5:1.000 for unrelated parents and 5.5:1.000 for first cousins. With 30 to 40% consanguineous marriages, this would mean that about 80 of the 140 affected children expected each year would be born from cousin couples and that 60 would come from non-related parents. In absence of consanguinity the incidence

in the same population would be 100 instead of 140 affected children each year. This tells us that to improve hemoglobinopathies prevention in communities with traditional consanguinity, discouraging consanguineous marriage is a less sensible choice than incouraging specific carrier diagnostics, using the elements and steps shown in Fig. VI-1 [2].

The prevention campaign

The aim of the prevention campaign in Iran is to allow parents who already have an affected child and seek more progeny to avoid more suffering and to reduce the socio-economic pressure on the family. In addition a prevention campaign will reduce the heavy burden on national healthcare represented by the large number affected children in need of very expensive medical care.
A campaign is therefore focused on informing of future couples, of all parents who had an affected child already and on their families (especially brothers and sisters). All of them are advised to be tested before marriage or before having children and to get prenatal diagnosis if they already had a sick child. Unfortunately, due to insufficient information many of them may get afraid of stigmatization and refuse information. Therefore also obstetricians are advised to test pregnant women for carrier status in early pregnancy, and if she appears to be a carrier to test also the husband. In order to save time the testing of husband and wife at the same time also is recommended. For couples at risk prenatal diagnosis is available and the techniques imply chorionic villus sampling (CVS) or cordocentesis.

The elements of prevention

Before starting any prevention campaign one must be aware of the frequency of the disease, and the heterogeneity of the molecular spectrum of DNA defects. In addition, knowledge on the genotype/phenotype correlation associated with the molecular spectrum must be present. Moreover, sufficient resources and technical know how must be available and last but not least, social, cultural and religious acceptance should be present.
A preventive program is required to offer the best possible service within an acceptable buget, which can be integrated into the already available basic health service. Training of primary health care doctors and obstetricians is needed to give basic information to the public, to identify carriers, to provide genetic counseling and prenatal diagnosis.

Steps in prenatal diagnosis

For a state of the art protocol in prenatal diagnosis the following steps are needed:

- Thorough non directive counseling of the couple at risk in full privacy
- Blood sampling of the parents and the affected child or children (if available) to be analyzed at the hematological, biochemical and molecular level to find the mutations causing the β-thalassemia trait in both parents and to predict the outcome of homozygosity or compound heterozygosity. In the affected child blood should be collected either a few days before, or two weeks after the transfusion.
- Chorionic villi sampling (CVS) can be performed starting from 10-11 weeks of gestation. The biopsy is usually taken under ultrasound guidance via a transabdominal approach. Alternatively the cervical approach may be utilized. The biopsy yields must be between 5-30 mg of tissue. An advantage of CVS is that abortion of affected fetuses can be done within the first trimester when it is technically easier and culturally acceptable.

Pre-implantation genetic diagnostics (PGD)

The use of artificial reproductive techniques provides access to gametes and pre-embryos and enables genetic diagnosis to be offered shortly after fertilization ex utero and before implantation [8, 9]. The PCR techniques have greatly improve the sensitivity of genetic diagnosis and permit analysis of minimal amount of material.

PGD can be offered as possible alternative to couples at risk that may have ethical, moral or emotional reasons accepting the abortion of a 3 months old affected fetus. Recently this technique has been proposed to parents seeking bone marrow transplantation for their child affected with β-thalassemia major, but not having an HLA identical sibling as a BM donor. In those cases a double analysis to exclude homozygosity and to match the HLA typing is needed and the chance to have a suitable fetus is 1:16. Many moral objections have been made to this PGD option and only few cases in which this technique has been used are known. The procedure is still an experimental research method and an international registry has been set up to monitor eventual effects on fetal and child development.

The Hormozgan experience

Figure VI-2: Map of Iran and the Hormozgan province

The Hormozgan region

A large share of the molecular work of this thesis is based on samples collected in the Hormozgan and Shiraz regions. The material collected in Hormozgan was used to establish the mutation spectrum of β and α-thalassemia, to investigate the occurrence of Hereditary Hemochromatosis (HH) and to study the survival analysis in the area of interest (publications 1, 2, 5 and 6). The sample materials collected in both Hormozgan and Shiraz were studied to compare the molecular spectrum and to define the background of HbH disease (publication 3). Part of these individuals represented the patient's population involved in the hydroxyurea therapeutic trial (publication 4).
The Hormozgan province represents a region of particular interest from the "gene-geographical" point of view and is therefore described in more details in the following paragraphs.
Hormozgan with 1.2 million inhabitants covers a costal area of 71.193 km^2 in the south of Iran facing part of the Persian Gulf including the islands of Hormoz, Larak, Qeshm, the Lesser Farud (Formerly Bani Forur), Farud, Abu-Musa, Kish, and Lavan. The major cities are Bandar Abbas, Bandar Lingeh, Jask, Haji-Abad, Roudan and Minab (Fig. VI-2).
A multi-ethnic population has lived in this region during the course of history. At least seven tribes are considered indigenous of this area. However, also these oldest inhabitants of Hormozgan present with both North African and South Asian phenotypes. Dravidian nomads, probably coming from India or the south of Pakistan, Sumerian merchants and other people from southern Mesopotamia, Akkadian and Semites, all settled in the territory in archaic periods. In 1628 Thomas Herbert wrote about the cosmopolitan nature of Bandar Abbas, where he reported seeing "English, Dutch,

Danish, Portuguese, Armenian, Georgian, Muscovite, Turkish, Arab, Indian, and Jewish merchants".
Finally, lots of recent eastern immigrants have been driven to Hormozgan by eight years of war between Iran and Iraq.
As discussed before, universal pre-marriage screening is mandatory in all Iran. However, the population at risk has a heterogeneous ethnic and geographical origin with variable carrier frequencies for β- and α-thalassemia and other hemoglobinopathies, making the situation complex.
In a one-year period (2001 to 2002) the "Thalassemia Center of Bandar Abbas" has registered 124 new cases of β-thalassemia major, a figure that is still an underestimation of the 140-150 cases theoretically expected. Less than 9% of these cases were first pregnancies, 21% were the second and third, and about 67% were the 4^{th} or later gravidity. Families with 13-14 children are not exceptional in the area.
At that time prenatal diagnosis was not accessible in Hormozgan. Therefore, from the 11 couples having an affected child at the first pregnancy, only two were previously screened and knew about their risk but rejected pre-marital counseling. Three were screened elsewhere but their thalassemia carrier status was misdiagnosed. The others couples were not screened at all because they did not marry officially but according to the local customs.

In conclusion

From the Hormozgan experience it appears relevant that the universal pre-marriage screening is not sufficient in an area with a high prevalence of hemoglobinopathies and ancient customes. In order to improve the effects of the public health authorities the following interventions are needed at different stages:

- Intervention in early pregnancy for women who have an affected child in their family and for those women that did not have any previous counseling.
- Intervention at the neonatal stage (screening) in order to reduce mortality or morbidity of newborns by early diagnosis and to stimulate retrospective primary prevention for the next pregnancy. This intervention is routinely done in immigration countries focusing on ethnic groups at risk (for example USA and England). It would be particularly relevant to organize a neonatal-screening program in areas of high prevalence (Hormozgan and Mazandran) and in the other provinces with high incidence.
- Intervention to create more Reference Hemoglobinopathies Laboratories in provinces with high frequency of thalassemia carriers. These laboratories should have skilled personnel able to diagnose hemoglobinopathy trait and to perform hematological, biochemical and molecular post and prenatal analyses.

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Chapter VII

Laboratory strategies for carrier diagnostics and prevention

Carrier diagnostics

Together with information, carrier diagnostics (screening) is one of the cornerstones of every prevention program for the hemoglobin disorders. The final goal is to be able to offer genetic counseling either before marriage, before conception or at early pregnancy to informed couples at risk that require this medical service (see also chapter VI, "primary prevention in Iran at present"). For this identification of the DNA mutations causing the disease is essential. Moreover, the understanding of the genotype/phenotype correlation of the various globin gene mutations is important in trying to predict the effects of interaction when several mutations are co-inherited. The commonest globin gene defect in Iran and their phenotypes are reported in detail in chapters III and IV and in publications 1, 2 and 3.

Screening

The aim of screening is the identification of thalassemia carriers. In fact in areas of low prevalence it is often rather a matter of routine diagnostics based on chronic microcytic hypochronic anemia as a clinical indication. Only when, due to high frequencies in a specific area, the whole population is examined without medical indication we can define this action as a screening.

In both carrier diagnostics and screening, hematological and biochemical methods can be applied as the cheapest and the fastest technology with the highest degree of directive information. DNA analysis identifies the specific mutation which can be used for risk assessment and prenatal diagnosis.

Hence, the quickest and easiest way to identify carriers is to observe their basic hematological parameters, which are automatically measured during blood analysis. Biochemical tests such as the automated separation of the Hb fractions on HPLC or on electrophoresis followed by manual estimation of the fractions will diagnose practically almost all carriers of β-thalassemia and abnormal hemoglobins.

The hematological diagnosis of Hemoglobinopathy carriers:

As mentioned above the standard indices of a β-thalassemia carrier will indicate a microcytic (MCV↓) hypochromic (MCH↓) state with or without anemia. Anemia will usually be present if the RBC count is lower than 5 million and patients do not compensate for their hypochromic anemia by raising the RBC production. Due to this compensation phenomenon, which seems to be often folic acid mediated, algorithms have been developed to distinguish microcytosis derived from α- and β-thalassemia from microcytosis due to iron deficiency [1].

Elevated HbA_2 and β-thalassemia. The postnatal HbA_2 level increases from about 0.5% at birth to the stable normal adults range of 2.5 to 3.4% at the age of 1 year.

A level higher than 4% measured starting from the second year of life is a diagnostic indication for β-thalassemia trait. The rate of HbA_2 elevation may give some indication on the type of β-thalassemia defect (mild β^+, β° or $\delta\beta$).

The great majority of β-thalassemia carriers, either induced by a β° or severe β^+ mutation, are characterized by a markedly elevated HbA_2 level in the range between 4.0 and 6.0% or higher. Higher levels of HbA_2 are reported in patients with the large deletions that remove the 5' promoter region of the β-globin gene [2]. Raised HbA_2 levels are also measured in some mutations of the third β-globin gene exon producing large but incomplete β-chains. These gene products, unsuitable for tetramer formation, become difficult to proteolysis and precipitate in bone marrow precursors. This results

in ineffective erythropoiesis and in a semi-dominant phenotype called Heinz Bodies hemolytic anemia [3].
Levels between 3.5 and 4.0% may be measured in particular cases when the thalassemic state is mild and induced by β^+-thalassemia mutations such as promoter, splicing, initiation codon or poly Adenylation signal defects.

Normal or low HbA_2 and β-thalassemia.

Technical artifacts may slightly reduce the HbA_2 level in the presence of iron deficiency. Iron deficiency should always be excluded as a cause of anemia at the very beginning of the analysis by by measuring ferritin, transferrin saturation or erythrocyte zinc protoporphyrin [1]. On the other hand the typically raised values of the heterozygous β-thalassemia are never lowered by iron deficiency below 3.5%. Only in some rare types of mild β-thalassemia the HbA_2 value can be as low as 3-3.5%. These mild mutations produce only a limited deficiency of β-globin production and may escape identification in a population screening. In the homozygous state and in combination with a severe β^+ or a β^0-thalassemia mutation these defects result in a mild thalassemia intermedia.
In some rare cases of classical high HbA_2 beta thalassemia genotype, a normal HbA_2 level can be measured if a coexisting δ-thalassemia or a δ-globin chain abnormality is present on the other allele.

Lower HbA_2 levels and α-thalassemia. When in a non iron deficient microcytic hypochromic patient the HbA_2 is lower than the normal range 2.5- 3.5% one should think of **α-thalassemia.** [1] On the other hand, combinations of α and β-thalassemia traits usually present with elevated HbA_2 levels. Due to the high frequency of both α- and β-thalassemia in some areas, α-thalassemia mutation analysis should always be done, especially in strongly hypochromic β-thalassemia carriers. The occurrence of α-thalassemia and the technology needed for confirmation of α-thalassemia defects are illustrated in publication 2 and 3.

High HbF β-thalassemia. Although HbF is not always elevated in adult carriers usually a slightly raised HbF level (1–3%) is measured.
When in a non-iron deficient microcytic hypochromic patient the HbA_2 is in the normal range 2.5- 3.5% and the HbF is elevated one should think of δβ, γδβ-thalassemia or hereditary persistence of fetal hemoglobin (HPFH).
Defining HPFH or δβ-thalassemia at the genotype level is a rather ambiguous matter. High expression of HbF can be induced by deletions on the β-gene cluster that in fact inhibit the expression of the β-gene even when they leave the gene intact. Due to the beneficial effect of the high HbF expression the lack of β-gene expression does not lead to severe or even intermediated phenotypes in these patients. In fact the only real HPFH are those that leave the expression of the β-gene intact and are induced by point mutations mostly on the promoters of the γ genes, as explained and summarized in chapter V.
For the reason explained above, it is important to discriminate between those β-gene expression defects, which can or cannot develop a severe pathological phenotype. The difference between the two conditions depends mainly from the quantity of HbF expression present. This can be insufficient in δβ-thalassemia but often sufficient in γδβ-thalassemia. Compound heterozygotes for HPFH and β-thalassemia may result in a silent or very mild phenotype in contrast to combinations of δβ-thalassemia and β°-thalassemia defects that may result in β-thalassemia intermedia or major.

How to diagnose abnormal hemoglobins

The most common and clinically relevant abnormal hemoglobins (HbS, HbE, HbC or HbD) can be putatively characterized by hemoglobin electrophoresis or HPLC. The latter is accurate, fast and estimates the fractions automatically.
It must be pointed out that a specific band on electrophoresis or peak on HPLC does not give a 100% identification of any Hb variant. Of course the chance that a HbS, C or E like fraction turns out to be another mutant is not high, but, taking this for granted without molecular confirmation is very risky, especially when counseling a couple supposed to be at risk. HbS is the only mutant that can be easily confirmed by the **sickle test** inducing the polymerization of the HbS between sealed microscope slides in the presence of sodium metabisulfite [4].
Thus, hematological, biochemical and molecular technologies are essential for the identification of the correct genotype. β-chain variants, such as HbS and HbE, are subject to a reduced expression in heterozygotes when α-thalassemia is co-inherited. In plain sickle cell trait the amount of HbS will be around 35–45%. The HbS expression will decrease to 30–35% in the presence of α^+-thalassemia trait (-α/αα) and drop further down to 25–30% in the presence of both α^+-thalassemia homozygosity (-α/-α) or α°-thalassemia heterozygosity (--/αα) [5].
For all the other mutants, common or rare, DNA analysis (DGGE, ARMS-PCR, DNA sequencing, or Restriction Enzyme Analysis) is needed.

The genotype determination Hemoglobinopathies carriers

A variety of hybridization protocols and polymerase chain reaction (PCR) based techniques have been developed to identify globin gene mutations. These techniques include Restriction Fragments Length Polymorphism (RFLP) analysis, Denaturing Gradient Gel Electrophoresis (DGGE), amplification refractory mutation system (ARMS), Dot-blot and Reverse Dot-blot analysis and Gap-PCR. Each method has its own advantages and disadvantages and all are recommended in The Best Practice Guidelines [6]. The choice depends obviously from the technical expertise available but also from the type and variety of mutations that are likely to be found in the area of interest or in a particular case.

β-Thalassemia

The mutation spectrum of β-thalassemia shows in many populations a limited number of prevalent mutations covering around 80% of the cases and a more or less large number of less frequent or rare mutations. As pointed out in chapter IV and in publication 1 more than 43 different β-thalassemia mutations have now been reported in the Iranian populations and the majority of them are, as expected, point mutations.
Usually common point mutations are analyzed by using allele specific detection methods such as reverse dot blot or by fragment identification and possibly pattern identification using DGGE [7]. If the mutation remains unidentified characterization by direct DNA sequence analysis will usually provide the identification.

Allele-specific oligonucleotide hybridization. Mutation detection by hybridization of allele-specific oligonucleotide probes (ASO's) was the first PCR method to be developed. For mutation screening a number of probes are required that will cover all (or at least the most frequent) defects found in the specific ethnic group including the (eventually polymorphic) wild type allele fragment. The amplified DNA fragment suspected to contain the mutation is than hybridized with the panel of specific

oligonucleotides. The genotype of the patient is determined by the presence or absence of the hybridization signal of both the mutation specific and the normal probe.

The Reverse Dot-Blot. The reverse dot-blot technique was developed to allow the identification of a number of common mutations in one hybridization reaction and it has been used for molecular characterization of globin gene mutations in many occasions [9-11]. The method is also suitable for non-isotopic labeling [8]. In this method, unlabelled ASO probes complementary to the mutant and normal DNA sequences are fixed to a nylon membrane in the form of dots or slots and labeled amplified genomic DNA is then hybridized to the filter using a two-step procedure with one nylon strip for the common mutations and a second one for the rare mutations (Fig. VII-1).
Non-radioactive labeled reverse hybridization kits are commercially available but designed for a limited number of mutations that do not cover the complete spectrum of the Iranian population (publication I in this thesis).

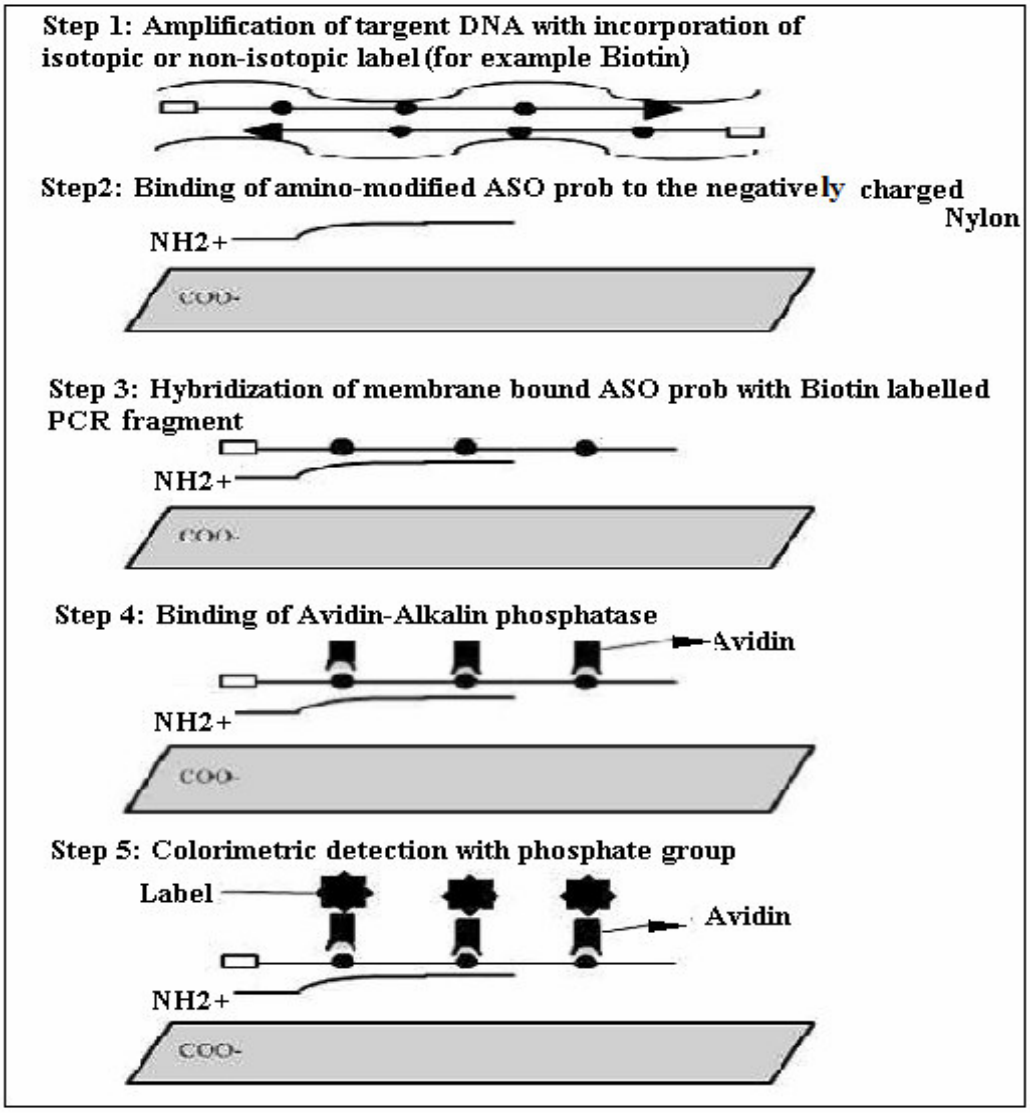

Figure VII-1. The representation of *Reverse Dot blots hybridisation* and detection step by step.

Allele-specific priming methods. These methods are based on the principle that a perfectly matched PCR primer is much more efficient in directing primer extension than a mismatched one. The most widely used technique is the amplification refractory mutation system (ARMS)[12]. In this method, the target genomic DNA is amplified using a common primer and an allele-specific forward primer with its 3'site complementary to the targeted mutation (the mutant primer) and a common reverse primer. The mutant primer, which has a mismatch with the normal sequence at the 3' terminal base has a second mismatch engineered at the second, third or fourth nucleotide to ensure it will not amplify normal DNA. As with the ASO approach, a panel of mutant-specific primers is required for mutation screening. ARMS reactions also contain two extra primers complementary to a different part of the β-globin gene included in the PCR to provide an internal control. The method is suitable for fast and inexpensive screening without the need for labelled primers. Fluorescent labelling of the common primer allows the quantitative estimation of the amplification products on an automated DNA fragment analyser [13].
If the normal and mutant ARMS primers for a specific mutation are co-amplified in the same reaction they compete with each other to amplify the target sequence. This technique, called competitive oligonucleotide priming, requires the two ARMS primers to be distinguishable in some manner. Fluorescent labels permit a diagnosis to be made by means of a colour complementation assay [14]. Alternatively, ARMS primers that differ in length can be used and thus a diagnosis can be made by estimation of the product sizes. This technique, called mutagenetically separated polymerase chain reaction (MS-PCR), has been applied for the prenatal diagnosis of β-thalassemia [15]. The advantage of this technique consists in the simultaneous screening of several mutations, in the inexpensive and non-radioactive alternative and in the simple and rapid procedure.

Restriction enzyme analysis of amplified products

This is a useful but limited technique based on the fact that some mutations abolish or create sites on which specific restriction enzymes wil cut the DNA, resulting in a change in size of the regular digested fragments. The presence or absence of the recognition site, and herewith the presence or absence of a specific mutation, is determined by the pattern of the digested fragments visualized on agarose or polyacrylamide gel electrophoresis. Unfortunately, only approximately 40 β-thalassemia mutations create or abolish a restriction site and only a limited number of "Iranian" mutations can be rapidly detected by restriction analysis of amplified DNA. These mutations are listed in table VII-1. Analysis of mutations that abolish a restriction sites should be provided of sufficient internal controls to guarantee that the loss of a restriction site is not due to an artifact.
Mutations that do not naturally create or abolish restriction sites may be diagnosed by another technique of amplification creating restriction sites (ACRS). This method uses primers that are designed to insert new bases into the amplified product in order to create a restriction enzyme recognition site adjacent to the mutation sequence (for a detailed description, see reference [16]).

Gap-PCR

The technique of gap-PCR can be used to detect α and β-globin gene mutations induced by deletions. Primers flanking the breakpoint sequences amplify a deletion-specific fragment that spans the deletion [17]. For large deletions, the distance between the two primers is too large to amplify normal DNA in the presence of the deletion and the

deletion allele is detected by amplifying between sequences spanning the breakpoints [18].
This technique can be multiplexed to detect more than one mutation at the time but in this case some difficulties can be expected. Control DNA is needed to validate the test and amplification problems of GC rich sequences in case of α-globin cluster may occur as well as allele-dropout (publication 2 and 3).
Gap-PCR can also be used to diagnose the δβ-thalassemia, Hb-Lepore and some HPFH like deletion defects. However, this technique can only be used for the deletions in which both the breakpoints on the DNA sequence have been characterized [25]. These involve six δβ-thalassemia deletions (the Spanish, Sicilian, Vietnamese and Chinese deletions, and the Turkish and Indian inversion/deletion mutations). Unknown deletions can only be characterized experimentally hybridizing with specific probes after restriction enzyme analysis with appropriate enzymes (Southern blot) followed by breakpoint sequencing.

Table VII-1 Iranian β-Thalassemia mutation detectable by restriction enzyme digestion

Mutation	Ethnic group	Enzyme
-88 C→T	African/Asian Indian	+*Fok*I
-87 C→G	Mediterranean	-*Avi*II
Initiation Cd T →C	Yugoslavian	-*Nco*I
Cd 5 (-CT)	Mediterranean	-*Dde*I
Cd 15 (-T)	Asian Indian	+*Bgl*I
IVSI-1 G→A	Mediterranean	-*BspM*I
IVSI-6 T→C	Mediterranean	+*Sfa N*I
IVSI-130 G→C	Turkish	-*Dde*I
IVSI-130 G→A	Egyptian	-*Dde*I
Cd 39 C→T	Mediterranean	+*Mae*I
	Mediterranean	-*Hph*I
	Mediterranean	+*Rsa*I

PCR methods for unknown mutations

A number of techniques have been applied for the detection of β-thalassemia mutations without prior knowledge of the molecular defect. One of the most widely used of these methods is denaturing gradient gel electrophoresis (DGGE). This method allows the separation according to the melting properties of DNA fragments differing by a single base change [7]. Direct DNA sequencing of that particular fragment will identify the mutation.

Heteroduplex analysis

Another approach is by heteroduplex analysis using non-denaturing gel electrophoresis [19]. These techniques create a unique heteroduplex pattern for each mutation that can be used diagnostically to identify known mutations. However, care is needed because of the additional presence of a β-globin gene framework polymorphism in the targeted fragment altering the heteroduplex pattern.

Direct DNA sequence

Originally two competing methods for the determination of DNA sequence were developed:

1. The Maxam-Gilbert method (20), in which a DNA sequence is end-labeled with P^{32}, attached to the nucleotide, and subsquently chemically cleaved to leave signature pattern of bands. Since the Maxam-Gilbert method is not frequently used, it will not be described further in this chapter.
2. The Sanger method [21], in which an oligonucleotide primer is annealed to a DNA sequence, which is then extended by DNA polymerase using a mixture of deoxynucleotide triphosphate (dNTP) and dideoxynucleotidetriphosphate (ddNTP).

The Sanger method starts with the annealing of a labeled primer to the DNA template, followed by the division of the sample into four aliquots in separated vials marked "G", "A", "T", "C" for the 4 different reactions. Subsequently the 4 deoxynucleotide triphosphate (dNTPs) are added to all 4 vials while the 4 dideoxynucleotide triphosphate (ddNTP ddGTP, ddATP, ddTTP and ddCTP) are added separately to the corresponding marked vials. The dideoxynucleotide containing a 3'H instead of a 3'OH group, acts as a sequence terminator and will be present in sufficient amount to be in partial competition with the deoxynucleotides. When the polymerase enzyme is added the 4 synthetic reactions will start. Part of each sequence reaction will stop when the specific dideoxynucleotide will be incorporated instead of a deoxynucleotide, while the synthesis will continue incorporating deoxynucleotides until the next specific dideoxynucleotide will be incorporated again. By this method 4 parallel DNA synthesis can be extended up to several hundreds base pairs. After denaturation of the products into single strand and polyacrylamide/urea gel electrophoresis the synthetic products of the 4 reactions will be separated by progressive length. Since the synthesized DNA contains a radiolabeled substrate the progression of the synthesis can be read base by base after exposure to X-ray film (fig. VII-2).

DNA sequence technology has evolved in the last decade and to date large DNA fragment can be very efficiently analyzed using an automated DNA sequencer utilizing fluorescence detection technology. By these methods the oligonucleotide primers are labeled with four different fluorescent dyes and four separate reactions are conducted in the presence of dNTPs and ddNTPs.

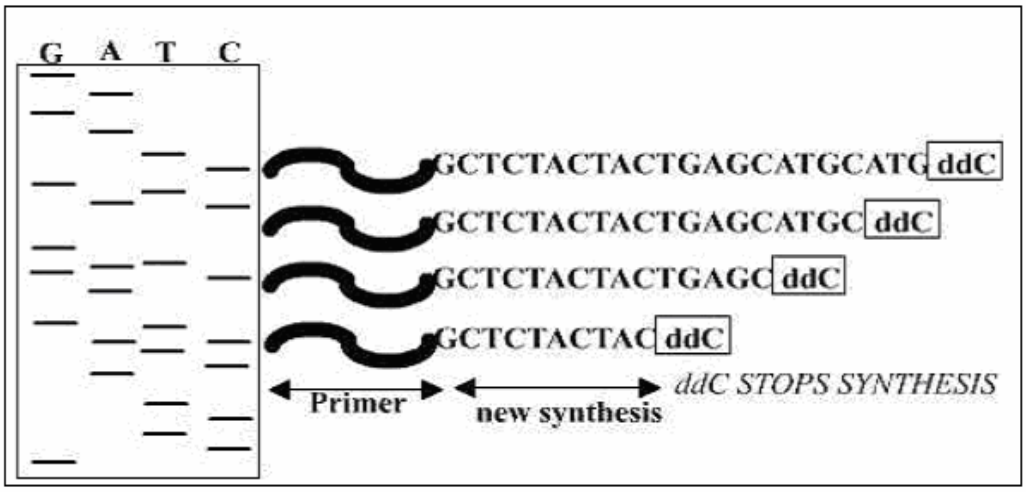

Figure VII-2. Line up of all four reactions. The sequence can be read 5' to 3'and from down to the top as G, C, T, C ….etc.

Micro array technology

DNA micro array technology is one of the latest tools for genetic analysis. Micro array "chips" offer the possibility of analyzing simultaneously analysis of thousands of predefined DNA sequences and can be applied to DNA diagnostics, gene expression analysis, and aneuploidy detection. The most significant application has been in monitoring expression profiles to deduce genes relevant to particular pathologies (by comparing cDNA extracts from tissues derived from normal or disease states). Detection of aneuploidy using chip technology would work in a similar fashion to that of expression analysis [22]. Pieces of genomic DNA from specific chromosomes act as probes on the slide and a competitive hybridization process between samples from known normal karyotype and unknown occurs. Aneuploidy detection using micro arrays is proving to be more difficult than expression analysis because copy number changes seen in aneuploidies are less well visible as changes in signal intensities than gene expression changes, which can vary by the order of magnitudes [23].

Several micro array methodologies for mutation analysis using micro arrays have also been described [37-40]. One of the more common examples of this is single base extension (also called SBE or minisequencing) in which an oligonucleotide probe is attached to the chip by its 5' end. Each spot on the surface of the chip can contain several million of these oligonucleotides probes. The oligonucleotide is complementary to the sequence of a disease causing gene and its 3' end terminates at the base before a known mutation site. When the surface of the chip is exposed to sample DNA, with DNA polymerase and di-deoxynucleotides triphosphates, the sample DNA acts as the template for the extension. By using labelled ddNTP in four different colors it is possible to determine which nucleotide was incorporated indicating the presence of the wild type or mutant sequence. Solid-phase minisequencing following whole genome amplification by primer-extension-preamplification (PEP), correctly genotyped single cells at 96% of the nucleotide positions analyzed [24]. Current drawbacks to using microarray technology in preimplantation genetic diagnosis (PGD) include high cost, poor reproducibility, complex and lengthy data analysis, and the absolute requirement for some form of whole genome amplification.

α-Thalassemia

As explained in chapter IV most α-thalassemia alleles result from deletions involving either one (α^+-thalassemia) or both the α-globin genes (α^0-thalassemia) [26]. Although the majority of mutations are deletions, about 20% of the carriers of mild α-thalassemia phenotype may result from point mutation defects. The deletion breakpoints of the seven most frequently occuring deletions have been sequenced and these alleles can now be diagnosed all together using Multiplex-PCR. If none of these mutations is found by Multiplex-PCR, direct sequencing of the α-globin genes to detect non-deletion types of α-thalassemia or Southern blot for large or unknown deletions is the second step.

The α^0-Thalassemia defects are mostly found in patients of Southeast Asian origin and are less frequent in Iran (see publication 3). Conversely α^+-Thalassemia defects can be very frequent in the country, especially the $-\alpha^{3.7}$ deletion being the predominant one in the Hormozgan and Fars province (see publication 2) and being the most common in the world.

The amplification of the α-globin gene cluster by polymerase chain reaction (PCR) has proved to be difficult, due to the high GC content of the α-globin gene. However, Multiple Gap PCR using the recently published primers [27] gives, with the addition of Betaine to the reaction mixture and the use of `hot start' amplification, good reproducible results.

In case of unknown deletions Southern blotting must be used [28]. This approach also detects α-gene rearrangements (the triple and quadruple α-gene alleles).
Alternatively, southern blot analysis using *Bam*HI, *Eco*RI, *Hind*III, *Xba*I and *Bgl*II restriction enzymes and hybridization with α- and ζ-globin gene probes can be used for detection of known and unknown α^0-thalassemia deletion. The technique is also useful as a second approach for the confirmation of prenatal diagnosis results initially derived from Gap-PCR analysis.

α-Thalassemia point mutations

The non-deletion α^+-thalassemia mutations can be identified by PCR techniques allowing the selective amplification of the α-globin genes [29]. Several of the non-deletion α^+-thalassemia mutations create or abolish a restriction site and may be analyzed by restriction enzyme digestion of the amplified product. For example, the Hb-Constant Spring mutation can be diagnosed by *Mse*I digestion [30]. In theory any of the techniques used for the diagnosis of β-thalassemia point mutations may be used for the diagnosis of the non-deletion α^+-thalassemia mutations. However, due to the high GC content, DGGE is less suitable for the α- than for the β-gene cluster. Therefore, Single Strand Conformation Analysis (SSCA) became a useful alternative [31] until more recently direct sequencing of the α_2- and α_1-genes separately became the method of choice [32]. No simple strategy to diagnose all the known mutations has been developed. The only published approach to date is a complex strategy involving the combined application of the indirect detection methods of denaturing gradient gel electrophoresis (DGGE) [31] and direct DNA sequencing [33].

How to diagnose abnormal hemoglobins

To date more than 23 hemoglobin variants have been described in Iran. The clinically most important ones, requiring routine diagnosis by DNA analysis methods, are HbS, HbD-Punjab, HbC, HbE, and HbO-Arab. The majority of the other abnormal variants are not clinically relevant and are usually given a putative identity based on their electrophoretic position or HPLC retention time. As already mentioned in this chapter, HbS is the only abnormal hemoglobin which can be recognized with absolute certainty using a non-molecular method (Sickle test). For the characterization of all the other mutants, common or rare, absolute certainty is obtained by direct DNA sequencing. However, in the absence of a DNA sequencer some alternatives to DNA analysis, applicable to the recognition of these mutants, are described below.
The HbS mutation, cd6 (A → T), abolishes the recognition site of the restriction enzyme *Dde*I at codon 6. Detection of the mutation by *Dde*I digestion of amplified globin gene fragment is the method of choice [30]. This test also gives a positive signal for the two β-thalassemia mutations cd6 (–C) and cd5 (–CT). Thus care must be taken when analyzing the genotypes of patients with possible Hb S/β-thalassemia. Care is also required in analyzing patients with possible HbS/HPFH or Hb S/δβ-thalassemia phenotypes, as they appear to have an Hb S/S genotype by *Dde*I analysis. For the same reason other PCR-based techniques used for the confirmation of the HbS mutation in carriers and patients can produce false homozygosity or false heterozygosity due to allele-drop-out by misamplification or deletions. A family study to determine the parental phenotypes is essential for the investigation of the genotypes of patients with sickle cell disease.
The Hb D-Punjab mutation, cd121 (G → C), and the HbO-Arab mutation, cd121 (G → A), abolishes an *Eco*RI site at codon 121. Diagnosis is carried out by *Eco*RI

digestion of amplified globin gene fragments [30]. However, this does not distinguish between the two variants and the result must be combined with HPLC or electrophoresis data to identify each variant in carriers.
The HbC mutation, cd6 (G →A), does not abolish the *Dde*I site at codon 6, thus DNA sequencing must be used to characterize the HbC mutation with absolute certainty [30].
The HbE mutation, cd26 (G → A), abolishes an *Mnl*I site and may be diagnosed by PCR amplification and restriction enzyme analysis of the product [31].

Neonatal Screening

The most effective protocol in primary prevention of hemoglobinopathies implies information, carrier diagnostics and counseling during the three most appropriate phases of life. Information and carrier diagnostics is suitable in the pre-marital and/or pre-conceptional phase but also in early pregnancy and to the parents of a diagnosed newborn when neonatal screening is done. The last phase is of course too late for primary prevention since the child is already born, but newborn diagnostics is favorable for morbidity prevention and early treatment of the affected child. Moreover it offers a better chance for retrospective primary prevention for the following children of the couple at risk.
Neonatal diagnostics is actually the only intervention that could be defined as a screening because no clinical indication is present at birth and virtually all newborns are controlled. A strategy for universal screening of neonates is recommended when the incidence is higher than 0.5 cases per 1.000 births [35]. Neonatal screening allows the early detection of sickle cell disease and the initiation of prophylactic antibiotic treatment at the age of 5-6 months reducing acute life-threatening infections and associated morbidity [36]. In the case of thalassemia major it leads to an early decision to come to an appropriate management of the disease, such as eventual bone marrow transplantation, if a severe phenotype is predictable.

The lab and prenatal diagnosis

Iranian authorities were suggesting in the past a different partner choice to young couples in the process of marriage. When couples at risk are diagnosed before pregnancy more prevention alternatives are still present. When they are diagnosed in early pregnancy, prenatal diagnosis remains the only alternative to be offered.
At present, this involves either chorionic villi sampling (CVS) from the 11^{th} week of gestation or much less common, amnio- or cordocentesis, to obtain fetal material for subsequent DNA analysis.
In Iran the uptake (proportion of couples who request prenatal diagnosis when informed about their risk) is variable depending upon the different ethnic, cultural, religious, and social backgrounds. Some other practical factors like gestational age, confidence to counselor, and knowledge about the consequence of affected child, may influence the effectiveness of primary prevention in different situations.

DNA analysis and prenatal diagnosis

As early discussed and presented in publication 1, thalassemia in Iran presents with a heterogeneous molecular background. At least, 43 different mutations can be expected to be found in the multi ethnic groups and an average of about 10% is still uncharacterized. Moreover, a rather broad spectrum of α-thalassemia deletion and point mutations is also present. Therefore a versatile procedure, which can identify all known

as well as unknown mutations causing β- and α- thalassemia point mutations, is required in prenatal diagnosis.

Denaturing gradient gel electrophoresis (DGGE) (as discussed early) seems to be a powerful technique by which one can identify all nucleic acid alterations.

On the other hand, the first half of the β-globin gene contains most of the underlying the β-thalassemia mutations in Iran (See chapter IV). In addition, the beginning of the second intron contains three di-allelic polymorphisms that define the β-globin gene framework. The identification of all mutations and/or polymorphisms within this region is possible using DGGE analysis of three separated PCR fragments.

Analysis of the framework polymorphisms facilitated assessment of allelic drop-out (ADO) in trial experiments. In addition this region has potential diagnostic value through linkage analysis, either by confirming the mutation or as an indirect method for diagnosing samples withβ-thalassaemia mutations localized outside the first half of the β-globin gene.

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