Toward prevention of hemoglobinopathies in Oman

Suha M Hassan

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Proefschrift

ter verkrijging van de graad van Doctor
aan de Universiteit Leiden,
op gezag van de Rector Magnificus
prof. mr.C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 22 september 2015

klokke 15.00 uur

door

Suha Mustafa Hassan
geboren te Muscat (Oman) in 1985

Promotie commissie

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ISBN No.: 978-94-6182-578-0

This study was partially conducted in Oman and partially in the Hemomoglobinopathies Laboratory, at the the department of Human and Clinical genetics at Leiden University Medical Center.

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ABBREVIATION LIST

Hb	Hemoglobin
HBP	Hemoglobinopathies
RBC	Red blood cells
SCD	Sickle cell disease
SCT	Sickle cell trait
HbS	Sickle hemoglobin
HbF	Fetal hemoglobin
HbA	Adult hemoglobin
HBB	Beta globin gene
LCR	Locus control region
IVS	Intervening sequence
HBA2	Alpha 2 globin gene
HBA1	Alpha 1 globin gene
HBD	Delta globin gene
MRE	Main regulatory element
BTM	Beta thalassemia major
G6PD	glucose-6 phosphate dehydrogenase
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
HPFH	High persistence fetal hemoglonin
CBC	Cell blood count
HPLC	High performance liquid chromarography
PCR	Polymerase chain reaction
HLA	Human leukocyte antigen
PD	Prenatal diagnosis
GP	General practitioner
PGD	Pre-implantation genetic diagnosis
CE	Capillary electropherisis

AIM OF THIS THESIS

Oman is one of the countries of the Arabian Peninsula that, in a few decades, has changed from a traditional nomadic culture to a modern industrialized society. In this rapid process, public health has made dramatic progresses fighting against diseases endemic in the country and is now facing the problem of severe hereditary recessive conditions like sickle cell disease (SCD) and beta thalassemia major (BTM). Carriers of this disease are frequent in the country because they were protected against Malaria during millennia of evolution and being in Oman consanguinity a socio-cultural habit, the incidence of children born with hemoglobinopathy is high in many areas of the country.

As shown by experiences made in other countries, management of these severe diseases consists of identifying the mutation spectrum, drawing a genotype-phenotype correlation, selecting the best possible supportive treatment and implementing primary prevention methods for couples at risk.

Oman has an extended network of structured hospitals where local patients receive care and treatment free of charge. Pre-matrimonial counseling clinics are widely distributed in the country; their policy is to identify carrier couples prior marriage, offering as the only option to alter the partner choice whenever a presumed genetic risk has been suspected at the protein level. However, healthy carriers of SCD and BTM may have progeny with striking variability in phenotype and response to therapy may depend on the inheritance of more or less severe mutation and other complex genetic factors. The precise genetic changes prevalent in the different regions of Oman and analysis of the genotype/phenotype relationship of the disease still remain inadequately studied in the country.

The aim of this thesis is to study in detail the molecular spectrum of globin gene mutations and other modifying factors among the different regions in the country by studying a large number of samples to cover the entire population to a) reflect the geographical and historical backgrounds of each region from the described mutations; b) document the mutation spectrum of β- and α- thalassemia to facilitate national screening and educational programmes; c) facilitates genetic pre-matrimonial counseling and testing for the purpose of better risk prediction; d) allow a more tailored treatment for the affected children based on a well characterized genotype and e) the possibility to offer prevention by prenatal diagnosis and the option of medical interruption of pregnancy when necessary, with approval by public health and religious authorities, as is the case in other modern Muslim countries.

Chapter 1 (General introduction) includes general knowledge on the hemoglobinopathies, the relevant genes, modifying factors, world prevalence and treatment options. Chapter 2 focuses on the country of Oman and gives a general view on the problem of hemoglobinopathies in Oman. Chapter 3 describes prevention options and the bottlenecks. Chapter 4 describes the general molecular spectrum of β-thalassemia and α-thalassemia in Oman. A broader spectrum of regional epidemiology and distribution of β-thalassemia and β-variants mutations among the different Omani subdivisions is outlined in Chapter 5. Chapter 6 presents two cases that were characterized during our studies; the first describing a novel Omani mild beta-thalassemia mutation; the second presenting an alpha gene variant in an Omani family that was assumed to lead to a lethal condition in the offspring. The spectrum of alpha-thalassemia in Oman including

large deletions and point mutations is described in Chapter 7. The molecular spectrum of delta-thalassemia in Omani cases associated with low HbA_2 levels is illustrated in Chapter 8. Chapter 9 describes the map of the haplotype distribution of homozygous HbS/S sickle cell disease as well as sub-haplotypes in otherwise identical beta clusters to support genotype/phenotype correlation. Chapter 10 studies the effect of Hydroxyurea treatment in Omani sickle cell disease in association with XmnI polymorphism. Alpha thalassemia whether a risk or a modulation factor in homozygous Omani HbS/S is underlined in Chapter 11. Chapter 12 describes the design of uni- and bi-directional barcoded new generation molecular methodology using Ion Torrent PGM to detect β-mutations in a cohort of Omanies with β-hemoglobinopathy to keep up with the new technologies and scientific advances. Chapter 13 focuses on the views of Omani carrier-couples towards prevention options in view of the ethnic, social and cultural background. Chapter 14 includes the final discussion and conclusion on hemoglobinopathies in Oman and presenting also the limitations encountered when offering modern prevention.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1 - GENERAL INTRODUCTION

1.1 Anemia

Anemia is a common clinical condition characterized by low levels of hemoglobin (Hb). The main causes of anemia include blood loss or insufficient production of red blood cells and/or Hb as a result of a pathological condition. Most common are iron or vitamins deficiencies (Folic acid and B12), which are generally easy to treat. More problematic can be anemia secondary to severe infections and hemolysis or to clinical conditions associated with disrupted bone marrow function. Anemia can be hereditary and caused by red cell enzyme defects, such as glucose-6 phosphate dehydrogenase deficiency (G6PD), or red cell membrane defects, such as congenital spherocytosis. However, the most common hereditary conditions causing anemia worldwide are the Hemoglobinopathies which are caused by abnormal synthesis of hemoglobin, the main focus of this thesis.

1.2 Hemoglobin

Hemoglobinopathies (HBP) are diseases characterized by changes in the production and function of the vital protein hemoglobin, one of the most essential proteins that transport oxygen from the lungs to all tissues, supporting oxidative metabolism. The hemoglobin molecule (Hb) is the main component of the red blood cells (RBC) and is a tetramer, consisting of two pairs of β-like and α-like globin chains. To fulfill the oxygen transport function, Hb tetramers have 4 oxygen binding prosthetic groups (hemes) each containing one iron atom (1) (Figure 1.1). The production of globin chains is coded by globin genes that have a strictly regulated pattern of expression in different tissues and stage of development (2, 3).

During early embryonic development, erythropoiesis takes place in the yolk sac, transitioning to the fetal liver and finally to the bone marrow as development proceeds through fetal and then postnatal life (4) (Figure 1.2). Furthermore with respect to hemoglobin expression there are also two main switches during prenatal development. The first switch from embryonic-to-fetal hemoglobin expression takes place early in gestation. The second from fetal-to-postnatal hemoglobin expression starts during the last months of gestation and is completed during the following 12 months of postnatal life (4).

At birth, the average content of the red blood cells consists of approximately 80% fetal hemoglobin Hb F ($\alpha_2\gamma_2$) and 20% postnatal hemoglobin Hb A ($\alpha_2\beta_2$).

After the age of 2 years and in normal conditions, HbA will make up »97% of the total circulating Hb, and HbA_2, a second postnatal Hb tetramer ($\alpha_2\delta_2$), will be expressed at a much lower level (»2.5%) while HbF expression will have almost disappeared, although it remains detectable at a very low levels (<1%).

All postnatal hemoglobins are synthesized in the bone marrow in the erythroid red cell precursors. After maturation erythroid cells loses their nucleus and are released in the peripheral circulation where, as young red cells (reticulocytes), they quickly mature into regular red cells and carry out their tasks for an average of 120 days before being replaced by new red cells.

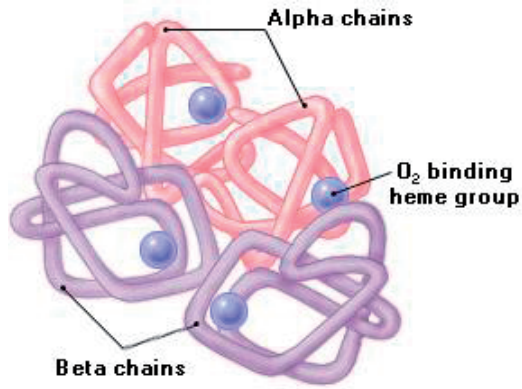

Figure 1.1. Schematic representation of the hemoglobin molecule consisting of two β and two α subunits, each containing an oxygen binding heme group. (Figure adapted from http://resources.med.fsu.edu/gsm/hp/program/section4/4ch5/asidpg16.htm).

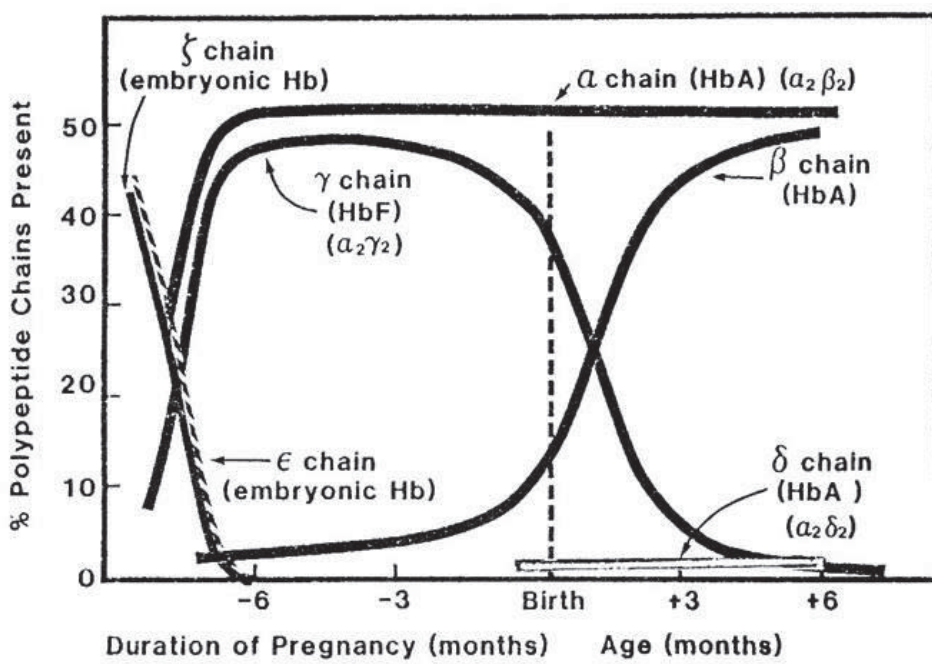

Figure 1.2. Depicting the expression of the globin genes during the switch from embryonic, fetal and postnatal stage. (Figure adapted from http://www.ilym.org/tiki-index.php?page=Plummer1983).

1.3 Hemoglobin evolution

The basic structure and function of the hemoglobin protein is highly conserved among different species making it an interesting model for evolutionary and biochemical investigation. Gametic mutations are the key of the evolutionary mechanism of all living organisms. The mechanism is based upon selection of random *de novo* mutations passed to the progeny to be tested for their advantage or disadvantage. Hemoglobin in its current form has a long evolutionary history. Globin genes coding for the basic protein in vertebrates are believed to be older than 500 million years (5). The relationship between the primary sequences and the three-dimensional structures shows that the human α- and β-globin genes family is derived from a monomeric myoglobin, the oxygen storage and delivery proteins still present in our muscular tissues (6). During the last 200 million years splitting between ancestral alpha and beta like genes, duplications and rare point mutation events have differentiated the ancestral proto-myoglobin gene into the current alpha- and beta- like genes present in the human gene clusters (7) (Figure 1.3).

1.4 Malaria and globin gene selection

As briefly mentioned above, evolution depends on many factors among which the most important are the adaptation to the environment and the fitness of the individuals in providing healthy progeny (8). Hemoglobinopathies (HBP) are caused by mutations on the globin genes that had probably not been particularly selected for until man developed agriculture and had to share the environment with malaria mosquitoes. Carriers of a globin gene mutation were advantaged in malarial-infested areas (9,10,11).

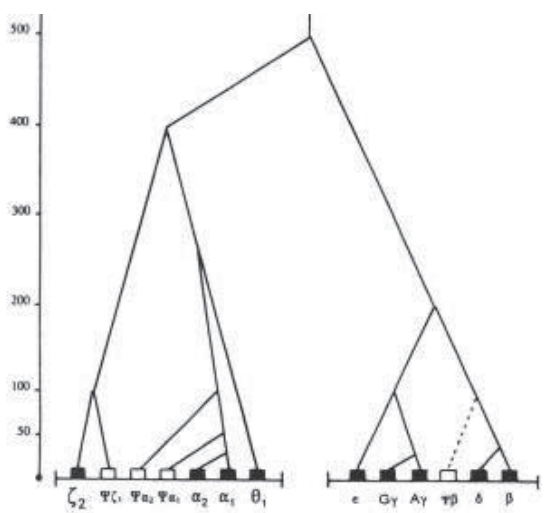

Figure 1.3. Evolution of 500 million years of the human globin gene clusters from proto-myoglobin to the actual α and β-like genes (From Giordano PC. Dissertation 1998). Active genes are in black, open boxes are non-active pseudo (ψ) genes.

Haldane observed that the geographical distribution of HBP and the distribution of malaria greatly overlap, and published the hypothesis that individuals carrying sickle cell disease (SCD) or thalassemia must have a selective advantage over non-carriers in terms of survival and reproduction in malarial-infested areas (12,13,14,15).

Regarding the protecting mechanisms, it has been postulated that the sickle cell hemoglobin (HbS) mutation confers a protection against malarial infection because the deoxy HbS cells infected with malaria become sickled and are subsequently destroyed by the T-cells and macrophages, reducing acute malarial infection in the carriers (16,17). Similarly, it has been shown that α-thalassemia protects against severe malaria by the same mechanism as HbS, ameliorating the pro-inflammatory effects of cytoadherence (18). In vitro studies have shown that there is reduced malarial growth in erythrocytes and enhanced removal of parasitized cells by the immune system in individuals that are thalassemia carriers or heterozygote for Hb S (19).

1.5 The human hemoglobin genes

The human globin genes are clustered on different chromosomes, the β- like genes on chromosome 11 and the α- like genes on chromosome 16. The beta cluster contains an inactive pseudo beta gene (ψβ), the embryonic epsilon (ε) gene as well as the fetal G-gamma (Gγ) (*HBG2*) and A-gamma (Aγ) *(HBG1*) and adult delta (δ) (*HBD*) and beta (β) (*HBB*) genes (20). As mentioned above, the main genes responsible for the hemoglobin composition in postnatal life (HbA) are the β and the α genes. The beta gene is 1.6 Kb in length. About 50 to 70 kb upstream of the beta globin gene is the locus control region (LCR) which regulates the activation and expression of the β globin gene cluster (21, 22).

The alpha cluster located on the telomeric region of chromosome 16, contains several inactive pseudo genes (ψζ, ψα2, ψα1) and a θ gene of unknown function, an embryonic zeta (ζ) gene and two identical α-globin genes (α_2 and α_1) (*HBA2* and *HBA1*) both 1.2 kb long. The main regulatory element (MRE) of the alpha globin genes is located about 60 kb upstream (23). All alpha- and beta- like genes consists of three coding regions (exons) and two introns known as intervening sequences (IVS) (Figure 1.4). The genes are linearly arranged 5' to 3' in the order expressed during development (Figure 1.5) (4). While the beta globin chains are needed for the formation of HbA only, the α-globin chains are involved in the embryonic hemoglobins Gower II (α_2/ε_2), the fetal hemoglobin (HbF (α_2/γ_2), and in both postnatal HbA_2 (α_2/δ_2) and HbA (α_2/β_2). This difference explains some of the postnatal pathology of beta gene defects and the pre- and post-natal pathology of alpha gene defects. The hemoglobin tetramers formed during embryonic, fetal and adult development are summarized in Table 1.1.

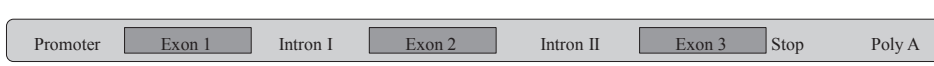

Figure 1.4. Schematic representation of the basic structure of the globin genes, showing the promoter, coding exons and introns and stabilizing polyA tail.

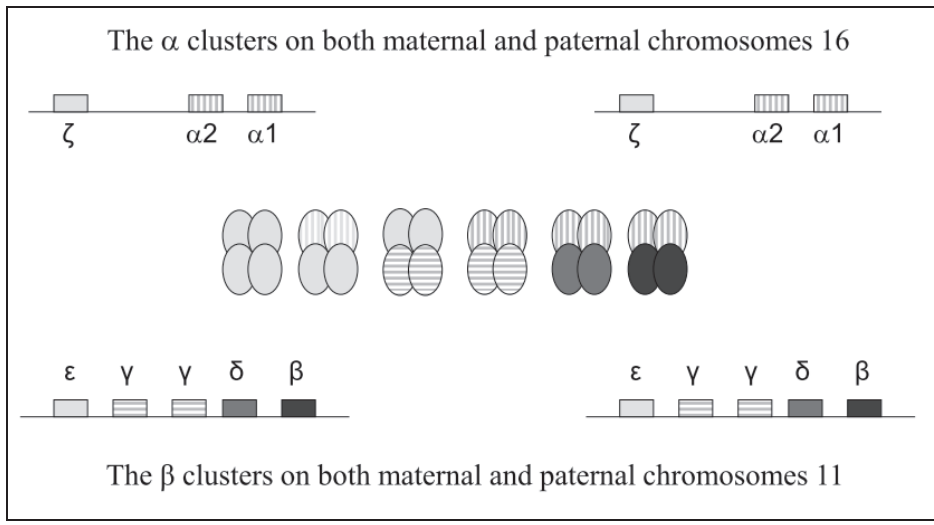

Figure 1.5. Schematic representation of the globin gene clusters on chromosome 16 and 11 respectively with the embryonic ζ and ε genes, the fetal γ genes, the embryonic, fetal and postnatal α genes and the postnatal δ and β genes. The corresponding Hb tetramers, Hbs Gower-I and -II, Hb Portland, Hb F, Hb A_2 and Hb A are depicted from left to right with their different globin chain compositions. Pseudo genes are not shown (Giordano PC, 2012, reference 24).

Table 1.1. Summary of the different hemoglobin synthesized during normal human developmental stages.

Embryonic hemoglobins	Fetal hemoglobins	Adult (post-natal) hemoglobins
Hb Gower I (ζ_2/ε_2) Hb GowerII (α_2/ε_2) Hb Portland (ζ_2/γ_2)	Hb F (α_2/γ_2)	Hb A (α_2/β_2) Hb A2 (α_2/δ_2)

1.6 The Hemoglobinopathies

Hemoglobinopathies (HBP) are the most frequent inherited autosomal recessive disorder in man. In an autosomal recessive disorder two copies of an abnormal gene must be present in order for the disease to develop. Both males and femailed can be carriers or affected.

A carrier couple will transmit the disease to their children in a Mendelian fashion, which means that statistically speaking, half of the progeny will be carriers of one of the two parental mutations and be unaffected like their parents (heterozygous), ¼ will not be carriers (normal) and ¼ will have both parental mutations and be affected by the disease (Figure 1.6). The severity of the disease will depend on the type of mutation and/or complex gene combinations and present with variable form of thalassemia.

As mentioned above, HBP are caused by mutations on the globin genes that, due to a selective advantage in the presence of malaria, have become endemic in the tropical and

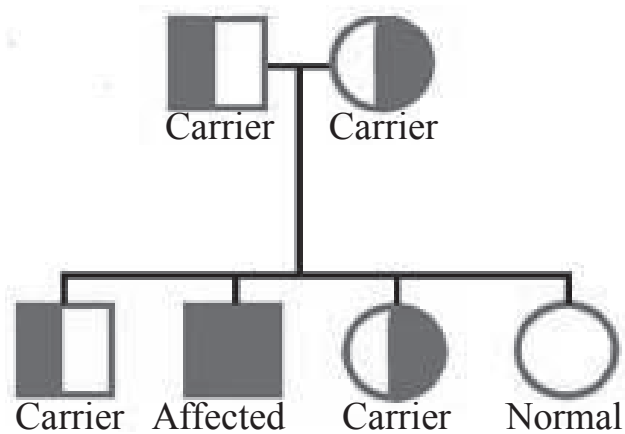

Figure 1.6. Inheritance of an autosomal recessive mutation causing SCD or BTM following a Mendelian fashion.

subtropical areas of the world. From there, they spread by ancient migrations and slavery and today, due to recent massive migrations, are found in many non-endemic areas worldwide (25).

Mutations on the globin genes can change the primary structure of the gene products causing abnormal hemoglobins, can decrease the expression of the gene causing thalassemia and anemia and finally some are silent (polymorphisms).

Most mutation events are limited to one or few nucleotides (point mutations) while other involves larger defects. Large deletions which eliminate parts of genes or even entire genes or locus control regions are associated with a reduced synthesis of the relevant globin chain and hence an expression of thalassemia or it can result in an elevated expression of Hb F (e.g HPFH deletions – section 1.13).

Point mutations can be associated with either thalassemia or abnormal hemoglobins, the latter typically caused by changes on the coding regions of the genes (exons). Point mutations associated with reduced globin synthesis may occur in all parts of the globin genes starting from the locus control regions, the promoter regions, the initiation and stop codons, the coding exons, the exon/intron splice junction sites, deeper intronic sequences, the 5' and 3' untranlsated regions and finally the poly A addition site. Point mutations may generate a stop codon directly or indirectly because of a frame shift due to a deletion or an insertion event of more than one nucleotide causing thalassemia. Exonic point mutations that change a particular amino acid lead to an abnormal hemoglobin, which may or may not have phenotypic consequences. Rarely exonic mutations affect the normal splicing of genes (for example HbE). Not all mutations affect the structure or function of the hemoglobin molecule and it is important to define the correlation between the mutation and the phenotype to predict the severity of the conditions in homozygous or compound heterozygous form.

Hundreds of point mutations causing Hemoglobinopathy have been characterized (26).

A minority are common in specific geographical areas or ethnic groups while many are rare and may or may not be associated with an ethnic origin. We investigated the common HBP's in Oman in chapter 4 to reaveal the common spectrum of HBP mutations.

1.7 The structural and the expression defects

As mentioned above, hemoglobin disorders are divided into two main groups: the structural (hemoglobin variants) and the expression defects (the thalassemias).

The structural hemoglobin variants result in most of the cases from point mutations causing single amino-acid substitutions mainly in the β or α globin chains (27) and have generally little or no adverse effect in the carriers. However, structural mutations may alter in some cases, the stability, the expression or the functional properties of the hemoglobin tetramer and lead to a clinical disorder in the carrier as well.

More than thousand structural hemoglobin variants have been described but the most common worldwide are Hb S, Hb C, Hb E and Hb D^{Punjab}.

Although all recessive, the homozygous state for Hb S as also the combination of HbS/β-thalassemia and HbS/HbC, E or D^{Punjab} variants results in sickle cell disease (SCD) (Table 1.2). The compound heterozygous state for Hb S and Hb C is associated with a clinically milder SCD while the other combinations are usually severe. The HbE variant, although mild in the carrier and in the homozygous state, it may result in both severe SCD and thalassemia major in combination with HbS and β-thalassemia mutations respectively (28) (Table 1.2).

Table 1.2. Cross table showing the combination of HbS and of other common β gene defects (double-lined squares) and of the common α gene traits and the genetic risk deriving from their combinations. (For rare, unknown Hb X variants: ? stands for unknown risk and ?! for possible risk.). Hb S: [β6(A3)Glu→Val, GAG>GTG; *HBB*: c.20A>T]; Hb E: [β26(B8)Glu→Lys, GAG>AAG; *HBB*: c.79G>A]; Hb C [β6(A3)Glu→Lys, GAG>AAG; *HBB*: c.19G>A]; Hb D: Hb D-Punjab [β121(GH4)Glu→Gln, GAA>CAA; *HBB*: c.364G>C] (courtesy of P.C Giordano).

Carrier of	β-Thal	HbS	HbE	HbC	HbD	α^+-Thal (- α/αα)	α°-Thal (- -/αα)	HbX
β-Thal	β-Thal major							
HbS	SCD	SCD						
HbE	β-Thal major	SCD	β-Thal minor					
HbC	β-Thal minor ?	SCD	β-Thal minor	HbC disease				
HbD	β-Thal minor	SCD	β-Thal minor	β-Thal minor	Normal			
α^+-Thal (- α/αα)	β/α^+ Thal minor	α^+-Thal minor	β/α^+ Thal minor	α^+-Thal minor	α^+-Thal minor	α^+/α^+ Thal minor		
α°-Thal (- -/αα)	β/α° Thal minor ?	α°-Thal minor	β/α° Thal minor ?	α°-Thal minor	α°-Thal minor	HbH disease	α°-Thal major	
HbX	?!	?!	?!	?	?	?	?	?

Expression defects of the β-globin genes, most of them recessive in the carriers, are virtually all associated with severe or intermediate forms of β-thalassemia, with SCD being the main concern for public health and prevention in endemic and immigration countries.

Due to the presence of 4 active alpha globin genes, structural mutations of these genes express at a lower percentage (<25%) and their phenotype is usually mild. Expression defects of the alpha genes (deletion or point mutations) express in mild to severe phenotypes depending from the number of genes affected. Loss of expression of all 4 alpha genes results in a lethal condition usually incompatible with post-natal life. When 3 alpha genes are not expressed the phenotype is severe or intermediate (HbH disease). Loss of expression of one or two alpha genes results in a very mild phenotype. Exceptions are those mutations causing unstable α-globin products or mutated Poly A addition signal sequences resulting in severe HbH diseases when in homozygous or hemizygous forms. An example of a possibly severe homozygous alpha-thalassemia case is described in Chapter 6.

1.8 Sickle cell disease: history and clinical condition

The disease long known in Africa with several local names (Abotutuo, Chwechweechwe, Nwiiwii or Nuiduidui), was firstly reported in 1910 by Herrick (29) as a severe hereditary condition affecting young children of African origin. Sickle cell disease or anemia (SCD or SCA) has probably expanded in West Africa, most likely before the desertification of the Sahara, which took place about 10,000 years ago (30). Herrick proposed the association with a cellular abnormality because of the peculiar elongated sickle like shape of the red cells observed in the blood smear of the patients.

In the 1930's, it was shown that the sickle shape was induced by low oxygen pressure and was reversible after oxygenation. Pauling et al. separated in 1949 the abnormal hemoglobin fraction and because of the sickle shape of the cells they called this hemoglobin HbS (31). Later, methods such as the starch gel electrophoresis, allowed an easy detection of HbS and of the other common Hb variants.

In the middle of the 50's, Ingram demonstrated, by separation and analysis of tryptic peptides on combined paper electrophoresis and chromatography (fingerprinting), that the glutamic acid residue at position 6 in the N-terminal peptide of the β chain was replaced by a valine (32). More than twenty years later DNA analysis and the identification of the β globin gene (33) allowed the characterization of the single amino acid substitution resulting from an adenine to thymine transversion at codon 6 (G*A*G→G*T*G) which results in the formation of hemoglobin S when the abnormal β-chains combine with the α-globin chains. To date, more than 1000 hemoglobin variants have been reported and new ones keep coming. The pathological implications of the common variants are well known and that of the rare ones is a matter of continuing research. One century later and the diseases caused by these recessive hereditary traits are quite well treated but not yet cured and therefore carrier identification and primary prevention are the main issues faced by public health.

Being a recessive condition, HbS carriers are mainly asymptomatic and their red cells do not sickle to any significant degree at normal venous oxygen tension. Only at a very low oxygen tension the erythrocytes will temporarily sickle when a carrier is exposed to high altitude (34), extreme exercise along with dehydration (17), and hypothermia in normoxic and hypoxic

conditions (35). Under environmental stress, HbS carriers are prone to a reduced red blood cell (RBC) deformability, which is thought to increase blood viscosity, raising the risk for vaso-occlusion events (36). Although it is very rare for sickle cell trait (SCT) subjects to experience any of these complications, it is of great importance that SCT individuals are aware of their carrier state to avoid potential genetic risk and risk of complications during anesthesia and pregnancy (37).

Patients affected with SCD suffer of a cascade of pathological events causing chronic and acute infarctions, excruciating painful crises, progressive organ and tissues damage and hemolysis (38), drastically shortening the RBC life span (39). HbS tend to polymerize when deoxygenated, resulting in sickle shaped cells causing chronic and acute infarctions (Figure 1.7). As a consequence of vaso-occlusion, dactylitis is one of the first symptoms observed in infants, affecting about 30% of patients in the first year of life (40). Other severe symptoms include splenic sequestration, acute chest syndrome, leg ulcers, avascular necrosis and gallstones. The disease is caused by different β-globin genotypes (always including at least one Hb S allele) and modulated by quite a few genetic and external causes. Therefore not all SCD patients have the same severe outcome of the disease and it is not easy to predict a genotype / phenotype correlation.

Chapters 9, 10 and 11 of this thesis are based on correlation studies between clinical severity of SCD with identical beta genotype and haplotype, subhaplotypes, XmnI polymorphism and response to hydroxyurea drug and finally in relation to alpha-thalassemia genotypes. These studies were conducted in order to draw associations between genotype and phenotype for better risk assessment and selection for the best treatment.

The life expectancy of well-treated SCD patients is considerably shorter than that of the general population and the survival greatly depends on the genotype. If well treated, male and female patients with intermediate SCD genotypes are reported to have a median life

Figure 1.7. Schematic representation of the vaso-occlusion caused by the formation of sickle cells in the post capillary veins under oxygen deprivation (adapted from http://www.cixip.com/index.php/page/content/id/559).

Table 1.3. Summary of the common and rare mutations in the *HBB* gene, including the common variants HbS, HbC, HbD^{Punjab}, HbE that may cause SCD or TM. The phenotype prediction is either described (!) or presumed (?). Adapted from Giordano, 2013 (reference 45).

Traits	Combinations	Predictions
HbS	S/S or S/β-thal	!
HbC	C/S intermediate	!
HbE	E/S severe or intermediate or E/β-thal	!
HbD^{Punjab}	D/S severe	!
HbO^{Arab}	O/S severe	!
Hb Lepore	L/S or L/β-thal severe or L/E	!
HbS Antilles (HbS + β23)	Dominant in carrier, severe in combination	!
HbC Ziquichor (HbS + β58)	S/S, C/S, D/S, E/S... or S/β-thal severe	!
HbC Harlem (HbS + β73)	S/S, C/S, D/S, E/S... or S/β-thal severe	!
HbS Providence (HbS + β82)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
HbS Oman (HbS + HbO^{Arab})	Dominant in carrier, severe in combination	!
HbS South End (HbS + β132)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
HbS-Sao Paulo (HbS + β65)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
Hb Ndjamena (HbS + β37)	S/S, C/S, D/S, E/S... or S/β-thal intermedia	?
HbS Travis (HbS + β142)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
HbS Cameroon (HbS + β90)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
HbS Wake (HbS + β139)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
HbS Jamaica Plain (HbS + β68)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
HbS Clichy (HbS + β8)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
HbS san Martin (HbS + β105)	S/S, C/S, D/S, E/S... or S/β-thal severe	?
$HbS/thal^{+}$ (HbS + -88C>T)	S/S or S/β-thal intermedia	?
HbC Rothschild (HbC + β37)	C/S intermediate	?
Hb Arlington Park (HbC + $HbN^{Baltimore}$)	C/S intermediate	?
Hb Corbeil (HbE + β104)	E/S intermediate, E/β-thal severe	?
$HbT^{Cambodja}$ (HbE + HbO^{Arab})	E/S intermediate, E/β-thal severe	?
Hb Cleveland (HbD^{Punjab} + β92)	D/S severe	?
Hb Korle-Bu	KB/S intermediate	?
All $β^{0}$-thalassemia defects	S/β-thalassemia severe	!
All $β^{+}$-thalassemia defects	S/β-thalassemia severe or intermediate	!?
All β-variants with thalassemic effect	S/β-thalassemia severe or intermediate	!?
GγAγδβ-deletions	Del/S, Del/E, Del/β-thalassemia severe	!?
Aγδβ-deletions	Del/S, Del/E, Del/β-thalassemia intermediate	!?
εγδβ-deletions	Del/S, severe	!?
δβ-deletions	Del/S, Del/E, Del/β-thalassemia intermediate	!?
HPFH point mutations	S/HPFH, β-thal/HPFH, HbE/HPFH mild/silent	!?

expectancy of 42 and 48 years respectively (41) whereas if untreated, the disease is usually lethal in childhood (42).

As shown in Table 1.2, patients with SCD can either be homozygous for the HbS allele (HbS/S), compound heterozygous with other common Hb variants or hemizygous with a β thalassemia mutation (HbS/β). Severe conditions can also result from less common combinations such as HbO^{Arab} (43) or with a list of rare traits (Table 1.3). Inheritance of HbS with β° thalassemia mutations results in absence of HbA and in the presence of HbS % similar to HbS/S condition but generally associated with marked microcytic anemia. When HbS is combined with a β^+ mutation, HbA is synthesized at a reduced rate, and with milder beta defect the SCD condition can be less severe (44). We describe an example of a mild beta-thal nucleotide transversion in association with HbS in chapter 6.

1.9 The thalassemias

As mentioned above, the thalassemias are defects caused by globin gene mutations affecting gene expression. Consequently, thalassemias are classified according to the particular globin chains ineffectively synthesized (46). The pathological conditions associated to thalassemia can be very variable. While generally asymptomatic and only associated with a mild microcytic anemia in the carrier, thalassemia can be lethal in utero and/or very severe in postnatal life depending from upon the genotype at the β-gene locus and to some extent other globin and non-globin genes. Because of the prenatal expression of the alpha genes, alpha thalassemias can present symptoms before birth in mild, severe or lethal forms, while beta thalassemias are usually asymptomatic until about six months after birth, at the time when the postnatal HbA normally takes over from the fetal HbF. In addition, patients may present with different phenotypes due to the kind of mutation or defect combinations. As previously mentioned, the majority of beta thalassemias are caused by point mutation defects. The majority of alpha- and a few beta- thalassemias are caused by large deletions. Alpha deletions may affect one or both alpha genes of the specific allele and homozygosity for two fully deleted genes is not compatible with life. Some large deletions of the beta genes may include the fetal and the embryonic genes in the beta globin gene cluster and in the heterozygous form may be severe in utero but transforming to a mild beta thalassemia heterozygosis in adult life (47).

1.10 The beta thalassemia: history and clinical conditions

How old thalassemia can be was shown by J. Lawrence Angel who in 1964 found porotic hyperostosis and thickening of the diploic space prominent in skeletal remains of children from the Bronze Age in Cyprus and Greece as a result of thalassemia major (9,48).

The disease has remained clinically unclassified until the 20th century probably due to the general high infant mortality and to the fact that the condition was not associated with the humoral theory of Greek medicine developed by Hippocrates which was rather philosophical but not that far from the truth.

Greeks believed that not only the blood itself but blood vessels carried air, food and emotions, while Romans associated all kind of unhealthy state with the stinking marshes that surrounded the city giving the actual name of the disease Malaria, meaning “bad air”.

Only in the 20th century two publications both published in 1925, one in Italy and the other in the USA, defined the severe form of the disease.

Rietti called the disease IERGA (Ittero Emolitico con Resistenza Globulare Aumentata) because of jaundice, hemolysis and the classical increased osmotic resistance of the red cells (49). Cooley and Lee reported the same pathology in children of Italian and Greek immigrants in the USA (50). Subsequently the disease was given many names such as Rietti-Greppi-Micheli disease, Cooley anemia, erythroblastic anemia and all this became confusing until the definition "Thalassemia" from the Greek word for sea was proposed in 1932 by Whipple and Bradford, due to the high incidence of the disease in Mediterranean populations (51).

At the same time, population geneticist J.B.S. Haldane, had elaborated the theory of malaria selection for thalassemia and his hypothesis was published with the first mathematical models for evolution due to selection (52). Between 1944 and 1947 Silvestroni and Bianco studied many affected families and defined the hereditary character of the disease (53). In 1955 Kunkel and Vallenius separated the HbA_2 fraction (54) and in 1957 Silvestroni and Bianco again demonstrated that an elevated HbA_2 fraction was present in the parents of affected children (55). From that time on, many developments have followed at the biochemical and molecular level and today thalassemia is perhaps the most well studied recessive disorder in man. As mentioned above, beta-thalassemia (β-thal) is mainly caused by prevalent or less common point mutations in the β-globin gene due to substitutions or deletional/ insertional defects. Defects can either lead to reduced synthesis of the β-globins (β^+) or complete absence (β^o). In distinction to the classical and common recessive forms of beta-thalassemia, some rare dominant beta globin mutations may result in the synthesis of extremely unstable beta globin variants causing intermediate thalassemia phenotype in the heterozygous state (56). Usually mutations in the promoter and 5' UTR affect gene transcription while mutations in the splice junction, 3' UTR and polyadenylation site affect mRNA processing and mutations in initiation codon or frame shift mutations affect mRNA translation. The very mild or silent mutations are associated with normal red blood cell indices and normal or borderline HbA_2 (57) raising difficulty in carrier screening at the hematological level. We describe in chapter 6 an example of a mild beta-mutation associated with near borderline HbA_2 %.

Clinically and hematologically, thalassemia is classified into three conditions of increasing severity; the mild beta-thalassemia carrier state, the thalassemia intermedia, and the severe thalassemia major.

As mentioned above, carriers of β-thalassemia are usually asymptomatic with a hematological parameters characterized by a mild anemia, reduced MCV and MCH values (60–70 fL and 19–23 pg), a raised level of HbA_2 (3.5–9.0 %), and normal or slightly elevated HbF (58).

Homozygous or compound heterozygous for severe mutations become severely anemic around six months of age, once the delayed expression of the normal HbF has become insufficient in absence of HbA. Children are usually diagnosed within the first two years of life due to severe anemia, hemolysis and failure to thrive. The hematological picture of a non-transfused patient will show a severe erythromorphology with numerous of erythroblasts, very low Hb, MCV and MCH values, absence of HbA with HbF and HbA_2 as the only Hb fractions present. In these conditions, patients will usually require an urgent blood

transfusion that will maintain the Hb value at a reasonable level for 2-3 weeks when the need for a continuous transfusion regime will become evident. Subsequently these patients will accumulate transfusional iron up to a toxic level and thus require continuous iron chelation therapy. Other complications occurring in transfusion-dependant patients include growth retardation, delay or failure of sexual maturation, cardiac disease and complications in liver or endocrine glands (59). Individuals who are regularly transfused and get state of the art management may survive beyond the age of 40. Cardiac complications are the cause of death in 71% of the patients with thalassemia major (60). The clinical presentation of thalassemia major is listed in Table 1.4.

Beta-thalassemia intermedia is a milder conditions that includes a very heterogeneous group ranging in severity from a transfusion free carrier-like state to the delayed severe transfusion-dependent type (61). Intermediate cases with the severe forms present severe clinical symptoms usually between the ages of 2 and 6 years. The mild condition may present with complications later in life or may go on without requiring blood transfusion till adulthood or during pregnancy. The intermediate state result from complex genotypes involving coinheritance of homozygous or compound heterozygous mild β-thalassemia alleles and may also result from the co-inheritance of alpha- and beta-thalassemia mutations or co-inheritance of additional alpha globin gene genotypes (triplicated or quadruplicated alpha globin gene rearrangements) when interacting with typical heterozygous beta-thalassemia (62).

Beta thalassemia subjects with no functional beta globin synthesis may present as beta intermedia due to amelioration by high Hb F expression caused by HPFH deletions (63) or rare mutations in the beta-LCR regulatory sequence (64). Essentially, the clinical severity of beta thalassemia major is related to the extent of imbalance between the alpha globin and non alpha globin chains (62). When the beta globin chains are reduced or absent, the free alpha chains precipitate and lead to oxidative damage of the cell membrane, hemolysis and ineffective

Table 1.4. List of the variable clinical presentation possibly seen in a β-thalassemia major individual divided per different age groups.

Age group	Clinical phenotype
6-24 months	Pallor and jaundice Failure to thrive Feeding problems Diarrhea Fever Spleen and liver enlargement
2+ years	Growth retardation Poor musculature Hepatosplenomegaly Leg ulcers Skeletal deformities Gallstones Thrombosis

erythropoesis (65). Although differentiation between thalassemia major and thalassemia intermedia is not always easy, it is needed to plan state of the art management and to avoid starting unnecessary transfusions (66).

In conclusion, analysis of both the alpha and beta genotypes and testing for the presence of ameliorating genetic factors is essential for risk prediction, treatment and / or prevention of the hemoglobinopathies. In order to define these factors in the Omani population we have described the beta-thalassemia spectrum in the country in an extensive study presented in chapter 5.

1.11 The alpha thalassemias

While humans have only two beta globin genes, one on the maternal and one on the paternal chromosome 11, the alpha gene cluster on chromosome 16 is characterized by two active alpha genes, two inherited on the maternal allele and two on the paternal, resulting in a total of four alpha globin genes.

As already mentioned, most α thalassemias are caused by deletions that may remove one or both α-globin genes of each allele. In the first case, when one of the two genes is still expressed, the condition is called α^+ thalassemia. When both α genes of the same allele are deleted the condition is called α° thalassemia. The presence of 4 alpha genes and the occurrence of partial expression of an affected allele make risk assessment somewhat complex. In general, carriers of α^+ or α° thalassemia will show mild anemia if any, with minimal to evident microcytosis (reductions in MCV values) and hypochromia (low MCH values) and sometimes a slight reduction in HbA_2 levels. However, the genetic risk of the two conditions will be very different. While an individual homozygous for α^+ thalassemia will not be affected and present with mild anemia, homozygosis for α° thalassemia will be a lethal condition and combinations of α° with α^+ conditions will result in the intermediate forms of variable severity called HbH disease.

The phenotype of Hb H patients is variable depending on the nature of the mutation. HbH caused by deletional mutations results in a moderately severe hypochromic microcytic anemia but clinically these patients are less severely affected than patients with HbH caused by point mutations who have a more severe manifestation and may require frequent hospitalization and recurrent blood transfusions (67).

The severe form α° homozygosis (Hb Bart's Hydrops Fetalis), which results from four defective α-globin genes, is a severe conditions leading to perinatal death. The common genotypes of these conditions are summarized in Table 1.5.

Most cases of alpha thalassemia trait in the world are caused by relatively large deletions rather than point mutations. The most common alpha thalassemia mutations are the $-\alpha^{3.7}$ kb rightward deletion (RW) followed by the $-\alpha^{4.2}$ kb leftward deletion (LW) which are both α^+ defects. The former involves the deletion of the 3' part of (*HBA2*) gene and 5' of (*HBA1*) gene, forming a hybrid gene while the latter consists of full removal of *HBA2* gene and 5' of the *HBA1* gene. Both defects are α+ conditions.

Other large deletions involving the alpha cluster include the $-\alpha^{20.5}$, the SEA, the Med I, the Thai and the Fil (68) (Figure 1.8). These deletions involve both alpha genes and are therefore called the α° thalassemia conditions associated with the severe forms of the disease. Non-

Table 1.5. The symbolic indication of the different alpha genotypes and the accompanied phenotype.

Genotype	No. of defective genes	Phenotype
(-α/αα)	1	Silent or mild carrier α^+
(αα/--)	2	Mild α° carrier
(-α/-α)	2	Mild homozygous α^+
(-α/--)	3	Intermediate (HbH)
(--/--)	4	Severe Hb Bart's (hydrops fetalis)

deletional alpha thalassemia mutations are also regularly found. Among the most common are the penta-nucleotide 5nt deletion (HBA2: c.95+2_95+6delTGAGG), the polyadenylation tail (+94) AATAAA>AATAAG mutation (HBA2: c.*94A4G) and Hb Constant Spring (CS) (HBA2:c.427T>C) (69). We have studied the spectrum of alpha-thalassemia in Oman including deletional and non-deletional mutations in chapter 7 to decipher the common and rare alpha mutations in the population.

Carrier identification of alpha thalassemia is important as it has a similar hematological picture to iron deficiency anemia (70). Carriers of alpha thalassemia are often wrongly diagnosed and empirically treated with iron therapy where in fact they only need information and eventually folic acid supplement.

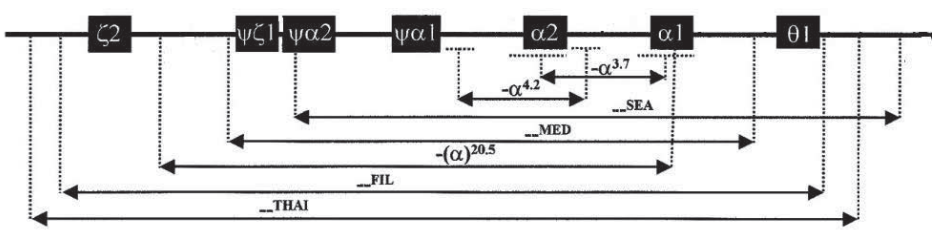

Figure 1.8. The common deletions of the alpha-globin gene cluster. The extent of each deletion is represented by an arrow line. Adapted from Tan et al, 2001 (reference 71).

1.12 Delta thalassemia

The delta globin gene coding for the globin chains needed to form hemoglobin A_2 (δ_2/α_2) is located on chromosome 11 between the β- and the γ-globin genes (see section 1.5). The δ gene (*HBD*) is similar to the β-gene but with a much less active promoter. Therefore, the δ gene expression in normal individuals is approximately 2.5–3.5% of the total hemoglobin resulting in a similar level of expression of HbA_2. Defects of the δ genes have no clinical significance but may compromise the diagnosis of β-thalassemia trait. Thalassemic or structural defects of the δ may reduce HbA_2 expression (<2%) and this in co-inheritance with a β-thal allele, may lead to diagnostic mistakes due to the reduction to normal ranges of the HbA_2 level that should

have been found elevated in that particular β-thalassemia carrier (72,70). Similarly, structural mutations of the delta globin genes may cause the splitting of HbA_2 in two fractions, and if the abnormal fraction is overlooked, it many cause a wrong estimation of the HbA_2 level as well.

As in β-thalassemia, also in δ-thalassemia, each population has its own spectrum of common mutations which should be taken into consideration when confirming or excluding a presumed carrier of β-thalassemia with less elevated or normal HbA_2 levels (73). For that we investigated cases with low HbA_2 readings to define the spectrum of δ-thalassemia in the Omani population in chapter 8.

1.13 Hereditary persistence of fetal hemoglobin (HPFH) and δβ-thalassemia

The clinical definition of hereditary persistence of fetal hemoglobin (HPFH) goes back to the time when molecular analysis of gene deletions was not yet available and in fact most of these defects are mild δβ-thalassemias resulting from large deletion in the β-gene cluster enhancing the HbF expression. Today, the real HPFH defects are considered those caused by non-deletion events, that is by point mutations in the promoters of the γ globin genes.

More than 40 different types of HPFH like δβ deletions with varying 5' and 3' breakpoints have been reported usually all mild in the carriers (74). These disorders are characterized by high HbF levels and can be distinguished from one another by comparing their clinical and hematological parameters and deletion break points (75). Individuals heterozygous for HPFH deletions present with elevated red blood cell (RBC) counts and with HbF levels ranging from (15-30%) (76). Rare cases homozygous for HPFH deletions have been described with near 100% HbF and very high RBC counts (77).

The elevated RBC counts are due in these cases to the higher oxygen affinity of the HbF tetramer which is delivering lesser O_2 to the tissues causing hypoxia in spite of the elevated Hb levels of these patients. Depending on the level of the HbF expression, deletions in combinations with β-thalassemia can be associated with thalassemia intermedia phenotypes (75) and in general compound heterozygous with β-thalassemia are clinically mild due to the high levels of γ-globin synthesis compensating for absence of β-globin chains, thus reducing the level of excess alpha globin chains.

1.14 Hb Lepore

Hemoglobin Lepore (Hb Lepore) defects are caused by unequal cross over between homologous regions on the δ and the β genes resulting in several δβ-fusion genes with β^+ thalassemia expression. Several Hb Lepore mutations have been described of different kind of δβ-globin hybrid chains (78). The reason why the δβ-globin hybrid chain is transcribed at a decreased rate is because it is controlled by the δ gene (*HBD*) promoter which is normally less efficient than the β gene (*HBB*) promoter. Due to decreased quantity of the δβ hybrid chains produced, the clinical phenotype in the heterozygote is associated with mild hypochromic, microcytic anemia with a normal or reduced HbA_2 and eventually elevated HbF level and a relatively low percentage of Hb Lepore (6-15%). Cases with homozygosity or compound heterozygosity of Hb Lepore and β-thalassemia, may present with a severe thalassemia major or intermedia phenotype (79). Therefore, molecular characterization is essential, especially in cases of risk prediction.

1.15 The classic modulating factors (β-globin cluster haplotypes, high HbF, XmnI polymorphisms and coexisting alpha thalassemia)

Thalassemia and sickle cell disease risk prediction is a complex matter. Different thalassemia mutations may have different reduction in expression, compensation with higher HbF expression can be present or absent, and coexisting alpha thalassemia or alpha gene triplications and duplications may ameliorate or aggravate the phenotype in both patients and carriers. As these and other still incompletely defined factors can either ameliorate or exacerbate the phenotype, genetic analysis of modulating factors helps the geneticist and the clinicians to predict the severity and prognosis of the disease. We have approached these elements in chapters 9, 10 and 11 of this thesis.

1.16 Haplotype

The β-globin haplotypes on which the mutation is carried was originally defined based on the pattern generated by restriction enzymes – so-called restriction fragment length polymorphisms or RFLPs (30). In the case of sickle cell disease (SCD), the HbS mutation has been described on different haplotype backgrounds, named after the regions in which they were first identified (80). The Asian (Arab-Indian) haplotype was first found in the Middle East and India (81). The Benin haplotype was first reported from central and West Africa whiles the Central African Republic (CAR) or Bantu haplotype from Central Africa around Angola and Congo. The Senegal haplotype was described on the Atlantic coast of West Africa (80).

Differential HbF expression is associated with the different haplotypes and may influence the clinical course of SCD. Thus analysis of β^S haplotype aids in predicting disease severity. The Senegal and Asian haplotypes are associated with higher levels of Hb F and a milder disease (82), while the CAR haplotype is associated with a more severe disease and the Benin and Cameroon haplotypes are intermediate in severity (30). Although several studies have been conducted to establish a clear relation between β^S-haplotypes and disease, the correlation remains unclear (83). Therefore we have charted the haplotypes and sub-haplotypes in homozygous Omani S/S patients and we have studied the correlation between different haplotype genotypes and SCD phenotype in chapter 9.

1.17 HbF

Hb F ($\alpha_2\gamma_2$) is the major ameliorating factor of beta thalassemia and sickle cell disease (84). Post-natal reactivation of fetal hemoglobin expression has been the aim of many studies. However, so far none means has been identified which permanently reactivates the expression of γ genes, to levels sufficient for therapeutic effect in postnatal life. Temporary reactivation however has been obtained to some extent by continuous use of different cytotoxic drugs, among which hydroxyurea (HU) is the only one without significant adverse effects, and is currently used routinely in the clinic (85).

The HbF expression is mainly regulated by elements linked to the β-globin gene cluster that switch off expression after birth. Some elements associated with the promoter sequences of the gamma globin genes are known to enhance HbF expression in postnatal life, among which the XmnI polymorphism on the promoter of the Gγ gene is the most important (86). High HbF

levels have also been observed significantly elevated in a number of bone marrow malignancies and slightly elevated during a pregnancy or at the laboratory which are likely due to a variety of mechanisms (87).

In case of sickle cell disease, high HbF concentration in the red cell dilutes the concentration of Hb S, increases the oxygen tension and inhibits Hb S polymerization (88), improving the phenotype significantly. In one study, reduced HbF level was associated with higher risk of stroke (89) and increased risk of brain infarcts in young children (90). However, these studies are often not sufficiently categorized; mixing ethnicity, genotypes, age of the patients, sample sizes and analytical approaches, all factors that are bound to bias the observations.

In our studies presented in chapters 9 and 10, we have tried to overcome this problem, categorizing patients more precisely and showing the correlation between genotype, haplotype and phenotype and the response to hydroxyurea treatment in the presence and absence of the XmnI polymorphism.

1.18 XmnI polymorphism and other natural beta cluster enhancers

As mentioned above, XmnI genotype variability can explain the considerable difference in HbF levels among patients and the variable responses to hydroxyurea in sickle cell disease and beta-thalassemia patients. As mentioned above, the XmnI -158 C>T mutation on the G-gamma gene promoter is one of the main single nucleotide polymorphism associated with high HbF%. It is usually silent in normal subjects but may become active in beta-thalassemia heterozygote under some hematopoietic stress and even fully active in cases of beta-thalassemia major and sickle cell disease, sometimes leading to notably raised levels of HbF production (91). Other HbF determinants not linked to the beta globin gene cluster have also been reported such as *HBS1L-MYB* intergenic region chr6q23, *BCL11A* mutations chr2p15, and *KLF1* mutations chr19p13.2 (92). The less common genetic variants influencing Hb F levels that are linked to the beta globin gene cluster are summarized in Table 1.6.

1.19 Coexisting alpha – thalassemia

Alpha-thalassemia may also modulate the phenotype of sickle cell disease (SCD) patients (82) by reducing the intracellular concentration of HbS, which in turn decreases the chance of polymerisation, cellular damage and hemolysis (93). Alpha thalassemia also lowers the MCV value and this should favor the rheology of the red cells and reduce the chance of HbS polymerization, the number of irreversibly sickled cells and the chance of infarctions (94). The modulating effect of α-thalassemia has been proposed to be proportional to the number of deleted α-globin genes (93) and the coinheritance of α-thalassemia has been reported to modify the phenotype of SCD by decreasing hemolytic rate, risk of stroke, pulmonary hypertension and leg ulceration while increasing the frequency of acute painful vaso-occlusive episodes and acute chest syndrome (95). However, also for alpha thalassemia the correlation studies are controversial and in our opinion this could be due to the fact that the studied cohorts are insufficiently categorized at the genotype level.

In our study presented in chapter 11 we have tried to overcome this problem by studying the effect of alpha thalassemia on well categorized cohorts of patients showing the correlation between genotype, haplotype and phenotype and the presence and absence of alpha thalassemia.

Table 1.6. New and known molecular determinants associated with elevated HbF. *=new mutations, **=common polymorphism present on many haplotypes (adapted from Amato et al, 2014, reference 87).

Mutation	Gene	Ethnic prevalence	HbF% in carrier
−4 bp (−225/−222)	Aγ	African	6–7
−202 C>G	Gγ	African	15–20
−202 C>T	Aγ	African	3
−198 T>C	Aγ	English	4–12
−196 C>T	Aγ	Italian/Chinese	21–15
−197 C>T*	Aγ	Italian	6
−195 C>G	Aγ	Brazilian	4–5
−175 T>C	Aγ	Afro American	17–38
−175 T>C	Gγ	Afro American, English, Italian	28–29
−161 G>A	Gγ	African	1–2
−158 C>T	Gγ	African and multiethnic	<1 unless stress
−158 C>T** (Xmn-I)	Gγ	Afro American	2–5
−117 G>A	Aγ	Mediterranean	8–10
−114 C>G	Gγ	Australian?	8.6
−114 C>T	Gγ	Japanese	11–14
−114 C>T	Aγ	Afro American	3–6
−114 C>G	Gγ	Australian ?	8.6
−13 bp (−114/−102)	Aγ	African	30
−113 A>G*	Aγ	Italian	6.5
−110 A>C	Gγ	Czechoslovakian	1%

By this we can again underline that the analysis of genotype, haplotype and modulating factors can help define the prognosis and the genetic risk allowing better counseling and rational based interventions before the onset of organ damage. Nevertheless, besides genetic and molecular modifiers, environmental factors such as physical activity, diet, toxins and socioeconomic status may also influence the clinical course of SCD (82). Understanding both the environmental and the genotype factors associated with the clinical variability between patients could dramatically improve patient care, and prevention.

1.20 Diagnosis of hemoglobinopathies

Hematology laboratories play an essential role in the diagnosis of sickle cell and thalassemia carriers and affected individuals. The two main basic methods of HBP diagnosis which are used in almost all laboratories are a) the complete blood count (CBC) which can detect hypochromic microcytic parameters, and b) separation and estimation of the hemoglobin fractions by high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). These methods can putatively detect heterozygosis, homozygosis or compound heterozygosis of the common conditions with great sensitivity and specificity.

Since carriers of the common HbS, C and D variants are often normochromic, CBC should not preclude Hb separation but should be done in parallel. By measuring the main hemoglobins; HbF, HbA and HbA_2, all carriers of "high HbA_2 beta thalassemia" will be diagnosed. Any other Hb fraction putatively identified, as HbS, C, E or D will detect carriers of the variant and/or combination of these traits. The CBC should always match the putative HPLC/CE diagnosis and if not, further examination should be done at the molecular level (96). Alpha thalassemia will be characterized by microcytosis in the presence of a slightly reduced HbA_2 but will need molecular confirmation, particularly in case of risk assessment. Only HbS can be confirmed by much simpler techniques such as sickle or solubility tests (24).

Although the provisional diagnosis of the common variants made by CBC and Hb separation are reliable, molecular confirmation is essential to decipher the underlined genotype, haplotype and modulating factors. The main method for molecular diagnosis is based on polymerase chain reaction (PCR) technique. The so-called GAP PCR (using primers complimentary to the breakpoint sequences amplifying a deletion-specific fragment that spans the deletion if present) is used for the common alpha deletions. Basic PCR reactions using specifically targeted gene primers followed by direct DNA sequencing are used to detect point mutations. Analysis of large deletions/insertions within the alpha and beta clusters can be performed by multiplex ligation-dependent probe amplification (MLPA). Figure 1.9 shows the basic flow chart of the methods used during HBP screening diagnosis in multi ethnic countries.

We have shown the validity of these technologies in our publications on the molecular spectrum in Oman reported in chapters 4 and 5. We have shown the possibilities of advanced screening technologies in our paper reported in chapter 12.

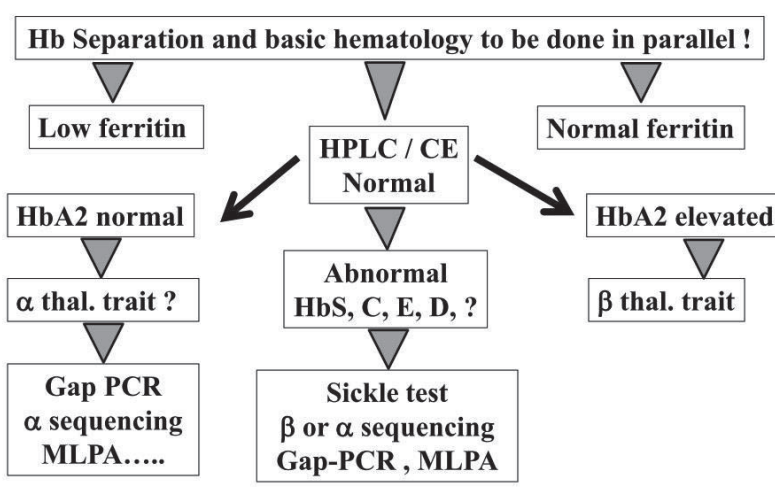

Figure 1.9. Flow chart of HBP screening/diagnosis (courtesy of P.C. Giordano).

1.21 Epidemiology in endemic countries (Worldwide epidemiology)

The World Health Organization (WHO) estimates that at least 5.2% of the world population is at risk for having children with a severe hemoglobin disorder (97), that over 7% of the pregnant women carry a hemoglobin variant (97) and that 300–400 thousand babies with severe forms of these diseases are born each year (98) (Figure 1.10). Although these conditions occur at their highest frequency in Africa and other tropical regions, population migrations have ensured that these conditions are now encountered in many non-endemic countries.

The most wide spread hemoglobin disorder is sickle cell disease (SCD). Thousands of children with this severe disease are born worldwide each year, largely in Sub-Saharan Africa (85%) (97), the Middle East and parts of the Indian sub-continent, where carrier frequencies are ranging from 5 to 40% (98). In India, around 40,000 SCD children are born each year. In America and Eastern Mediterranean the figure is estimated at 10,000 while 2000 are born in Europe (99). It is calculated that 10,000-15,000 SCD patients are living in the United Kingdom and France (100) while it is estimated that worldwide 1.1% of the couples are at risk of having children with a severe hemoglobin disorder, and that 2.7 per 1,000 conceptions are affected (101).

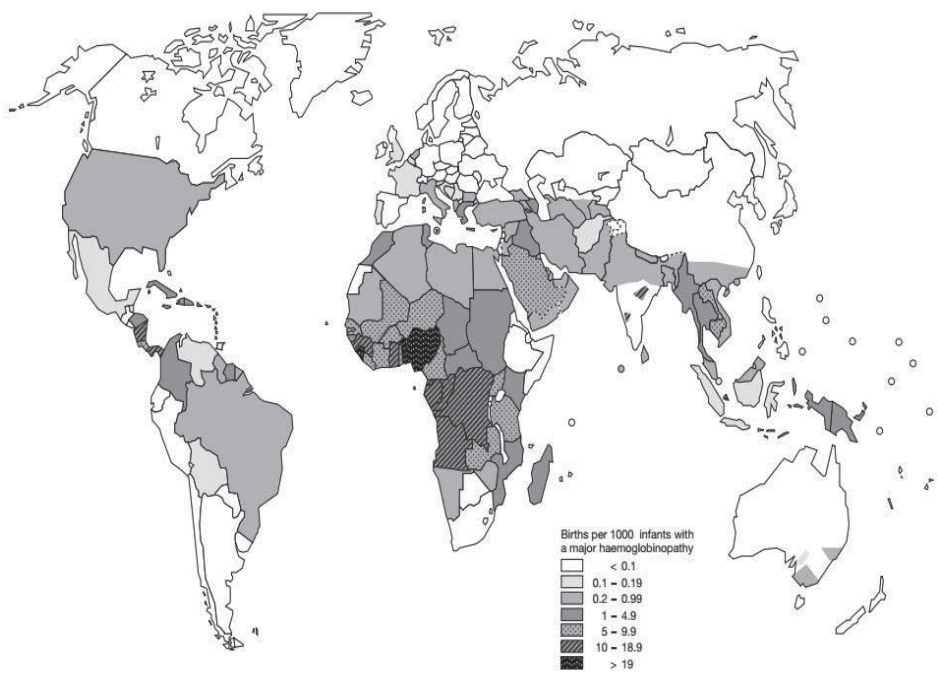

Figure 1.10. Geographical map showing the distribution of HBP disorders around the world in terms of expected affected infants per 1000 births. Adapted from (http://www.who.int/genomics/public/Maphaemoglobin.pdf).

Globally, a total of 269 million persons are estimated to carry thalassemia (β-thalassemia and α-thalassemia) (98). High incidences of thalassemia are reported for the Mediterranean region, parts of Africa, the Middle East, the Indian sub-continent, the Pacific Islands and in South-East Asia where carrier frequency for β-thalassemia and α-thalassemia ranges from 1 to 20% and from 10 to 20% respectively (98). However, α thalassemia do not pose a huge global health problem as β-thalassemia (98) because 20% of the world population carry α+ thalassemia (99) which is a less problematic condition for global public health than the α° thalassemias (due to large deletion of the α-globin cluster) which has a restricted distribution, occurring mainly in South-East Asia and the Mediterranean basin.

High prevalence of beta-thalassemia is present in Mediterranean populations, the Middle-East, Central Asia, India and Far East with the highest incidences reported in Cyprus, Sardinia and South East Asia (102). Due to migration, β-thalassemia is now common in Europe, North and South America, Caribbean, and Australia.

1.22 Treatment

Beta- thalassemia major patients rely entirely on regular blood transfusions to avoid the complications of severe anemia. Blood transfusions allows normal development throughout childhood but lead rapidly to iron overload that can be fatal in the second decade of life. For that reason, iron-chelating therapy has been developed. A drug named Deferoxamine mesylate was first introduced in the 1960s as a standard iron chelating therapy to improve the survival of thalassemia patients (103).

The pathophysiology in SCD is quite different to that of Thalassemia Major. In sickle cell disease children, immunizations through vaccination are the key to prevent the body from infections due to their damaged spleens that are unable to protect the body from bacteria. Much effort has been and continues to be directed to design drugs that could promote HbF synthesis, prevent red cell dehydration, and inhibit HbS polymerization. Only hydroxyurea has been proven thus far to be a useful drug to alleviate the symptoms of SCD such as acute vaso-occlusive complications, episodes of acute pain, acute chest syndrome, along with blood transfusion in highly symptomatic patients (104). Besides raising HbF %, hydroxyurea also reduces red cell–endothelial interaction and decreases erythrocyte density (105). However, it is estimated that 40% of patients do not respond to hydroxyurea treatment at all (106). Furthermore, long-term exposure to hydroxyurea treatment still raises concerns due to its side effects such as myelosuppression and the potential risk of malignancies (106).

Bone marrow transplantation (BMT) is the only cure available for severe Hemoglobinopathy (HBP), but it is only used in patients when a sibling donor with identical human leukocyte antigen (HLA) is matched. Its use is limited by the toxicity and morbidity associated with the procedure, the difficulty in finding a suitable family donor (107) and the availability of transplant centers. Bone marrow transplantation has been carried out from unrelated donor, as long as the HLA genotype is near-compatible, and some patients have become transfusion free following BMT (108). Cord blood transportation from an unaffected HLA compatible newborn also offers a good chance of curing an affected sibling (109). Moreover, hematopoietic stem cells and homologous recombination techniques are being actively investigated using affected mouse models with beta-thalassemia to correct the molecular

defect by transferring a normal gene via a suitable vector or transfecting the embryonic stem cells from the affected mice with a DNA fragment containing a normal and active beta globin gene construct (110).

REFERENCES

1. Hardison RC. Evolution of Hemoglobin and its Genes. Cold Spring Harb Perspect Med. 2012;2(12):a011627. doi: 10.1101/cshperspect.a011627.
2. Maniatis T, Goodbourn S, Fischer JA. Regulation of inducible and tissue-specific gene expression. Science. 1987;236(4806):1237–1245.
3. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bulletin of the World Health Organization. 2001;79(8):704–712.
4. Bauer DE, Kamran SC, Orkin SH. Reawakening fetal hemoglobin: prospects for new therapies for the β-globin disorders. Blood. 2012;120(15):2945-2953.
5. Wajcman H, Kiger L. Hemoglobin, from microorganisms to man: a single structural motif, multiple functions. C. R. Biol. 2002;325(12):1159–1174.
6. Wittenberg BA, Wittenberg JB. Myoglobin-mediated oxygen delivery to mitochondria of isolated cardiac myocytes. Proc Natl Acad Sci. 1987;84(21):7503–7507.
7. Dickerson RE, Geis I. Hemoglobin: Structure, Function, Evolution and Pathology. Menlo Park, CA: The Benjamin/Cummings Publishing Co., Inc, 1983.
8. Balgir RS. Community expansion and gene geography of sickle cell trait and G6PD deficiency, and natural selection againstmalaria: experience from tribal land of India. Cardiovasc Hematol Agents Med Chem. 2012;10(1):3-13.
9. Hershkovitz I, Ring B, Speirs M, Galili E, Kislev M, Edelson G, Hershkovitz A. Possible Congenital Hemolytic Anemia in Prehistoric Coastal Inhabitants of Israel. American Journal of Physical Anthropology 1991;85(1):7-13.
10. Flint J, Harding RM, Boyce AJ, Clegg JB. The population genetics of the haemoglobinopathies. Baillieres Clin Haematol 1993;6(1):215–262.
11. Allison AC. Protection afforded by sickle cell trait against subtertian malaria infection. Br Med J. 1954;1(4857):290-294.
12. Haldane JB. A mathematical theory of natural and artificial selection 1924. Bull Math Biol. 1990;52(1-2):209-240.
13. Haldane JB. The theory of natural selection today. Nature. 1959;183(4663):710-3.
14. Haldane JB. The relation between density regulation and natural selection. ProcR Soc Lond B Biol. 1956;145(920):306-308.
15. Haldane JB. Natural selection in man. 1956-1957;6(3):321-332.
16. Allison AC, Eugui EM. A radical interpretation of immunity to malaria parasites. Lancet, 1982;320(8313):1431-1433.
17. Martin TW, Weisman IM, Zeballos RJ, Stephenson SR. Exercise and hypoxia increase sickling in venous blood from an exercising limb in individuals with sickle cell trait. Am J Med. 1989;87(1):48-56.
18. Krause MA, Diakite SAS, Lopera-Mesa TM, Amaratunga C, Arie T, Traore K, Doumbia S, Konate D, Keefer JR, Diakite M and Fairhurst RM. α-thalassemia impairs the cytoadherence of Plasmodium falciparum-infected erythrocytes. PLoS One. 2012;7(5):e37214.
19. Ayi K, Turrini F, Piga A, Arese P. Enhanced phagocytosis of ring-parasitized mutant erythrocytes: A common mechanism that may explain protection against falciparum-malaria in sickle-trait and beta-thalassemia-trait. Blood. 2004;104(10):3364– 3371.
20. Stamatoyannopoulos G. Control of globin gene expression during development and erythroid differentiation. Exp Hematol. 2005;33(3):259-271.
21. Moon AM, Ley TJ. Conservation of the primary structure, organization, and function of the human and mouse β-globin locus-activating regions. Proc Natl Acad Sci, USA. 1990;87(19):7693–7697.
22. Noordermeer D, de Laat W. Joining the loops: beta-globin gene regulation. IUBMB Life. 2008;60(12):824-833.
23. Higgs D, Wood W, Jarman A, Sharpe J, Lida J, Pretorious IM, et al. A major positive regulatory region located far upstream of the human α-globin gene locus. Genes and Devel. 1990;4(9):1588–1601.
24. Giordano PC. Strategies for basic laboratory diagnostics of the hemoglobinopathies in multi-ethnic societies: interpretation of results and pitfalls. Int J Lab Hematol. 2012 Dec 7. doi: 10.1111/ijlh.12037.
25. Amato A, Grisanti P, Lerone M, Ponzini D, Di Biagio P, Cappabianca MP et al. Prevention strategies for severe hemoglobinopathies in endemic and nonendemic immigration countries: the Latium example. Prenatal Diagnosis. 2009;29(12):1171-1174.

26. HbVar: A database of human hemoglobin variants and thalassemias. http://globin,cse.psu.edu/cgi-bin/hbvar/counter.
27. Steinberg MH et al. Disorders of hemoglobin. New York, Cambridge University Press, 2001.
28. Rees DC, Styles L, Vichinsky EP, Clegg JB, Weatherall DJ. The hemoglobin E syndromes. Annals of the New York Academy of Sciences, 1998;850:334–343.
29. Herrick JB. Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. Yale J Biol Med. 2001;74(3):179-84.
30. Adekile AD. Historical and anthropological correlates of β^s haplotypes and α- and β-thalassemia alleles in the Arabian Peninsula. Hemoglobin; 1997; 21(3):281–296.
31. Pauling L, Itano HA, Singer SJ, Wells IC. Sickle cell anemia, a molecular disease. Science. 1949;111:543–548.
32. Ingram VM. A specific chemical difference between globins of normal and sickle-cell anemia hemoglobins. Nature. 1956;178(4573):792-794.
33. Maniatis T, Hardison RC, Lacy E, Lauer J, O'Connell C, Quon D, et al. The isolation of structural genes from libraries of eucaryotic DNA. Cell. 1978;15(2):687-701.
34. Murano T, Fox AD, Anjaria D. Acute splenic syndrome in an African-American male with sickle cell trait on a commercial airplane flight. The Journal of Emergency Medicine. 2013;45(5):161–165.
35. Kark JA, Posey DM, Schumacher HR, Ruehle CJ. Sickle-cell trait as a risk factor for sudden death in physical training. N. Engl. J. Med. 1987;317(13):781–787.
36. Brandao MM, Fontes A, Barjas-Castro ML, Barbosa LC, Costa FF, Cesar CL, et al. Optical tweezers for measuring red blood cell elasticity: application to the study of drug response in sickle cell disease. Eur J Haematol. 2003;70(4):207–211.
37. Taylor MY, Wyatt-Ashmead J, Gray J, Bofill JA, Martin R, Morrison JC. Pregnancy loss after first trimester viability in women with sickle cell trait: time for a reappraisal? Am J Obstet Gynecol. 2006; 194(6):1604-1608.
38. Hebbel RP. Reconstructing sickle cell disease: a data-based analysis of the "hyperhemolysis paradigm" for pulmonary hypertension from the perspective of evidence-based medicine. Am J Hematol. 2011;86(2):123-54.
39. Bunn HF. Pathogenesis and treatment of sickle cell disease. N Engl J Med. 1997; 337: 762-769.
40. Gill FM, Sleeper LA, Weiner SJ, Brown AK, Bellevue R, Grover R, et al. Clinical events in the first decade in a cohort of infants with sickle cell disease. Cooperative Study of Sickle Cell Disease. Blood. 1995;86(2):776-83.
41. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med. 1994;330:1639-1644.
42. Serjeant GR. Natural history and determinants of clinical severity of sickle cell disease. Curr Opin Hematol. 1995;2(2):103-108.
43. Lal A, Vichinsky EP. Sickle cell disease. In: Hoffbrand AV, Catovsky D, Tuddenham EGD, Green AR (eds) Postgraduate haematology, 6th edn. Wiley-Blackwell, Chichester, 2011, pp 109–125.
44. Kinney TR, Ware RE. Compound heterozygous states. In: Embury H, Hebbel RP, Mohandas N, Steinberg MH (eds). Sickle cell disease: basic principles and practice. New York: Raven Press, 1994:437-51.
45. Giordano PC. Strategies for basic laboratory diagnostics of the hemoglobinopathies in multi-ethnic societies: interpretation of results and pitfalls. Int J Lab Hematol. 2013; 35(5):465-479.
46. Weatherall DJ, Clegg JB. The thalassaemia syndromes. Oxford, Blackwell Science, 2001.
47. Harteveld CL, Osborne CS, Peters M, van der Werf S, Plug R, Fraser P, et al. Novel 112kb (epsilonGgammaAgamma) deltabeta-thalassaemia deletion in a Dutch family. Br J Haematol. 2003;122(5):855-858.
48. In "The People of Lerna: Analysis of a Prehistoric Aegean Population" by J. Lawrence Angel . Author(s) of Review: Richard Jantz *American Anthropologist*, New Series. 1973;75(4):1106-1107.
49. Rietti F. Sugli itteri emolitici primitivi. Atti Accad Svi Med Nat. Ferrara Sez II, 1925; 2:14.
50. Coley TB, Lee P. A series of cases of splenomegaly in Children with anemia and peculiar bone changes. Am J. Dis Child. 1925;30:447.
51. Whipple GH, Bradford WL. Racial and Familiar anemia of Children. Am J. Dis Child. 1932;44:336.
52. J.B.S. Haldane. The Causes of Evolution. 1932. 1990 edition ISBN 0-691-02442-1.
53. Sivestroni E, Bianco I. Microcitemie e morbo di Cooley. Boll Atti Accad Med Roma. 1945-46;71:3.
54. Kunkel HG, Vallenius G. New hemoglobin in normal adult blood. Science. 1955; 122: 288.
55. Silvestroni E, Bianco I. Studio Biochimico, elettroforetico e spettrofotometrico nei malati di di anemia microcitica costituzionale e di morbo di Cooley. Il Prog Med. 1957; 13:705.
56. Thein SL: Dominant beta thalassaemia: molecular basis and pathophysiology**.** Br J Haematol. 1992;80(3):273-277.

57. Ristaldi MS, Murru S, Loudianos G, Casula L, Porcu S, Pigheddu D, et al. The C-T substitution in the distal CACCC box of the beta-globin gene promoter is a common cause of silent beta thalassaemia in the Italian population. Br J Haematol 1990;74(4):480–486.

58. Galanello R, Melis MA, Ruggeri R, Addis M, Scalas MT, Maccioni L, et al. Beta0 thalassemia trait in Sardinia**.** Hemoglobin. 1979;3(1):33-46.

59. Borgna-Pignatti C, Galanello R: Thalassemias and related disorders: quantitative disorders of hemoglobin synthesis**.** In Wintrobe's Clinical Hematology. Lippincott Williams and Wilkins. 2004;42(11):1319-1365.

60. Borgna-Pignatti C, Cappellini MD, De Stefano P, Del Vecchio GC, Forni GL, Gamberini MR, et al. Survival and complications in thalassemia. Ann N Y Acad Sci. 2005;1054:40-47.

61. Ho PJ, Hall GW, Luo LY, Weatherall DJ, Thein SL. Beta-thalassaemia intermedia: is it possible consistently to predict phenotype from genotype? Br J Haematol 1998;100(1):70–78.

62. Galanello R, Paglietti ME, Addis M, Melis MA, Tuveri T, Furbetta M, et al. Pitfalls in genetic counselling for beta-thalassemia: an individual with 4 different thalassemia mutations. Clin Genet 1988;33(3):151–155.

63. So CC, So AC, Chan AY, Tsang ST, Ma ES, Chan LC. Detection and characterisation of beta-globin gene cluster deletions in Chinese using multiplex ligation-dependent probe amplification. J Clin Pathol 2009;62(12):1107–1111.

64. Beris P, Kitundu MN, Baysal E, Oner C, Lanclos KD, Dimoyski AJ, et al. Black beta-thalassemia homozygotes with specific sequence variations in the 5′ hypersensitive site-2 of the locus control region have high levels of fetal hemoglobin. Am J Hematol 1992;41(2):97–101.

65. Olivieri N, Weatherall DJ. Clinical aspects of β-thalassemia. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL, editors. Disorders of hemoglobin, genetics, pathophysiology, and clinical management. Cambridge, England: Cambridge University, 2001:277–341.

66. Yavarian M, Karimi M, Bakker E, Harteveld CL, Giordano PC. Response to hydroxyurea treatment in Iranian transfusion-dependent beta-thalassemia patients. Haematologica. 2004;89(10):1172-1178.

67. Laosombat V, Viprakasit V, Chotsampancharoen T, Wongchanchailert M, Khodchawan S, Chinchang W et al. Clinical features and molecular analysis in Thai patients with HbH disease. Ann Hematol. 2009;88(12):1185-1192.

68. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of alpha-thalassaemia deletions and alpha-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol. 2000;108(2):295-299.

69. Harteveld CL, Losekoot M, Haak H, Heister GA, Giordano PC, Bernini LF. A novel polyadenylation signal mutation in the alpha 2-globin gene causing alpha thalassaemia. Br J Haematol. 1994;87(1):139-143.

70. Tzetis M, Traeger-Synodinos J, Kanavakis E, Metaxotou-Mavromati A, Kattamis C. The molecular basis of normal HbA2 (type 2) beta-thalassemia in Greece. Hematol Pathol. 1994;8(1-2):25–34.

71. Tan ASC, Quah TC, Low PS, Chong SS. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for α-thalassemia. Blood. 2001;98(1):250-251.

72. Lacerra G, Scarano C, Lagona LF, Testa R, Caruso DG, Medulla E, et al. Genotype-phenotype relationship of the δ-thalassemia and Hb A_2 variants: Observation of 52 genotypes. Hemoglobin. 2010;34(5):407–423.

73. Phylipsen M, Gallivan MV, Arkesteijn SG, Harteveld CL, Giordano PC. Occurrence of common and rare δ-globin gene defects in two multiethnic populations: thirteen new mutations and the significance of δ-globin gene defects in β-thalassemia diagnostics. Int J Lab Hematol. 2011;33(1):85-91.

74. Giardine B, van Baal S, Kaimakis P, Rierner C, Miller W, Samara M, *et al.* HbVar database of human hemoglobin variants and Thalassemia mutations: 2007 update. Hum Mutat 2007;28(2):206.

75. Thein SL, Wood WG. The molecular basis of β thalassemia, δβ thalassemia and hereditary persistence of fetal hemoglobin. In:Steinberg MH, Forget BG, Higgs DR, Weatherall DJ, eds. Disorders of Hemoglobin. Cambridge, UK: Cambridge University. 2009:323–56.

76. Rochette J, Craig JE, Thein SL. Fetal hemoglobin levels in adults. Blood Rev 1994; 8(4): 213-224.

77. Bernards R, Flavell RA. Physical mapping of the globin gene deletion in hereditary persistence of foetal haemoglbin (HPFH). Nucleic Acids Res. 1980;8(7):1521-1534.

78. Harteveld CL, Wijermans PW, Arkesteijn SG, Van Delft P, Kerkhoffs JL, Giordano PC. Hb Lepore-Leiden: a new delta/beta rearrangement associated with a beta-thalassemia minor phenotype. Hemoglobin. 2008;32(5):446-453.

79. Forget BG. The molecular basis of beta thalassemia, delta beta thalassemia, and hereditary persistence of fetal hemoglobin. In: Steinberg MH, Forget

BG, Higgs DR, Weatherall DJ, eds. Disorders of hemoglobin: genetics, pathophysiology, and clinical management. 2nd ed. New York: Cambridge University Press; 2009;323–356.

80. Serjeant GR. Geography and the clinical picture of sickle cell disease. An overview. Ann N Y Acad Sci. 1989;565:109–119.
81. Rahgozar S, Poorfathollah AA, Moafi AR, Old JM. Beta S gene in Central Iran is in linkage disequilibrium with the Indian-Arab haplotype. Am J Hematol. 2000;65(3):192–195.
82. Serjeant GR. Natural history and determinants of clinical severity of sickle cell disease. Curr Opin Hematol. 1995;2(2):103-108.
83. Steinberg MH. Genetic etiologies for phenotypic diversity in sickle cell anemia Scientific World Journal. t2009;9:46–67.
84. Kato GJ, Gladwin MT, Steinberg MH. Deconstructing sickle cell disease: Reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev. 2007;21(1):37-47.
85. Fard AD, Hosseini SA, Shahjahani M, Salari F, Jaseb K. Evaluation of novel fetal hemoglobin inducer drugs in treatment of β-hemoglobinopathy disorders. Int J Hematol Oncol Stem Cell Res. 2013;7(3):47-54.
86. Galarneau G, Palmer CD, Sankaran VG, Orkin SH, Hirschhorn JN, Lettre G. Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. Nat Genet. 2010;42:1049-1051.
87. Amato A, Cappabianca MP, Perri M, Zaghis I, Grisanti P, Ponzini D, et al. Interpreting elevated fetal hemoglobin in pathology and health at the basic laboratory level: new and known γ-gene mutations associated with hereditary persistence of fetal hemoglobin. Int J Lab Hematol. 2014;36(1):13-19.
88. Noguchi C, Schechter AN, Rodgers GP. Sickle cell disease pathophysiology. In:Higgs DR,Weatherall DJ, Eds. Baillière's Clinical Haematology: The Haemoglobinopathies. London: Baillière Tindall. 1993;6:57–91.
89. Powars DR, Schroeder WA, Weiss JN, Chan LS, Azen SP. Lack of influence of fetal hemoglobin levels or erythrocyte indices on the severity of sickle cell anemia. J Clin Invest. 1980;65(3):732-740.
90. Wang WC, Pavlakis SG, Helton KJ, McKinstry RC, Casella JF, Adams RJ, et al. MRI abnormalities of the brain in one-year-old children with sickle cell anemia. Pediatr Blood Cancer. 2008;51(5):643-646.
91. Thein SL, Menzel S. Discovering the genetics underlying foetal haemoglobin production in adults. Br J Haematol. 2009;145(4):455-467.
92. Satta S, Perseu L, Moi P, Asunis I, Cabriolu A, Maccioni L, Demartis FR, Manunza L, Cao A and Galanello R. Compound heterozygosity for *KLF1* mutations associated with remarkable increase of fetal hemoglobin and red cell protoporphyrin. Haematologica, 2011; 96: 767-770.
93. Steinberg MH, Embury SH. Alpha-thalassemia in blacks: Genetic and clinical aspects and interactions with the sickle hemoglobin gene. Blood. 1986;68(5):985-990.
94. Ballas SK. Effect of α-globin genotype on the pathophysiology of sickle cell disease. Pediatr Pathol Mol Med. 2001;20(2):107–121.
95. Steinberg MH. Genetic etiologies for phenotypic diversity in sickle cell anemia. ScientificWorld Journal. 2009;9:46–67.
96. Traeger-Synodinos J, Harteveld CL, Old JM, Petrou M , Galanello R, Giordano P, Angastioniotis M, De la Salle B, Henderson S and May A. EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies. European Journal of Human Genetics. 2015;23:426–437.
97. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bulletin of the World Health Organization 2008;86(6):480–487.
98. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bulletin of the World Health Organization. 2001;79(8):704–712.
99. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Dewi M, et al. Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. Lancet. 2013;381(9861):142-51.
100. Streetly A, Latinovic R, Henthorn J. Positive screening and carrier results for the England-wide universal newborn sickle cell screening programme by ethnicity and area for 2005-07. J Clin Pathol. 2010;63(7):626-9.
101. World Health Organization. Global epidemiology of haemoglobin disorders and derived service indicators, http://www.who.int/bulletin/voluines/86/6/06-036673/en/.
102. Weatherall DJ, Clegg JB, Higgs DR, Wood WG. The hemoglobinopathies. In: Scriver CR, Beaudet Al, Sly WS, Valle D, Vogelstein B, editors. The metabolic and molecular bases of inherited disease (OMMBID). Chapter 101. New York, NY: McGraw-Hill, 2002.
103. Olivieri NF, Brittenham GM. Iron-Chelating Therapy and the Treatment of Thalassemia.The Journal of American society of hematology. 1997;89(3):739-761.

104. Charache S, Dover GJ, Moore RD, Eckert S, Ballas SK, Koshy M, et al. Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia. Blood.1992;79(10):2555-65.

105. Ware RE. How I use hydroxyurea to treat young patients with sickle cell anemia. Blood. 2010;115(26):5300–5311.

106. Amrolia PJ, Almeida A, Halsey C, Roberts IA, Davies SC. Therapeutic challenges in childhood sickle cell disease. Part 1: current and future treatment options. Br J Haematol. 2003;120(5):725-736.

107. Bhatia M, Walters MC. Hematopoietic cell transplantation for thalassemia and sickle cell disease: past, present and future. Bone Marrow Transplant 2008;41:109-17.

108. La Nasa G, Argiolu F, Giardini C, Pession A, Fagioli F, Caocci G, et al. Unrelated bone marrow transplantation for beta-thalassemia patients: The experience of the Italian Bone Marrow Transplant Group. Ann N Y Acad Sci 2005;1054:186-195.

109. Orofino MG, Argiolu F, Sanna MA, Rosatelli MC, Tuveri T, Scalas MT, et al. Fetal HLA typing in beta thalassemia: implications for haemopoietic stem-cell transplantation. Lancet 2003;362(9377):41-42.

110. Sadelain M, Boulad F, Galanello R, Giardina P, Locatelli F, Maggio A et al. Therapeutic options for patients with severe beta-thalassemia: the need for globin gene therapy. Hum Gene Ther. 2007;18(1):1-9.

CHAPTER 2

OMAN: THE COUNTRY AND HEMOGLOBINOPATHIES

OMAN: THE COUNTRY AND HEMOGLOBINOPATHIES

2.1 Geography

The Sultanate of Oman with Muscat as the capital city, is a Middle Eastern country, located in the Arab Peninsula. It covers a total land area of approximately 309,500 km^2 consisting of varying topographic features. Valleys and desert account for 82 % of the land, mountain for 15% and the remaining 3% covers the long 1700 km coastline. Oman is flanked by the Arabian Sea, the Gulf of Oman, the Persian Gulf and the Rub Al Khali desert. The top northwestern part of Oman is bordered by United Arab Emirates, by Saudi Arabia to the west and by Yemen to the southwest. In the past, the country's contacts with the rest of the world were by sea. Today Oman's strategic location on the Musandam Peninsula facing the Strait of Hormuz, plays a vital role for the transit of world crude oil transport. The Rub Al Khali desert forms a barrier between Oman and Saudi Arabia due to difficulty in travelling across. Oman's climate is hot and dry in the interior but humid along the coast and Southern Dhofar.

2.2 Economy

Historically, Oman has always been on the trade routes between the Middle East, India and Africa. Oman's most prominent economical role was in trading and seafaring activities in Zanzibar, East of Africa, and India. Nowadays Oman's main trading partners are United Arab Emirates, China, Korea, Thailand, Italy, Germany, the United Kingdom and the United States of America. Economically, Oman is a middle-income country that is heavily dependent on crude oil, natural gas production, agriculture and fishery.

2.3 Population

According to the last census (2013), Oman's population has risen to 3.87 million inhabitants including more than 500 thousand foreigners coming from Egypt, India, Pakistan, Bangladesh and the Philippines. The population growth rate is about 2% while birth and mortality rates are about 24/1000 and 3/1000 respectively. The median age is 24.7 years, life expectancy is around 74 years and infant mortality rate is 14.5 per 1000 live births. About 50% of the population lives in the capital city, Muscat, and in the Al Batinah coastal region, northwest of the capital. The main spoken language is Arabic but other languages such as English, Baluchi, Sawahili and Hindi are also spoken based on population tribal origins.

2.4 The origin of the Omani population (historic migrations)

The Omani population is heterogeneous, with mixed ethnicity tracking their ancestral roots to neighboring countries via tribal migrations and trading contacts. The main ethnicities are Arab, Baluchi, Sindi and African. Arabs migrated from what was known as Arabia since the 9th century BC onward and settled in Oman. Baluchis originated from Pakistan and the Iranian coasts. Sindhi tribe descends from Indian sailors while others originated from Africa, due to the historical trade between Oman, Zanzibar and Mombasa favored by the Indian Oceans monsoons. Oman was a Portuguese colony from 1508 to 1741, and when the Portuguese were forced out, Oman became an empire that expanded to Zanzibar. This lasted until 1861, when Zanzibar was separated from Omani control. Also many Omanis migrated to India, Pakistan and Iraq looking for a better life. Not until 1971 when native locals started coming back to Oman,

a modern government was established and the wealth of the oil industry was used to aid the people in the country.

Understanding population heterogeneity is important to study the mutation spectrum of disease such as hemoglobinopathies (HBP), which in turn is essential for molecular diagnosis and prevention. We have extensively studied the correlation between ethnicity and molecular spectrum of HBP in Oman. Our results are outlined in chapters 4-8.

2.5 The Omani tribes and their geographical distribution

The north and south parts of the country became united only about 100 years ago. Today the country is divided into eight different governorates (Muhafathat); Muscat, being the capital center, Al Dakhiliyah, Al Batinah, Al Wusta, Al Sharqiyah, Al Dhahirah, Musandam and Dhofar (Figure 2.1). As mentioned above, Oman has a multi-ethnic society in which Arabs constitute the majority. Non-Arab descendants such as the Baloch, who originally migrated to Oman from Iran and Pakistan over several centuries, currently live in Muscat and in the Al-Batinah region. A significant number of tribes of Sindi ethnicity, South Asian ancestry and African origin live in Muscat, along the coasts of Al-Batinah and in the interior regions (1). Some inhabitants of Persian origin are also present in the Musandam region. The existence of multi-origins among the Omani populations would be of a great intrest when studying the molecular spectrum of a disease such as hemoglobiopathies which was identified in this thesis.

Muscat

Muscat is the capital with an area of approximately 1500 km². The city has been known since the second century AD and is considered as one of the oldest in the Middle East. Today Muscat is a large city with residential, commercial and industrialized districts.

Musandam

Musandam is the northernmost area of the country adjacent to Strait of Hormuz, between the Gulf of Oman and the Persian Gulf. This strategic area of 1800 km² is on a vital transit point for the world oil transportation. Musandam is separated from the rest of the country by mountains and a territory belonging to United Arab Emirates.

Al Batinah

Al Batinah is a highly populated coastal area that runs from the lower northern part of Oman, below Musandam, to just above Muscat.

Al Dakhiliyah

Al Dakhiliyah occupies a distinctive location with a belt of mountains on the western side and desert in the south.

Al Wusta

Al Wusta is a flat rock desert in the center of Oman and is populated by the Bedouins.

Al Dhahirah

Al Dhahirah lies towards the west of the coastal areas. It is separated from Muscat by the western Al Hajar Mountains.

Al Sharqiyah

Al Sharqiyah is the eastern region of Oman. It is considered a natural reserve in the country.

Dhofar

Dhofar, at the bottom south and bordering Yemen is a mountainous area covering 99,300km².

Figure 2.1. Map of Oman.

2.6 Religious and cultural practice (consanguinity)

Conversion to Islam in Oman occurred during the lifetime of the prophet Muhammad in the 7th century and the Arab tribes living in Oman were among the first people to embrace Islam. Nowadays the majority of the populations belong to the Abadhi subgroup, comprising 75% of the Muslims in the country. The second Muslim subgroup (the Sunnies) and the third (the Shia'a) are considered a minority in Oman. Omanis tend to be strict observant of their religious

obligations. The government follows the Abadhi subgroup doctrine and appoints a Mufti who has the authority to maintain the Islamic fatwas for the whole country. Culture is deeply influenced by the Arabian traditions and Islamic religion and differs slightly between different groups based on ethnicity, social and tribal stratification.

Consanguineous marriages are very common among Omani as it is perceived to ensure trust and to strengthen family bonds. According to the Oman National Health Survey, 52% of the marriages in Oman are consanguineous (2). Moreover, another large study conducted by Rajab and Patton (3) among more than 60,000 couples reported that 35.9% of the marriages take place between first or second cousins and another 20.4% between members of the same tribes, giving a rate of consanguineous marriage of over 50%. Another study calculated that marriages between first cousins was 34 % and the total consanguinity rate including second cousin relationships and beyond was 58 % (4).

2.7 Hemoglobinopathies in Oman

Hemoglobinopathies are the most frequent autosomal recessive disease in Oman where malaria was endemic. Malaria eradication activities started in Oman in 1971 and included house and open space spraying operations. These measures were followed by rapid reduction in malarial incidence. Most of sickle cell disease (SCD) cases in Oman are found in agricultural areas consisting of a continuous chain of villages situated along the coast, which provided favorable conditions for malaria transmission. One exception is Dhofar, an area with higher rainfall than other regions, abundance of mosquitoes and tropical vegetations but no SCD. The reason is probably due to the presence of a strain of mosquitoes (Anopheles Coustiani) that does not allows the malaria parasite to complete its life cycle (sporogony) efficiently.

In the past, the majority of the Omani patients with severe SCD or β-thalassemia major were not surviving infancy. With advanced health management, early diagnosis and comprehensive treatment, the survival of patients with hemoglobin disorders has significantly improved. Nevertheless, these patients are in need of intensive therapy with poor life expectations and always in need of special support by public health services which in terms have to face economical stress for treatment coverage that is free of charge in Oman.

A comprehensive national program was developed in 1999 in Oman aiming at forming a national committee and a unit in each region specialized in educating and training all regional teams through seminars and workshops and developing a registry document of all cases. By 2003, counseling services were incorporated in community education through mass media taking all ethical, legal and social issues into consideration. Collaboration with experts and reference laboratories of neighboring countries was undertaken, as well as consulting with international western countries was made in order to establish and design a controlled program (Diagnostics and management of genetic blood disorders in Oman, 2008, ISBN number 978-90-807039-3-3).

In spite of the public health improvements made in Oman, patients with hemoglobin disorders are still born at a high rate. For this, a comprehensive knowledge on hemoglobinopathies in Oman had to be derived to prevent the occurrence of such severe conditions and to give a better life to the affected patients.

As previously mentioned, understanding the molecular and genomic make up is essential to be able to improve the health care system, to offer effective prevention programs and

counseling to the families helping them in making the right choice to avoid getting infants with severe diseases.

For this we have studied in this thesis the molecular spectrum of beta and alpha gene defects essential for diagnosis, early screening and risk assessment of the young Omani generation (chapters 4, 5 and 7).

2.8 Mutation spectrum and variability in prevalence in Oman

The annual birth rate in Oman consists of 82, 000 newborn (5). According to Al Riyami et al. the birth prevalence of β-hemoglobinopathies is 3.1/1000 live births while the national incidence of sickle cell disease (SCD) is 2.7/1000 among 2-5 years old children (6).

Various studies have been conducted thus far to calculate the prevalence of different hemoglobin disorders in Oman generating similar or discrepant figures. The first Omani population study on hemoglobinopathies was done by White et al. in 1986 on a relatively small cohort looking for cases with α-thalassemia. Homozygous α^{+}-thalassemia was found at a frequency of 0.39. In 1993 (7), White et al, studied a larger cohort (n=952) and found a higher frequency of homozygous α^{+}-thalassemia (0.45). The frequencies of β-thalassemia trait and sickle cell carriers (SCT) were also estimated to be 0.015% and 6.1% respectively.

From January 2001 to December 2004, more than 30,000 blood samples were collected by Adly et al. and analyzed using the sickle cell solubility method for HbS detection and high performance liquid chromatography (HPLC) for beta gene variant analysis (8). The total prevalence rate of hemoglobinopathies (HBP) was found to be around 8.1%; SCT 7.5 %, SCD 0.46% and 0.102% for other β gene defects (HbD, HbE, HbS-Oman, beta-thalassemia) (8).

In 2003 a large national survey was undertaken by Oman's Ministry of Health in which a total of 6103 households were interviewed and 6,342 children under 5 years of age were screened. The total HBP prevalence rate was 9.5%; SCT (6%), β-thalassemia trait (2%), BTM (0.07%) and SCD (0.2%), Hb D (0.6%), Hb E (0.3%) and Hb C traits (0.02%) respectively (4). Compound heterozygosis of HbS with other abnormal Hb was detected at a very low prevalence (4). When the prevalence of SCT was compared between the different regions, variability in frequency was noted. Al Sharqiya had the highest prevalence (13.9%) followed by Al Batinah (10.8%), Al Dakhiliya (9%), Muscat (8%) Musandam (4.7%) and Al Dhahira (3.9%). The prevalence of β-thal trait was the highest in the Al Batinah region (5.4%) followed by Muscat (2.8 %), Al Sharqiya (2.3%), Al Dakhiliya (2 %), Al Dhahira (1.7 %) and Musandam (1.6 %). The prevalence of SCT and β-thal trait in Dhofar was very low (0.2 %) and no cases were detected in Al-Wousta region (4).

During a neonatal screening program on cord blood samples in 2009, a total of 7,837 Omani neonates were analyzed for complete blood counts and for hemoglobin profile by HPLC. No case with Hb H disease (-α/αα) was detected, α-thalassemia traits were observed with a frequency of 48.5% while β-globin-defects accounted for 9.5% of the samples (4.8% SCT, 2.6% β-thal trait, 0.9% Hb E trait, 0.8% Hb D trait, 0.08% Hb C trait, 0.3% SCD and 0.08% homozygous β-thal) (9). It was also found that the birth rate of newborns affected with major hemoglobinopathies was 4.3/1,000 live births (3.5/1,000 for SCD and 0.8/1,000 for β-thalassemia) (9).

Recently, it was reported that about 400 patients with β-thalassemia major (BTM) and 3,000 with SCD are treated in different hospitals in Oman (10). Around 10 % of Omanis are HbS carriers (10), 2–3 % carries a defect causing β-thalassemia and 45 % are carriers of α-thalassemia (7).

At the genetic level, one study was carried on to decipher the molecular spectrum among beta thalassemia patients which was conducted in 1998 where fifteen different β-determinants were identified (11). Due to the historical migration of the local and trading links with neighbouring countries, ethnic admixing is expected in the country and thus broader spectrum of hemoglobinopathy mutation was needed and this was looked at in (Chapters 4-7).

In year 2000, a study was conducted on Omanie sickle cell disease with the aim to identify the coexisting haplotypes for genetic epidemiology purposes to demonstrate the uni- or multicentric origin and genetic flow of sickle cell mutation in northern part of Oman (12). However, no genotype-phenotype correlation studies were drawn which is important for risk assessment and treatment selection. This association was studied and extended in (Chapters 9-11).

The high birth rate of affected neonates results in huge suffering for patients and families, increasing the financial burden on health resources and for this reason, great attention should be given to appropriate cost-effective prevention strategies and measures. In addition, state of the art medical care has improved the survival age of HBP's and, in absence of prospective primary prevention, the number of patients requiring care in the country is likely to multiply by three times in the next 20 years with a stable population of at least 1,000 patients with BTM and 7,000 with SCD.

Such an increase correlates directly with an increased health burden as patients are largely hospital dependent. At present, the cost of treating all Omani patients with a HBP disorder is estimated to be about 10 million US$/year. In the absence of primary prospective prevention, this will rise to at least $30 million in the next 20 years. Considering the rising costs of treatment, other authors have recently published much higher figures for beta thalassemia reaching levels of 2 million US$ per life treatment for a single patient (13).

To find a sensible solution to this problem, we have evaluated the attitudes of Omani carrier couples towards prenatal diagnosis and medical abortion in chapter 13, showing that primary prevention of hemoglobinopathies by prenatal diagnosis and selective pregnancy termination could be culturally acceptable also in Oman

2.9 The role of public health and religious authorities

Considerable time is needed to establish a developed program for the control and management of severe disorders such as hemoglobinopathies.

As mentioned above, the first community genetic program in Oman was introduced in 1999 by trained regional teams as the national program for the control of genetic blood disorders. The program consists of providing the best possible patient care, raising community awareness through education programmes, screening couples at risk and offering genetic counselling (14). During the last decade, health care in Oman has shown great achievements in medical services in both the preventive and the curative fields and in 2001, Oman was ranked number 8 by the World Health Organization. Although efforts are made by public health centers to raise awareness regarding the burden caused by hemoglobin disorders, a sector of the population still remain unaware of the disease and more efforts should be made by the responsible authorities to inform couples at risk on the meaning of prevention and treatment of these incurable disease.

Especially β-thalassemia major patients need intensive treatment as they present with continuous life-threatening problems. As briefly mentioned above, the disease poses a significant burden not only on patients and families but also on public health due to high costs of medications and treatments, requiring constant highly qualified multidisciplinary care, life-long and frequent blood transfusions and very expensive chelation therapy which are offered for free to all Omani citizens.

Bone marrow transplant (BMT) as the only "curative" option was introduced in Oman in 1995 with a two-bed unit in one of the specialised hospitals. The infrastructure can perform only a few transplants per year, whereas hundreds of patients, of which approximately 72% suffer from β-thalassemia major, remain on the waiting list (15). Although in Oman, due to large families, the possibility of finding human leukocyte antigen (HLA) matched sibling is high, the BMT capacity is insufficient.

Alternatively, primary prevention not only spares the suffering and the burden of expensive treatment, with little hope to become cured, but is also highly cost-effective as it has been shown in many country with a considerable carrier frequency, where offering this option to couples at risk has become established (11).

In Oman, premarital carrier screening service for sickle cell disease and β-thalassemia carrier state is available in all primary health centers. Nonetheless, the service is not mandatory as it is in neighboring Arab countries such as Saudi Arabia (16) and Bahrain (17) and where the incidence of severe hemoglobinopathies has gradually decreased since the carrier screening test has become compulsory prior to marriage.

A study done in the Al Batinah region to estimate the knowledge and the attitude of the population towards premarital testing showed that majority of the responders believed that premarital testing is necessary in Oman. While about half of them supported the option of making this screening mandatory by law about one third was less in favour of being tested before marriage (18). This shows that the level of awareness in the general population still needs more attention by the health affair directorates who are responsible for the development, control and implementation of health policies (10).

Moreover, religious authorities should be made aware of the severity of the condition and of the burden of the disease for an affected child and the family in order to allow free decisions concerning prenatal diagnosis (PD) and pregnancy termination (if indicated) based upon serious medical grounds. Given that religion still contextualises decision making about termination of pregnancy, it is important for policy makers and public health providers to consult the country's main religious scholars to discuss the issue of offering PD to all at-risk couples in the first trimester of pregnancy. Although Islam is one religion, permitting medical abortion in one Islamic country and forbidding it in another is not logical in spite of the fact that it could be explained by the differences of the sub-religion practiced in each country, which includes Shiaa, Sunni and Abadhi. Coming to a common decision that precludes or diminishes human suffering and public health problems which affect a large share of the population in many Muslim countries is therefore highly needed.

2.10 The involved parties (pediatricians, hematologists, laboratories, general practitioner, ethics, politics, insurances)

Multidisciplinary teams are needed to care for patients with severe hemoglobinopathies. Pediatricians, general practitioners (GP) and midwifes are crucial partners of the families that require their patients and their pregnancies, to be managed along with the aid of hematologists, geneticists, laboratory doctors and technicians. Chronic complications manifest 6 months after birth, aggravate with age and need to be managed by an integrated team of devoted specialists including psychologists.

Dedicated medical teams have shown that comprehensive care focusing on the education of patients and their families, may improve physical growth and decrease acute events in sickle cell disease (SCD) children (19). Screening for hemoglobinopathies is usually community based, starting with the GP or the midwife who sees the couple before marriage or the woman early in pregnancy respectively. In case of individual premarital testing, when one partner is found to carry a relevant hemoglobinopathy, the other partner will be offered testing, and if both are found to be carriers, the couple will be offered genetic counseling. This also involves the efforts of a team that will have to rely upon in the work of molecular geneticists and counselors that will provide all data and advice needed for informed reproductive choice (20).

The Omani Ministry of Health follows the Islamic perspectives of medical ethics and world health organization medical policy. The concept of informed consent has been implemented. The patient is given full autonomy to accept or reject a treatment (e.g.: Hydroxyurea for SCD patients) or to choose or not for primary prevention in full patient confidentiality. Although many international health insurance companies exist in Oman, it is not compulsory to have one as it is the case in many countries in the world. Omani nationals receive free treatment in public hospitals thus locals rarely seek to apply and pay for a private medical insurance. Foreign immigrants residing in the country may face financial issues if not insured and since many immigrates who come to Oman, are from countries endemic for hemoglobinopathies (Egypt, Indian, Pakistan, Indonesia and the Philippines), Oman may face the same problem being faced by other western immigration countries (21) in which it may be difficult to reach carriers at risk in the immigrant populations and unable to provide them with sufficient counseling information and thus prevention.

2.11 The implementation of our research

Social medicine is point of care in Oman, a country in which public health has dramatically improved in the last few decades. However, recessive genetic diseases such as hemoglobinopathies remain a substantial burden in the country. Prevention can be dramatically improved by offering prenatal diagnosis and for this, comprehensive molecular studies are required. As mentioned, Oman is a country with a wide range of ethnic groups (3) and thus an extensive spectrum of mutations is expected. Knowledge of the distribution of these mutations is essential in health care planning and management of diseases with a complex molecular pathology such as the hemoglobin disorder. For this, an accurate database with all patients and carriers in all regions with hematological and molecular analysis is needed to aid in the treatment and prevention programs. Advances in this field will be a breakthrough in applied public health science and will put Oman at the state of the art level in treatment and prevention of hemoglobinopathies among Arab countries and worldwide. The aim of this thesis is to

contribute to this process, to reduce the birth prevalence of sickle cell disease, beta- and alpha-thalassemia in the country, with a relevant gain in public health as well as a substantial reduction in treatment related expenses for severely affected patients that are and might dramatically increase in the near future if primary prospective prevention is not implemented (22).

REFERENCES

1. Islam MM. The practice of consanguineous marriage in Oman: prevalence, trends and determinants. J Biosoc Sci. 2012;44(5):571-594.
2. Al-Riyami A, Afifi M, Al-Kharusi H, Morsi M. National Health Survey 2000. Volume 2. Reproductive Health Survey. (2000) Ministry of Health, Muscat.
3. Rajab A, Patton MA. A study of consanguinity in the Sultanate of Oman. Ann Hum Biol. 2000; 27(3):321–6.
4. Al Riyami A, Ebrahim GJ. National Genetic Blood Disorders Survey - Ministry of Health, Sultanate of Oman. J Trop Pediatr. 2003;49(1):1–20.
5. Rajab A, Patton M. Development and use of a National Haemoglobinopathy Register in Oman. Letter to the Editor. Community Genet. 1999;2(1):47–48.
6. Al-Riyami AA, Suleiman AJ, Afifi M, Al-Lamki ZM, Daar S. A community-based study of common hereditary blood disorders in Oman. East Mediterr Health J. 2001;7(6):1004–11.
7. White JM, Christie BS, Nam D, Daar S, Higgs DR J. Frequency and clinical significance of erythrocyte genetic abnormalities in Omanis. Journal of Medical Genetics. 1993;30(5):396-400.
8. Adly G, A Rajappa A. Haemoglobinopathies encountered at Khoula Hospital, Oman. Sultan Qaboos Univ Med J. 2008;8(1):59–62.
9. Alkindi S, Al Zadjali S, Al Madhani A, Daar S, Al Haddabi H, Al Abri Q, et al. Forecasting hemoglobinopathy burden through neonatal screening in Omani neonates. Hemoglobin. 2010;34(2):135–44.
10. Rajab A, Al Rashdi I, Al Salmi Q. Genetic services and testing in the Sultanate of Oman. Sultanate of Oman steps into modern genetics. J Community Genet. 2013;4(3):391-397.
11. Daar S, Hussain HM, Merghoub T, Kr ishnamoorthy R. Spectrum of b -thalassemia mutations in Oman. Ann N Y Acad Sci. 1998;850:404–406.
12. Daar S, Hussain M, Gravell D, Nagel RL and Krishnamoorthy R. Genetic Epidemiology of HbS in Oman: Multicentric Origin for the bS Gene. American Journal of Hematology. 2000; 64:39–46.
13. Koren A, Profeta L, Zalman L, Palmor H, Levin C, Zamir RB, et al. Prevention of β-thalassemia in Northern Israel – a cost benefit analysis. Mediterr J Hematol Infect Dis. 2014; 6(1): e2014012. doi: 10.4084/MJHID.2014.012.
14. Rajab A, Jaffar MA. Genetic diseases in the Sultanate of Oman: public health perspective. Genetic disorders in Arab populations: a 2008 update. In: Tadmouri GO, Taleb Al-Ali M, Al- Khaja N (eds) Genetic disorders in the Arab World: Oman. Centre for Arab Genomic Studies, Dubai, 2008.
15. Dennison D, Al Kindi S, Pathare A, Daar S, Nusrat N, Ur Rehman J, et al. Hematopoietic stem cell transplantation in Oman. Bone Marrow Transplantation. 2008;42(1):109–113.
16. Alswaidi FM, Memish ZA, O'Brien SJ, Al-Hamdan NA, Al-Enzy FM, Alhayani OA, et al. At-risk marriages after compulsory premarital testing and counseling for β-thalassemia and sickle cell disease in Saudi Arabia, 2005–2006. J Genet Couns. 2012;21(2):243–255.
17. Al-Arrayed S. Campaign to control genetic blood diseases in Bahrain. Commun Genet. 2005;8(1): 52–55.
18. Al Farsi OA, Al Farsi YM, Gupta I, Ouhtit A, Al Farsi KS, Al Adawi S. A study on knowledge, attitude, and practice towards premarital carrier screening among adults attending primary healthcare centers in a region in Oman. BMC Publish Health. 2014 ;14:380.
19. Rahimy MC, Gangbo A, Ahouignan G, Adjou R, Deguenon C, Goussanou S, et al. Effect of a comprehensive clinical care program on disease course in severely ill children with sickle cell anemia in a sub-Saharan African setting. Blood. 2003;102(3):834– 838.
20. Weatherall D, Hofman K, Rodgers G, Ruffin J, Hrynkow S. A case for developing North–South partnerships for research in sickle cell disease. Blood. 2005;105(3):921–923.
21. Amato A, Grisanti P, Lerone M, Ponzini D, Di Biagio P, Cappabianca MP, et al. Prevention strategies for severe hemoglobinopathies in endemic and nonendemic immigration countries: the Latium example. Prenat Diagn. 2009;29(12):1171-4.
22. Giordano PC, Harteveld CL, Bakker E. Genetic epidemiolgy and preventive healthcare in multiethnic societies: the hemoglobinopathies. Int J Environ Res Public Health. 2014;11(6):6136-6146.

CHAPTER 3

PREVENTION IN ENDEMIC AND NON-ENDEMIC IMMIGRATION COUNTRIES

CHAPTER 3: PREVENTION IN ENDEMIC AND NON-ENDEMIC IMMIGRATION COUNTRIES

Hemoglobinopathies were originally endemic only in tropic and sub-tropic areas of the Old World but in the past centuries sickle cell disease (SCD) and thalassemia have spread world-wide as a consequence of slave trade, colonization and recent migrations.

National programs consisting of information, screening and counseling have been implemented in many endemic countries in order to prevent the birth of children with severe hemoglobinopathies, while emerging countries are beginning to acknowledge the importance of starting prevention strategies focusing at population screening, genetic counseling and developing reference centers to identify healthy couples at risk (1).

Primary prevention can be prospective if offered before conception or retrospective if made available after the birth of the first affected offspring (2). Different prevention strategies are available that are differently accepted in various cultures, influenced by social and religious background, economy and politics. In some countries religion plays a major tolerant or restrictive role when prevention involves pregnancy termination, in other the strategy involves mandatory premarital screening.

In some cultures carrier screening may result in changing the candidate partner if the diagnosis is made prior marriage but if the couple at-risk still decide to marry, the options they have are to a) avoid getting pregnant, b) ask for prenatal diagnosis (PD), c) opt for a non-carrier gamete donation or d) choose to adopt. However not all these options are widely accepted and most couples at-risk would opt for PD if legally permitted or pre-implantation genetic diagnosis (PGD) (3). Some cases may even take the risk and hope for a healthy child. When newborn screening (NBS) is available, it may help these couples to plan for the best-tailored treatment and to reduce the risk of the severe symptoms in case of an affected child. It is evident that the earlier carrier detection is done, the more options are available for primary prevention (4).

Most endemic countries with long prevention experience focus on early carrier detection while non-endemic countries with large populations of immigrants at risk offer mainly NBS. Although NBS is not an efficient option for primary prevention, it allows secondary (morbidity) prevention and eventually retrospective or even prospective primary prevention for the following child if the NBS procedures are properly managed and all new born carriers are reported. This is the case in the UK and to some extend in Holland where, besides national NBS, early pregnancy screening is also offered universally or regionally.

A positive aspect in hemoglobinopathy screening is that carriers can be identified by simple, fast and relatively inexpensive hematological and biochemical tests which most laboratories can offer and afford. This is fairly unique amongst human genetic diseases.

Once couples at risk are identified or suspected, accurate molecular characterization might be required to avoid misdiagnosis that can lead to potential pitfalls in genetic counseling and prenatal diagnosis when it is offered, especially in countries where the spectrum of mutations is very heterogeneous and the conditions are widespread (5).

Below we describe the available prevention option in endemic and non-endemic countries and highlight the bottlenecks of carrier diagnostics and early screenings in Muslim Arab societies and in Oman in particular.

3.1 Prevention of severe hemoglobinopathies in different countries

Prevention of hemoglobinopathies is offered to couples that are at risk for getting a child with a severe phenotype and that, although treatable at great expenses, it is associated with heavy suffering and premature death.

The most relevant hemoglobinopathies are those caused by defects on both beta genes, such as beta-thalassemia major and sickle cell disease. Being most forms of alpha thalassemia mild due to the presence of 4 alpha genes (6), prevention is only relevant for couples who are carriers of severe alpha genotypes (usually alpha-zero deletions) that are at risk of getting a child with hydrops fetalis, or for couples with combinations of alpha zero carriers and certain alpha^{+} defects that might cause severe HbH disease. Severe hemoglobinoapthy conditions for which prenatal diagnosis is generally justifiable are summarized in Table 3.1.

Table 3.1. Six categories of severe HBP states, for which genetic counseling, and possibly PD is needed. The first 3 being the most common, adapted from Traeger-Synodinos, 2013 (38).

Category of hemoglobin disorder	Most frequent causes
Thalassemia major	Severe β- and/or δβ-thalassemia mutations
Sickle cell disease	S/S, S/C, S/β-thal, S/D, S/O^{Arab}, S/Lepore, S/E etc.
Hb E/ β-thalassemia	Co-inheritance of β-thal mutations with HbE
Hb Lepore and other thal variants	Co-inheritance of β-thal and Hb variants with thal or instable phenotype
Hb Bart's Hydrops Fetalis syndrome	Homozygous α°-thalassemia deletions
Hb H intermediate or severe	Interaction of α°-thalassemia deletions with severe nondeletion α-thal mutations or homozygous severe nondeletional α-thal mutations

3.1.1 Pre-conception carrier diagnostics/screening and premarital counseling

The first prevention strategy which has been made available in endemic countries is premarital carrier screening followed by genetic counseling, where couples at risk are provided with information for a reproductive decision making. Detecting couples at risk before marriage has been made mandatory in several Middle Eastern and Muslim countries like Iran (7), the UAE (2), Saudi Arabia (8) and Tunisia (9) (Table 3.2). Premarital testing resulted in dramatic decrease of affected births either preventing at-risk marriages through adapting partner choice, remaining childless (10) or prenatal diagnosis where it is offered. Successful examples of preconception counseling have been reported in many countries (9, 11) with Sardinia and Cyprus as the first endemic countries to develop awareness and screening to detect carriers (1, 12) with significant decline in the incidence of thalassemia major.

Early carrier detection in high schools is also a good example of prospective prevention at the preconception level (13). This has been shown to be effective in some countries such as Canada (14) and in Southern France (Marseille) (15) and Latium (Italy) (4). High school screening

resulted in a dramatic fall of beta-thalassemia major over the years in these regions. Identified carriers kept the information and remembered their status, despite the time lapse between screening at school and reproduction time, had their partner tested and inquired about prevention options (4, 14, 15). High school screening has been carried out as a national project in some Arab countries including Bahrain (16) and Egypt (17), aiming at raising awareness among the young generation. However, in Egypt, low acceptability was observed due to limited number of premarital centers and high costs. Knowing the individuals carrier status as early as school age, allows sufficient time to understand the consequences of getting an affected child in the future and to come up with a decision calmly after considerate thinking. Although school screening programs sounds feasible, they are not widely implemented. A drawback includes the risk of ethnic discrimination between the students, since the recessive diseases may be more common in people of specific ethnic origin or tribe compared to others (2).

Should premarital screening be made compulsory in Oman? And if so, should a marriage at risk be interfered by the legal system? In China, premarital genetic examination was mandated by law in 1995 and couples at risk were not permitted to marry without contraception or tubal ligation (18). This rather awkward law was redrawn as a flagrant violation of medical ethics and human rights, and premarital testing became voluntary in 2003 (19). It should be emphasized that in western countries in particular, many couples have children without a formal or legal marriage. This is unlike in Muslim communities where couples are not allowed to have children unless legally married. Although premarital screening has shown some positive outcomes in regions where the test has been mandatory, on its own is not sufficient enough to be the basis of a national prevention program if prenatal diagnosis and medical abortion are not considered as part of the process.

3.1.2 Carrier diagnostics early in pregnancy, prenatal diagnosis and pregnancy termination

In most cases of recessive inherited disorders, healthy carrier couples discover their risk only when an affected child is born. Alternatively, specific testing early in pregnancy may allow prospective prevention. At this point however, only two choices are left, accepting the risk or asking for prenatal diagnosis (PD) and medical abortion in case of an affected fetus.

As mentioned above, PD is offered to the pregnant mother mainly by testing fetal cells obtained by amniocentesis (after the sixteenth week of gestation) or a few weeks earlier in pregnancy by chronioc villi sampling (20). In most developed countries comprehensive programs for PD to address severe inherited conditions are available (21). In case of hemoglobinopathies, PD is mostly relevant in countries endemic for beta-thalassemia major and sickle cell disease. For alpha-thalassemia, as most forms compatible with postnatal life are mild (6), PD, as mentioned above, is only indicated for couples who are at risk of having a child with hydrops fetalis or severe HbH disease. In some cases, attempts to keep alive a homozygous alpha°-thalassemia fetus have been made by intrauterine transfusions and post-natal chronic transfusions (22). Due to the ambivalent effectiveness of such management, this practice is subject to ethical question. Examples on hemoglobinopthy (HBP) cases on which PD may be necessary is summarized in Table 1.

PD and medical abortion has shown to be the most effective prevention option available either as a national program or on request in endemic countries such as Hong Kong, Taiwan, India, Iran, Maldives and Singapore as well as non-endemic countries such as Australia, New Zealand, North America and parts of the Caribbean (23). A remarkable success in the decline of β-thalassemia major cases in regions where high thalassemia carrier rate exist were observed in northeast Thailand (24), Greece (25), Sardinia (26) and Cyprus (27).

Screening early in pregnancy is not offered routinely at the national level in Arab countries. This might be because it is socially not always accepted or due to lack of education about PD among the health workers and the population at large (1). Moreover, screening early in pregnancy leaves medical abortion as the only prevention option. This option however, is not legally or religiously permitted in many Muslim countries since, as previously mentioned, most Islamic institutions prohibit pregnancy termination at any time, unless the life of the pregnant woman is threatened. In some countries, (see below) it is allowed before the 120th day of gestation under specific circumstances (28).

Under civil law, selective abortion of an affected fetus is permissible in some Muslim countries such as Tunisia (29), Egypt (30) and Iran (31) with the last representing one of the classic examples of dramatic reduction of severe HBP incidences (32). Another recent prevention success have been reported from Northeastern Iraq (33) with 65% reduction in number of affected births over five years of implementing premarital screening and PD for HBP's. Although the Quran does not explicitly state whether termination of pregnancy is permissible in case of a severely affected fetus, PD and selective abortion remains a controversial issue in different Muslim Arab cultures (28,34,35). Some countries such as Iran and Iraq are led by a Shiaa scholar while others such as Egypt by a Sunni scholar. Oman is the only country led by an Abadhi scholar and all have different views towards PD in terms of legality of medical abortion. A release of a fatwa in each country based on the sub-religion (Islamic sector) is necessary as it provides guidance necessary to facilitate decision-making to those who need to base their decision upon their religious believe. Moreover, when the carrier status of the couples is found during pregnancy, it might be too late for any decision, or the time to make a decision may be too limited to understand the disease and the decision may be difficult to take under stressful circumstances (15).

Deciding to interrupt a pregnancy, even if in an early fetal stage, is a difficult and an emotional process for most couples involving their moral feelings and religious believes (2). On the other hand, knowing that the fetus will have a shorter life, devastated by a severe progressive disease that can be treated but not cured and with the high likelihood of a premature death, involves parent's responsibility as well as anxiety and fear to cope with a severely affected child. Moreover, the cost of PD can be an obstacle as well as the procedure itself, which is considered invasive and carries a risk of 1-2% of a miscarriage. The recent discovery of free fetal DNA in maternal plasma has opened a new possibility of noninvasive PD by identifying informative SNPs within a gene cluster. The method may offer an alternative to couples at risk but is only applicable in on average 50% of the cases and for HBP is thus far only done on a research basis (36).

Pre-implantation genetic diagnosis (PGD) may represent an alternative prevention option to PD. It involves testing either the oocytes before *in vitro* fertilization or the early

embryo immediately after *in vitro* fertilization, allowing pre-selection and transfer of normal pre-embryos to the women uterus (11). PGD is permissible under Islamic law, provided that the sperms and oocytes are from the husband and wife (37). The main advantage over PD is the avoidance of terminating an affected pregnancy. However, PGD is costly, laborious, psychologically and physically burdening to the woman and requires highly experienced operators for the procedure and pregnancy rates rarely surpass 30–35% (38).

Studies of the applicability of PGD have been reported mainly from Greece (39), Sicily (40), Scotland (41) Hong Kong (42), the United Kingdom (43) and Sardinia (44). In some countries PGD is only restricted to cases of infertility.

We have interviewed Omani couples at risk and studied their attitude towards PD and medical abortion. Our results reported in chapter 13 show that if PD was legalized and approved by the Omani religious authorities, this option would be most probably accepted by the Omani couples at risk as the alternatives of choice to prevent severely affected progeny, and leading to a dramatic reduction in the number of affected births in the country.

3.1.3 Newborn screening

Newborn screening (NBS) for hemoglobinopathy (HBP) allows the identification of affected infants soon after birth, allowing secondary (morbidity) prevention, offering tailored management through prophylactic treatment and vaccination and comprehensive care prior to the development of severe clinical complications (3). National systematic newborn screening for HBP has been implemented in many countries during the last decade (45) including Arab countries such as Bahrain (46), and the UAE (47). In North America (48), the UK (49) The Netherlands (50) France and other northern European countries NBS is either national or regional (51,52,53). As a consequence of NBS one may expect that parents of affected infants would become informed and aware of their risk and would follow the available prevention methods for subsequent pregnancies and an eventual decline in the number of affected birth should be noticed. However, this was not the case in The Netherlands where after 7 years of applying newborn screening, no reduction of HBP incidence has been noticed. Possible explanations could be due to counseling directing toward treatment rather than prevention, lack of awareness in poorly informed parents (54) and potential conflict of interest on the part of clinicians and institutions (55).

In a study conducted by Al Kindi et al (56) on cord blood samples from Omani neonates, high prevalence of HBP in newborns was confirmed, emphasizing the need of implementing neonatal cord blood screening as a national strategy program in the management and prevention of HBP. Although newborn screening cannot identify and alert couples at risk when they have a non-carrier newborn child, it could be a useful secondary and primary preventative tool in Oman (57).

Donor insemination and gamete donation are not acceptable in the traditional Islamic culture and gamete donation is forbidden as it involves the use of egg and / or sperm that are not from the husband and wife, meaning that the born child belongs to one parent only and to the donor person. Adoption, although encouraged in Islam, it has many ethical and religious implications.

Ongoing screening programs and their status in some Arab countries are summarized in Table 3.2.

Table 3.2. Available HBP preventive carrier screening methods for; premarital, prenatal and neonatal screenings and their status in some Arab countries. (NA = service not available). Note: newborn screening is mainly implemented for SCD and G6PD.

Country	Premarital	Prenatal & medical abortion	Neonatal
Oman	Optional	NA	optional
UAE	Mandatory	NA	mandatory in Abu Dhabi
KSA	Mandatory	NA	optional
Bahrain	Mandatory	NA	mandatory
Qatar	Mandatory	NA	optional
Kuwait	Mandatory	NA	???
Lebanon	Mandatory	NA	optional
Jordan	Mandatory	Optional	mandatory
Palestine	Mandatory	Optional	???
Iraq	Mandatory	Optional	???
Egypt	Optional	optional	optional
Tunisia	Mandatory	optional	mandatory

3.2 The molecular spectrum of mutations and pitfalls in hemoglobinopathy diagnosis

The molecular basis of hemoglobinopathies is extremely heterogeneous. Over a thousand different mutations are reported worldwide and many of them may interact in homozygous or compound heterozygous modes, producing a wide range of disorders of varying degrees of severity (Table 3.1). The mutations are regionally specific, with each country having a characteristic spectrum of abnormal hemoglobins and thalassemia mutations. The mutation spectrum in Oman is already quite heterogeneous and the recent migration of foreign laborers is bound to expand the ethnic profile of the Omani population with different variants and defects may arise in unexpected combinations.

The usual screening of carrier couples at risk of having children with a β-thalassemia major or sickle cell disease (SCD) or sickle cell thalassemia (β/S) is achieved through basic hematology methods such as cell blood count (CBC) together with hemoglobin separation and measurement on High Performance Liquid Chromatography (HPLC) or Capillary Electrophoresis (CE). However, in some cases the interpretation by these basic methodologies can be compromised and are insufficient to give provisional results, especially in cases when multiple mutation genes are co-inherited or rare variants are involved (58).

Abnormal hematology readings and Hb separations should always be further investigated during premarital screening and risk prediction, including for the large share of non-Omani citizens residing in the country which were excluded in this project.

As mentioned in Chapter 1, the standard reading of HPLC value of 30-40% HbS will usually indicate a HbS carrier status (59). Nonetheless, some rare abnormal hemoglobins (at least 33 variants) co-migrate or co-elute like HbS with identical retention time on HPLC (60, 61).

Although the provisional diagnosis of HbS carrier made by traditional HPLC or CE is quite robust, and a second confirmatory test such as solubility test or the simple sickle test might be sufficient in most cases, in rare cases DNA analysis is essential to avoid pitfalls and to confidently report the risk assessment when more complex genotypes are present. (58).

The pitfalls: Few examples on problematic cases are for instance those of carriers of unstable hemoglobin variants either undetectable or with isoelectric point and hydrophilic interaction identical to HbA and not separable with any of the available technologies. These variants may cause severe conditions in combination with β-thalassemia or HbS (58) and will never be diagnosed unless DNA analysis is done. The differential diagnosis of hemoglobin variants in homozygous or hemizygous states (Hb variant/β-thalassemia) is also crucial as the conditions cannot always be diagnosed by basic hematology methods. In adults, the microcytic hypochromic red cell indices and the HbA_2 measurement might help to discriminate to some extent, but not during newborn screening when HbA_2 is not yet expressed.

Another problematic cases can be those in which a β°-thalassemia point mutations is present in one parent while an unknown deletion in the β-globin gene cluster (normal HbA_2 and elevated or normal HbF) is present in the other. This may result in a progeny affected with mild or intermediate or a severe phenotype, depending from the type of deletion (with or without significant HbF expression). Deletions involving not only the b but also both γ-globin genes may result in severe compound heterozygosity due to the absence of the compensatory effect of fetal hemoglobin (62).

As mentioned in Chapter 1, association of β- and δ-thalassemia may also lead to misdiagnosis. That's why it is important to consider δ-thalassemia during β-thalassemia screening when borderline/normal HbA_2 value are observed risking false-negative results in the detection of couples at-risk (63).

Although the α^+-thalassemia trait is very common in Oman and it does not represent a major burden for public health in the native population, coexisting α-thalassemia could make the diagnosis more complex (58). For instance, α^+-thalassemia is important to predict to some extend the modulation and prognosis of the β-thalassemia and SCD patients. Conversely, α° thalassemia common in immigrant populations living in Oman may either improve β thalassemia major, or generate intermediate or severe phenotypes in couples of Asian origin.

Defective α-globin genes alongside the common mutations in any population generates atypical combination of these mutations with variable phenotype severity making molecular diagnostics at the DNA levels crucial especially in risk assessments. Moreover, alpha-thalassemia is not always easily diagnosed at the hematological level as it does not have specific characteristics on electrophoresis, HPLC, or CE except for a marginal reduction in HbA_2 expression (64) or in case of α-variants with known peaks (e.g.: Hb Constant Spring) and HbH disease, which may be associated with a rapidly eluting but unstable and disappearing HbH (β4) fraction (65). On the other hand α thalassemia can be easily diagnosed if screened at birth by the presence of detected Hb Bart's (γ4) fraction.

Finally, although most of the hemoglobin variants are either rare or silent and few semi-dominant or highly unstable, they are often not easily detectable with any of the available

basic technologies and need to be characterized at the molecular level (66). Then, in order to overcome most of these pitfalls, it is necessary to cover the full mutation spectrum in the country and keep in mind that new, rare or unexpected mutations will continue to be found in a multi-ethnic population and that consanguinity will increase the chance of homozygosis for rare conditions.Some examples of basic diagnostics and possibly associated pitfalls using the common parameters are given in Table 3.3.

Table 3.3. Summary of the basic hematological parameters used to obtain a provisional or definitive diagnosis of the most common HBP traits and factors that may influence the interpretation of the results.

Hematological reading	Diagnosis interpretation/ discrimination	External factors that may alter the hematological value
RBC	↓= Iron deficiency , ↑= thalassemia	Folic acid insufficiency
MCV – MCH	↓= Iron deficiency or thalassemia	Coexisting vitamin B12 deficiency
Erythromorphology	Typical in Thalassemia	Old specimen
Osmotic fragility	Lower in Thalassemia	Old specimen
Sickle test	Positive in trait and disease	Old specimen, technical failure
Elevated (HbF)	δβ- or γδβ-thalassemia or HPFH	Raise due to HbA1c overlapping
RDW	↓= thalassemia , ↑= iron deficiency	Cardiac and hepatic conditions
Elevated HbA_2	β-thalassemia, HbE, Hb Lepore	HIV patients / glycated HbS
Boarder line HbA_2	Normal HbA_2 β-thalassemia	δ defect or α-gene variants
Low HbS value	Coexisting α-thalassemia in HbS carrier [HbS<35-40%→(-α/αα), HbS~25-35%→(-α/-α) or (--/αα), HbS<25%→HbH (-α/--)] or non HbS variant eluting at the same spot or iron depletion.	Transfusion with an HbS blood donor carrier in a HbA/A individual and vice versa.
Low HbC or HbE	Coexisting HbH disease	HbH instability
Hb Bart's (γ_4) at birth	Hb Bart's of 0-5%→(-α/αα), 5-10%→(-α/-α) or (--/αα), 10-30%→HbH (-α/--).	Old sample

3.3 The process of changing attitude

As mentioned, the attitudes of the Arab-Muslim countries toward genetic screening, prenatal diagnosis (PD) and medical abortion is changing in some areas as more people are educated and aware of the consequences. In other countries screening and primary prevention remains limited due to many factors such as low economic resources, limited premarital screening centers, religious beliefs, culture and traditions, literacy, education and government policy.

If no preventative measures are taken in these endemic regions, in time the cost of treatment would consume the entire health budget of the country. However, before establishing any prevention option, it is necessary to assess the interest of the community in a preventive service and to generate professional genetic counselors that understand the sensitivity and the

ethical issues in handling prevention options such as PD and pregnancy termination and finally is essential to have policy makers aware of the magnitude of the problem.

Efforts are then needed to establish a comprehensive infrastructure for pre-conception clinics as an essential community based primary prevention measures (11) and to stimulate general practitioner and midwifes to change their passive attitude of "no complaints = no actions" into an active behavior of referral for carrier diagnostics for prevention purposes. Finally, to avoid stigmatization, awareness campaigns should be directed to the whole of the population rather than to immigrants or tribes/ethnic groups at high carrier frequency.

A number of studies have examined the attitude of Muslim couples at risk towards early genetic screening, PD, medical abortion and PGD and variable results were obtained. A recent study conducted in Saudi Arabia, showed that 90% of the couples at risk who were diagnosed prior marriage and advised to choose a different partner, proceeded with their marriage intention (8). When these couples were questioned, around 50% thought it is a good thing to undergo a screening test but before the engagement stage because of the difficulties in cancelling familial commitment, the wedding ceremony preparation, and because of social stigma (8). Similar cultural feelings have been observed in neighboring Middle Eastern countries, as well as the desire to have early preventive genetic services to reduce the incidence of HBP in these regions with high frequency of consanguineous marriages. Studies from Saudi Arabia (67) and Lebanon (68) have shown that couples considered PGD to be preferable, as their opinion towards PD was influenced by the religious authorities in the country.

Not all Muslim cultures accept the Fatwa of the official Islamic jurisprudence allowing selective abortion within the first trimester of pregnancy in case of an affected foetus (Council of the World Islamic League, 15-22/07/1410 Hijri/ 10-17 February 1990). However, all Muslim jurists, have agreed regardless the different Islamic streams that PGD for genetic disorders is permissible provided that the gametes are from husband and wife and this on the basis that IVF does not conflict with God's desire nor is considered a modification of God's creation, but rather a way of treatment because PGD and embryo selection is done when embryos are only at the eight-cell stage (69). The question is why should PD, followed by early medical abortion in vivo within 120 days of conception be unacceptable to all Islamic sectors, while PGD which could be considered in fact an early medical abortion in vitro be acceptable? In both cases there is interruption to fetal growth to prevent the birth of a severely affected child.

3.4 Diagnostics and management of hemoglobinopathies in Oman

Since sickle cell disease (SCD), β- and α- thalassemia are the most common autosomal recessive gene disorders in Oman, premarital testing for hemoglobinopathies (HBP) is provided as a national program for identifying couples at risk. The service is voluntary and is offered free of charge to the Omani citizens. The first line of diagnostic tool is based on the measurement of the hematological parameters (CBC) and on the separation and estimation of the hemoglobin fractions on high performance liquid chromatography (HPLC).

A normal individual after the age of 2 will present with about 96-97% HbA, ±3% HbA_2 and <1%HbF. Any change in this pattern will be anomalous and might indicate an HBP disorder. Frequent traits are confirmed by simple additional analysis, solubility test for HbS, alkaline or acid electrophoresis for common traits (HbD, HbE, HbC). If the variant peak cannot be

confirmed biochemically, DNA test is requested. Figure 3.1 summarizes how the value of HbA_2 is interpreted in the Omani population.

Molecular analysis is used to confirm a diagnosis when hematological and biochemical parameters are complex or unclear. The main technologies involved are direct DNA sequencing for the β- and α- globin genes. Gap-PCR is used to detect the common α-thal deletions when microcytic and hypochromic red cell indices are observed. Multiple ligation probe amplification (MLPA) is the ultimate solution for defining unknown deletion defects of either the β- or α-globin genes cluster and should be considered to be implemented in the genetic laboratory.

For the development and quality of a molecular laboratory one needs to keep up with new technologies and scientific advances. Robust and fast screening methods should be used once premarital testing for HBP becomes mandatory in the country. The referral hematology laboratory should have more than one method for hemoglobin analysis besides HPLC, for example Capillary Electrophoresis (CE). The serum ferritin test should be replaced by the zinc protoporphyrin method and be made mandatory on all samples. Serum ferritin is usually normal or elevated in β-thalassemia carriers who have increased iron absorption in the intestine but might also be borderline or low in α-thalassemia carriers. Moreover, ferritin is an acute-phase protein and might be falsely elevated in the presence of a coexisting infection (58). In some clinics, genetic counseling is not provided by a certified professional genetics counselor, but by a local health practitioner who has sufficient experience and training to convey the results. Efforts should be directed to have more professional specialists in the field.

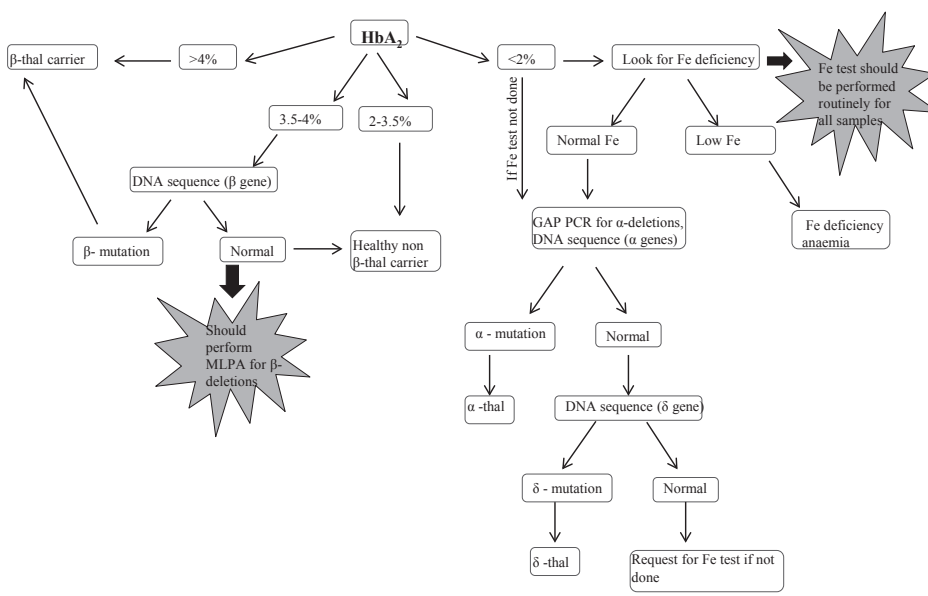

Figure 3.1. The criteria used to diagnose HBP related cases based on HbA_2 value, indicating when DNA analysis is performed. Tests highlighted in gray are needed and should be considered for future implementation in Oman.

Currently prevention of SCD and β-thalassemia major (BTM) in Oman involves either choosing another partner, accepting the risk or seeking for prenatal diagnosis (PD) or PGD abroad for those who can afford the costs. A typical premarital screening scenario includes the following stages: signing a consent form at the first consultation, sending a blood sample to the nearest hematology laboratory, sending a sample for DNA analysis if diagnosis cannot be confirmed by hematology tests, receiving results (by fax) and finally reporting the findings to the couples by either arranging a counseling session or conveying results over the phone.

Severely affected patients are treated in the major hospitals. Thalassemia patients are offered monthly blood transfusion and chelation therapy. Sicklers are regularly immunized and admitted when severe symptoms strike. Recently, Hydroxyurea treatment has been introduced but is given to only very severe cases of SCD following stringent criteria.

The majority of the society of modern Oman may eventually accept selective abortion if religiously permitted. Prevention will spare a lot of human suffering to children and families as well as the huge expenses to the country necessary for treatment of the ever growing number of patients affected with these incurable diseases. Efforts were made to open a constructive dialogue with the main religious authority in Oman (country's Muftee) in order to discuss the issue of PD and medical abortion in case of a severely HBP affected fetus. However, to date, the religious authority has not agreed to make this service legal as yet.

A SUMMARY OF THE PATIENTS AND METHODS

As mentioned at the beginning in "Aim of this thesis" the intention of this study was to investigate all possible factors involved with the implementation of a national prevention strategy for Oman and in other Muslim countries also endemic for HBP. For this I have studied at the biochemical, molecular and clinical level a cohort of 722 Omani subjects (1444 alleles), affected with BTM, SCD, alpha thalassemia or presumed to be carriers of HBP. Details of all patients and methods used for analysis are included in the publications summarized in Chapters 4-13.

I have provided the widest spectrum of common and rare mutations in Oman and the prevalence of specific mutations more common in specific areas of the country (Chapters 4, 5, 6, 7 and 8),

I have studied the factors involved in the modulation of severity of SCD such as haplotype and coexisting alpha thalassemia and the value of these factors for prognostics, risk assessment, state of the art treatment (genotype / phenotype correlation) and for pharmacogenomics (Hydroxyurea treatment) (Chapters 9, 10 and 11).

I have tested the application of next generation technology in identifying large number of at risk cases in a rapid period of time (Chapter 12)

Finally I have studied the attitudes of the Omani couples towards PD and medical abortion (Chapter 13).

I believe that this study has provided new information on different aspect essential for the implementation of a modern, national diagnostics, treatment and prevention program for HBP in Oman.

REFERENCES

1. Angastiniotis M, Modell B, Boulinzhenkov V. Prevention and control of haemoglobinopathies. Bulletin of the World Health Organization. 1995;73(3):375-386.
2. Giordano PC, Harteveld CL, Bakker E. Genetic epidemiology and preventive healthcare in multiethnic societies: the hemoglobinopathies. Int J Environ Res Public Health. 2014;11(6):6136-46.
3. Traeger-Synodinos J, Harteveld CL, Old JM, Petrou M, Galanello R, Giordano P, et al. EMQN Best practice guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies. Eur J Hum Genet. 2014 Jul 23. doi: 10.1038/ejhg.2014.131.
4. Amato A, Cappabianca MP, Lerone M, Colosimo A, Grisanti P, Ponzini D, et al. Carrier screening for inherited haemoglobin disorders among secondary school students and young adults in Latium, Italy. J Commun Genet. 2014;5(3):265-8.
5. Eram SM, Azimifar B, Abolghasemi H, Foulady P, Lotfi V, Masrouri M, et al. The IVS-II-I (G>A) beta^{0}-thalassemia mutation in cis with HbA_2-Troodos delta116(G18) Arg>Cys (CGC>TGC) causes a complex prenatal diagnosis in an Iranian family. Hemoglobin. 2005;29(4):289-292.
6. Yi JS, Moertel CL, Baker KS. Homozygous alpha-thalassemia treated with intrauterine transfusions and unrelated donor hematopoietic cell transplantation. J Pediatr. 2009;154(5):766 –768.
7. Zeinalian M, Nobari RF, Moafi A, Salehi M, Hashemzadeh-Chaleshtori M. Two decades of pre-marital screening for beta-thalassemia in central Iran. J Community Genet. 2013;4(4): 517-522.
8. Alswaidi FM, Memish ZA, O'Brian SJ, Al-Hamdan NA, Al-Enzy FM, Alhayani OA, et al. At-risk marriages after compulsory premarital testing and counseling for β-thalassemia and sickle cell disease in Saudi Arabia. J Genet Couns. 2012;21(2):243–255.
9. Chaabouni-Bouhamed H. Tunisia: communities and community genetics. Community Genet. 2008;11(6):313-323.
10. El-Tayeb EN, Yaqoob M, Abdur-Rahim K, Gustavson KH. Prevalence of beta-thalassaemia and sickle cell traits in premarital screening in Al-Qassim, Saudi Arabia. Genet Couns. 2008;19(2):211–218.
11. Czeizel AE, Gasztonyi Z, Kuliev A. Periconceptional clinics: a medical health care infrastructure of new genetics. Fetal Diagn Ther. 2005;20(6):515-518.
12. Angastiniotis MA, Hadjiminas MG. Prevention of thalassaemia in Cyprus. Lancet 1981;1(8216):369–71.
13. Amato A, Grisanti P, Lerone M, Ponzini D, Di Biagio P, Cappabianca MP, et al. Prevention strategies for severe hemoglobinopathies in endemic and non endemic immigration countries: the Latium example. Prenatal Diagnosis. 2009;29(12):1171-1174.
14. Mitchell JJ, Capua A, Clow C, Scriver CR. Twenty-year outcome analysis of genetic screening programs for Tay-Sachs and beta-thalassemia disease carriers in high schools. Am. J. Hum. Genet. 1996;59(4):793–798.
15. Lena-Russo D, Badens C, Aubinaud M, Merono F, Paolasso C, Martini N, et al. Outcome of a school screening programme for carriers of haemoglobin disease. J. Med. Screen. 2002;9(2):67–69.
16. Al-Arrayed S, Hafadh N, Amin S, Al-Mukhareq H, Sanad H. Student screening for inherited blood disorders in Bahrain. East Mediterr Health J. 2003;9(3):344–352.
17. El-Beshlawy A, Youssry I. Prevention of hemoglobinopathies in Egypt. Hemoglobin. 2009;33(1):14–20.
18. Hesketh T. Getting married in China: pass the medical first. BMJ. 2003; 326(7383): 277-279.
19. Ebrahim SH, Lo SS, Zhuo J, Han JY, Delvoye P, Zhu L. Models of preconception care implementation in selected countries. Matern Child Health J. 2006;10(5):37-42.
20. Kan YW, Chang JC. Molecular diagnosis of hemoglobinopathies and thalassemia. Prenat Diagn. 2010;30(7):608-610.
21. Liao C, Mo QH, Li J, Huang YN, Hua L, Li QM, et al. Carrier screening for alpha and beta-thalassemia in pregnancy: the results of an 11-year prospective program in Guangzhou Maternal and Neo natal hospital. Prenat Diagn. 2005;25(2):163–171.
22. Chmait RH, Baskin JL, Carson S, Randolph LM, Hamilton A. Treatment of alpha(0)-thalassemia ($--^{SEA}/--^{SEA}$) via serial fetal and post-natal transfusions: Can early fetal intervention improve outcomes?. Hematology. 2014 (Epub ahead of print).
23. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bulletin of the World Health Organization. 2008;86(6):480–487.
24. Yamsri S, Sanchaisuriya K, Fucharoen G, Sae-Ung N, Ratanasiri T, Fucharoen S. Prevention of severe thalassemia in northeast Thailand: 16 years of experience at a single university center. Prenat Diagn. 2010;30(6):540-6.
25. Theodoridou S, Alemayehou M, Prappas N, Karakasidou O, Aletra V, Plata E, et al. Carrier screening and prenatal diagnosis of hemoglobinopathies. A study of indigenous and immigrant couples in northern Greece over the last 5 years. Hemoglobin. 2008;32(5):434-9.

26. Cao A, Rosatelli MC, Monni G, Galanello R. Screening for thalassemia: a model of success. Obstet Gynecol Clin North Am. 2002;29(2):305–328.
27. Zlotogora J. Population programs for the detection of couples at risk for severe monogenic genetic diseases. Hum Genet. 2009;126(2):247-253.
28. Al-Aqeel AI. Islamic ethical framework for research into and prevention of genetic diseases. Nat Genet. 2007;39(11):1293-1298.
29. Chaabouni H, Chaabouni M, Maazoul F, M'rad R, Jemaa LB, Smaoui N, et al. Prenatal diagnosis of chromosome disorders in Tunisian population. Ann Genet. 2001;44(2):99–104.
30. El-Beshlawy A, El-Shekha A, Momtaz M, Said F, Hamdy M, Osman O, et al. Prenatal diagnosis for thalassaemia in Egypt: what changed parents' attitude? Prenat Diagn. 2012; 32(8):777–782.
31. Samavat A, Modell B. Iranian national thalassaemia screening programme. BMJ. 2004; 329(7475):1134–7.
32. Nikuei P, Hadavi V, Rajaei M, Saberi M, Hajizade F, Najmabadi H. Prenatal diagnosis for beta-thalassemia major in the Iranian province of Hormozgan. Hemoglobin. 2008;32(6):539–45.
33. Al-Allawi NA, Jalal SD, Ahmed NH, Faraj AH, Shalli A, Hamamy H. The first five years of a preventive programme for haemoglobinopathies in Northeastern Iraq. J Med Screen. 2013; 20(4):171-176.
34. El-Hazmi MA. Islamic teachings of bioethics in relation to the practice of medical genetics. Saudi Med J. 2007;28(12):1781–1787.
35. Asman O. Abortion in Islamic countries-legal and religious aspects. Med Law 2004;23(1):73-89.
36. Mavrou A, Kouvidi E, Antsaklis A, Souka A, Kitsiou Tzeli S, Kolialexi A. Identification of nucleated red blood cells in maternal circulation: a second step in screening for fetal aneuploidies and pregnancy complications. Prenat Diagn. 2007;27(2):150-153.
37. Lavery SA, Aurell R, Turner C, Castello C, Veiga A, Barri PN, et al. Preimplantation genetic diagnosis: patients' experiences and attitudes. Hum Reprod. 2002;17(9):2464-2467.
38. Dreesen J, Destouni A, Kourlaba G, Degn B, Mette WC, Carvalho F, et al. Evaluation of PCR-based preimplantation genetic diagnosis applied to monogenic diseases: a collaborative ESHRE PGD consortium study. Eur J Hum Genet. 2014;22(8):1012-8.
39. Traeger-Synodinos J. Preimplantation genetic diagnosis, an alternative to conventional prenatal diagnosis of the hemoglobinopathies. Int J Lab Hematol. 2013;35(6):571-579.
40. Chamayou S, Guglielmino A, Giambona A, Siciliano S, Di Stefano G, Scibilia G, et al. Attitude of potential users in Sicily towards preimplanataion genetic diagnosis for beta-thalassaemia and aneuploidies. Hum Reprod. 1998;13(7):1936-1944.
41. Miedzybrodzka Z, Templeton A, Dean J, Haites N, Mollison J, Smith N. Preimplantation diagnosis or chorionic villus biopsy? Women's attitudes and preferences. Hum Reprod. 1993; 8(12):2192–2196.
42. Hui PW, Lam YH, Chen M, Tang MH, Yeung WS, Ng EH, et al. Attitude of at-risk subjects towards preimplanatation genetic diagnosis of alpha- and beta-thalassaemias in Hong Kong. Prenat Diagn. 2002;22(6):508-511.
43. Snowdon C, Green JM. Preimplantation diagnosis and other reproductive options: attitude of male and female carriers of recessive disorders. Hum Reprod. 1997;12(2):341-350.
44. Palomba ML, Monni G, Lai R, Cau G, Olla G, Cao A. Psychological implications and acceptability of preimplantation diagnosis. Hum Reprod. 1994;9(2):360-362.
45. Bain BJ. Neonatal/newborn haemoglobinopathy screening in Europe and Africa. J Clin Pathol. 2009;62(1):53-6.
46. Al-Arrayed S, Al-Hajeri A. Newborn screening services in Bahrain between 1985 and 2010. Adv Hematol. 2012; 2012:903219.
47. Al-Gazali LI, Alwash R, Abdulrazzaq YM. United Arab Emirates: communities and community genetics. Community Genet. 2005;8(3):186-196.
48. Hoppe C. Newborn screening for Hemoglobinopathies in the U.S. Journal compilation, Special Issue, International Journal of Laboratory Hematology. 2009;31(1):27–28.
49. Streetly A, Latinovic R, Hall K, Henthorn J. Implementation of universal newborn bloodspot screening for sickle cell disease and other clinically significant haemoglobinopathies in England: screening results for 2005–7. J Clin Pathol. 2009;62(1):26–30.
50. Kaufmann JO, Demirel-Güngör G, Selles A, Hudig C, Steen G, Ponjee G, et al. Feasibility of nonselective testing for hemoglobinopathies in early pregnancy in The Netherlands. Prenat Diagn. 2011;31(13):1259–1263.
51. Giordano PC. Starting neonatal screening for haemoglobinopathies in The Netherlands. J Clin Pathol. 2009;62(1):18–21.
52. Bardakdjian-Michau J, Bahuau M, Hurtrel D, et al. Neonatal screening for sickle cell disease in France. J Clin Pathol 2009; 62(1): 31–33.
53. Gulbis B, Cotton F, Ferster A, Ketelslegers O, Dresse MF, Rongé-Collard E, et al. Neonatal haemoglobinopathy screening in Belgium. J Clin Pathol. 2009;62(1):49-52.
54. Kaufmann JO, Krapels IP, van Brussel BT, Zekveld-Vroon RC, Oosterwijk JC, van Erp F, et al. After the

introduction into the national newborn screening program: who is receiving genetic counseling for hemoglobinopathies in the Netherlands? Public Health Genomics. 2014;17(1):16-22.

55. Kaback MM. Population-based genetic screening for reproductive counseling: The Tay-Sachs disease model. Eur J Pediatr. 2000;159:S192–S195.
56. AlKindi S, Pathare A, Al-Madhani A, Al Zadjali S, Al Haddabi H, Al Abri Q, et al. Neonatal screening Mean haemoglobin and red cell indices in cord blood from Omani neonates. SQU Med J. 2011;11(4):462-469.
57. Thuret I, Sarles J, Merono F, Suzineau E, Collomb J, Lena-Russo D, et al. Neonatal screening for sickle cell disease in France: evaluation of the selective process. J Clin Pathol. 2010;63(6):548-51.
58. Giordano PC. Strategies for basic laboratory diagnostics of the hemoglobinoapthies in multi-ethnic societies: interpretation of results and pitfalls. Int J Lab Hematol. 2013 ;35(5):465-79.
59. Harteveld CL, Ponjee G, Bakker-Verweij M, Arkesteijn SG, Phylipsen M, Giordano PC. Hb Haaglanden: a new nonsickling β7Glu>Val variant. Consequences for basic diagnostics, screening, and risk assessment when dealing with HbS-like variants. Int J Lab Hematol. 2012;34(5):551–5.
60. Moradkhani K, Riou J, Wajcman H. Pitfalls in the genetic diagnosis of HbS. Clin Biochem. 2013;46(4-5):291-299.
61. Szuberski J, Oliveria JL, Hoyer JD. A comprehensive analysis of hemoglobin variants by high-performance liquid chromatography (HPLC). International Journal of Laboratory Hematology. 2012;34(6):594-604.
62. Phylipsen M, Amato A, Cappabianca MP, Traeger-Synodinos J, Kanavakis E, Basak N, et al. Two new beta-thalassemia deletions compromising prenatal diagnosis in an Italian and a Turkish couple seeking prevention. Haematologica. 2009;94(9):1289-92.
63. Li J, Xie XM, Zhou JY, Li DZ. Co-inheritance of β- and δ- thalassemia compromising prenatal screening in a Chinese souple seeking prevention. Fetal Diagn Ther. 2011;30(1):73-6.
64. Stephens AD, Angastiniotis M, Baysal E, Chan V, Fucharoen S, Giordano PC, et al. International council for the standardisation of haematology (ICSH). ICSH recommendations for the measurement of haemoglobin A_2. Int J Lab Hematol. 2012; 34(1):1–13.
65. Papassotiriou I, Traeger-Synodinos J, Vlachou C, Karagiorga M, Metaxotou A, Kanavakis E, et al. Rapid and accurate quantitation of Hb Bart's and Hb H using weak cation exchange high performance liquid chromatography: correlation with the alpha-thalassemia genotype. Hemoglobin. 1999;23(3):203–11.
66. van den Berg HM, Bruin MC, Batelaan D, van Delft P, van Zwieten R, Roos D, et al. Hb Nijkerk: a new mutation at codons 138/139 of the beta-globin gene inducing severe hemolytic anemia in a Dutch girl. Hemoglobin. 1999;23(2):135-44.
67. Alkuraya FS, Kilani RA. Attitude of Saudi families affected with hemoglobinopathies towards prenatal screening and abortion and the influence of religious ruling (Fatwa). Prenat Diagn. 2001;21(6):448-451.
68. Farra C, Nassar AH, Usta IM, Salameh P, Souaid M, Awwad J. Acceptance of preimplantation genetic diagnosis for beta-thalassemia in Lebanese women with previously affected children. Prenat Diagn. 2008;28(9):828-832.
69. El-Hashemite N. Genetic Malformation in Children, its Causes, and the Islamic View in Preventive Procedures (in Arabic Language). Dar Al-Hekma: London. 1995.

CHAPTER 4

EXTENDED MOLECULAR SPECTRUM OF β- AND α- THALASSEMIA IN OMAN

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Hemoglobin. 2010;34(2):127-134

ABSTRACT

Sickle cell disease is known to be very common in the Omani population, although data are limited concerning β-thalassemia (β-thal). We report the molecular background of 87 unrelated patients from the Sultanate of Oman, diagnosed with β-thal major (β-TM), β-thal intermedia (β-TI) or minor. Diagnosis was based on clinical and hematological data and confirmed by molecular analysis. We found 11 different β-thal determinants in our cohort, which consists of subjects from different regions of Oman. Six of these mutations have not been previously reported in the Omani population. The prevalence of α-thal single gene deletions (-$\alpha^{3.7}$ and –$\alpha^{4.2}$) in the same cohort was very high (58.3%). These data will contribute to the implementation of a country-wide service for early molecular detection of hemoglobinopathies and for providing genetic counseling following premarital screening.

INTRODUCTION

Hemoglobinopathies are the most frequent autosomal recessive disease in man. The most common conditions are caused by mutations in the β- or the α-globin genes coding for the postnatally expressed Hb A subunits. Mutations may either change the primary structure of the gene product [abnormal hemoglobins (Hbs)] or impair the expression of the mutated gene (thalassemia). Expression defects are subdivided into α- and β-thalassemia (α- and β-thal), resulting from the defective synthesis of α- and β-globin chains, respectively. Carriers of β-thal (thalassemia minor) are mostly asymptomatic with mild anemia, while patients with β-thal major (β-TM) are severely affected, transfusion-dependent and in need of continuous iron chelation therapy. Patients who are carriers of α-thal (−α/αα, −α/−α and − −/αα) are mildly anemic, while patients with Hb H disease (−α/− −) present with an α-thal intermedia (α-TI) picture with enlarged spleen/liver, and in some cases, may need regular or occasional blood transfusions, whereas homozygous α^0-thal (− −/− −) is lethal. But for a successful bone marrow transplant, these diseases cannot be cured, and for most of these patients the only alternative is intensive treatment until premature death. More than 80% of the α^+-thal determinants are common deletions such as the $-\alpha^{3.7}$ and $-\alpha^{4.2}$. Conversely, more than 95% of the β-thal cases are due to point mutations, of which only a few are prevalent and population-specific, while many others are rare. Population isolates usually present with a narrow spectrum of mutations, while admixed populations are characterized by a large number of mutations (1).

After sickle cell disease (SCD), β-TM is the second most common severe hemoglobinopathy in Oman, a country with a population of 2.6 million inhabitants, of which 73% are native Omanis and 27% are foreign immigrants. The annual birth rate is ±50,000 newborn, of which 250 have β –TM and are in need of intensive therapy and the figures continue to rise every year. Although there has been a significant improvement in social conditions and medical care for these patients during the last three decades, life expectation and quality of life still remains poor with severe human suffering and patients remain in continuous need of burdening therapy provided by public health services which, being free of charge in Oman, represents a considerable share of public health expenses. Prevention being better than treatment, the aim of this study was to investigate the molecular spectrum of β-thal in order to enable the study of the geographical prevalence of the different thalassemia defects occurring in the different regions of Oman. Knowledge of this molecular spectrum is necessary for early diagnosis and prevention of morbidity and mortality of these most debilitating inherited disorders.

MATERIALS AND METHODS

The examined cohort consisted of 87 unrelated individuals of both genders previously diagnosed with β-TM, β-TI or minor or with sickle cell disease. Of these, 84 were Omani subjects and three were immigrants. Age ranged from 1 to 44 years old (1963–2006). Blood was collected in EDTA and patients gave written consent for the procedures to be performed according to the local ethical regulations. Routine analyses were performed in The Netherlands, by measurement of Hb fractions on a high performance liquid chromatography (HPLC) apparatus (Variant, Bio-Rad Laboratories, Hercules, CA, USA) and on a capillary electrophoresis (CE) machine (Capillarys,

Sebia, Paris, France), as described elsewhere (2). All patients were analyzed at the molecular level. DNA extraction was done by high salt technologies, either manual or automated, using the Puregene DNA Purification System (Gentra Systems, Minneapolis, MN, USA) (3). Mutation analyses of α-thal included multiplex polymerase chain reaction (gap-PCR) as described by Liu et al. (4). Point mutation analysis of the β-globin gene was done on a GeneAmp9700 (Applied Biosystems, Foster City, CA, USA) using the QIAGEN® Multiplex PCR kit (Cat. no. 206143; Qiagen GmbH, Hilden, Germany), as previously reported (5). DNA sequencing was done on an ABI PRISM™ 3730 Genetic Analyzer (Applied Biosystems) using ABI PRISM® Big Dye Terminators v2.0 Cycle Sequencing kit according to the manufacturer's instructions.

RESULTS

Routine analyses of samples freshly collected in Oman showed either the pattern of transfused β-thal patients, of sickle cell disease, either homozygous or in combinations Hb S [β6(A3) Glu→Val]/ β-thal, or of carriers of these traits. All patients were examined at the molecular level.

In total we examined 174 alleles and found 11 different β-thal determinants.

In the non Omani, four codon 39 (C>T) and two codons 41/42 (–TTCT) alleles were found. In the 168 alleles from Omani subjects, 83 chromosomes with β-thal point mutations were characterized with nine different "Omani" mutations.

The IVS-I-5 (G>C) was the most prevalent (73%), while Hb Monroe [IVS-I (–1) or codon 30 (G>C), β30(B12)Arg→Thr], codon 5 (–CT) and IVS-I (–25 bp) 3' end mutations were the second in frequency (4.5%) together with codon 39 found in immigrants. Codons 8/9 (+G) was third in prevalence (3.3%) and codons 41/42 fourth at 2.2% but in immigrants only. The remaining four mutations were observed at lower frequencies (1.1%).

We have observed six β-thal mutations that were not found in the previous study by Daar et al. (6) on 99 Omani patients in 1998. Conversely, we have not found nine other mutations that were observed by Daar et al. (6). The IVS-I-5 mutation was the most predominant in both studies which complement each other, providing an extended spectrum of the β-thal determinants in the Sultanate of Oman. The data are summarized in Table 4.1.

Table 4.2 shows the distribution of eight Omani β-thal mutations in four regions. The IVS-I-5 mutation was predominant in Batinah, Muscat and Dhakhiliyah (73.7, 86.6 and 83.3%, respectively), while the same mutation was absent in Musandam with Hb Monroe being the most prevalent. Although the number of alleles studied is too small to provide a precise assessment, these preliminary results seem to indicate that the distribution of the mutations is not uniform and further studies are required to confirm the data (Table 4.2).

We have examined the same 87 individuals for the presence of the common α-thal deletions by gap-PCR (4). In three cases the analysis failed due to technical reasons. In 35 patients no α-thal deletion was found. In 25 cases, the $-\alpha^{3.7}$ deletion was found in the heterozygous state (29.8%) and in 22 in the homozygous state (26.2%). One patient was heterozygous for the $-\alpha^{4.2}$ deletion (1.2%) and one patient presented with compound heterozygosity for the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions (1.2%). Thus, the prevalence of α-thal alleles in this cohort was 58.3%. The data are summarized in Table 4.3.

Table 4.1. Comparison of the molecular spectrum of β–thalassemia mutations in Oman with the data of Daar et al. (6). [a] : Found in immigrants only

	Present study (chromosomes)		Daar et al. (6) (chromosomes)	
Mutations	***n***	**%**	***n***	**%**
IVS-I-5, G>C	65	73.0	122	61.6
Codon 5, -CT	4	4.5	1	0.5
Codon 30, G>C, Hb Monroe	4	4.5	0	0.0
IVS-I (-25bp) 3' end	4	4.5	11	5.5
Codon 39, C>T [a]	4	4.5	2	1.0
Codons 8/9, +G	3	3.4	0	0.0
Codons 41/42, -TTCT [a]	2	2.3	0	0.0
IVS-I-1, G>A	1	1.1	2	1.0
Codon 41, -C	1	1.1	0	0.0
Codon 16, -C	1	1.1	0	0.0
Codon 44, -C	0	0.0	19	9.6
Hb Dhofar, β58(E2) Pro→Arg	0	0.0	13	6.6
619 bp deletion	0	0.0	8	4.0
IVS-II-1, G>A	0	0.0	7	3.5
Codons 36/37, -T	0	0.0	2	1.0
Codon 15, G>A	0	0.0	1	0.5
Codon 37, -G	0	0.0	1	0.5
Codon 30, G>A	0	0.0	1	0.5
IVS-I-110, G>A	0	0.0	1	0.5
Hb E, β26(B8) Glu→Lys	0	0.0	1	0.5
Unknown	0	0.0	6	3.0

DISCUSSION

We herein describe eleven different β-thalassemic mutations present in Omanis and three in immigrant subjects. The imported mutations need to be taken into consideration for two reasons: *(i)* because of their significant contribution (from India, The Philippines, Egypt and others) in the past to the present day spectrum of mutations in the Omani population; *(ii)* given the actual percentage (27%) of the immigrant community within Oman, health professionals and authorities must be aware of the issues in future planning strategies (7).

As Daar et al. (6) reported, we also found that the IVS-I-5 mutation is the most common (73%) in Oman but with uneven distribution across the region. This mutation is most prevalent in the northern regions of Oman Batinah, Muscat and Dhakhiliyah (Table 4.2). The second most common molecular defects are the Hb Monroe, codon 5 and the IVS-I (–25 bp) 3'end mutations

with a prevalence of 4.5%. These data are different from those reported by Daar et al. (6) in which Hb Monroe was not found and the codon 5 mutation was observed at a much lower frequency (0.5%). Moreover, the remaining mutations were observed at lower frequencies and six were not observed by Daar et al. (6), while nine mutations reported by these investigators were not observed by us. This is probably due to differences in the ethnic composition of the cohorts and to the relatively small number of individuals studied. Larger surveys are needed

Table 4.2. Regionwise variation in the distribution of Omani β–thalassemia mutations

Region	Omani alleles studied	Omani mutations found	Type of mutation	Regional prevalence (%)
Musandam	4	3	Codon 30, G>C, Hb Monroe	75.0
		1	Cd 8/9, +G	25.0
Batinah	19	14	IVS-I-5, G>C	73.7
		4	IVS-I (-25bp) 3' end	21.0
		1	Codon 30, G>C, Hb Monroe	5.3
Muscat	53	46	IVS-I-5, G>C	86.6
		3	Codon 5, -CT	5.6
		2	Codons 8/9, +G	3.8
		1	Codon 16, -C	1.9
		1	Codon 41, -C	1.9
Dakhiliyah	6	5	IVS-I-5, G>C	83.3
		1	IVS-I-1, G>A	16.7
Total	82	82	8	

Table 4.3. Spectrum of α-thalassemia mutations in Oman

(a) Allele frequencies

Mutation	Allele frequency (%)	Prevalence (%)
$-\alpha^{3.7}$ (rightward)	41.7	
$-\alpha^{4.2}$ (leftward)	1.2	
Total	42.9	58.3

(*b*) Genotype frequencies

Mutation	*n*	Genotype frequency (%)
Rightward homozygotes	22	26.2
Rightward heterozygotes	25	29.8
Compound rightward/leftward	1	1.2
Leftward heterozygote	1	1.2

to obtain a better picture regarding prevalence and distribution of thalassemic mutations in a multi-ethnic society such as Oman.

Geographical and cultural factors separate the tribal populations of Oman causing isolation and the high degree of consanguineous marriages. Consanguinity doubles the chance of generating couples at risk and if a partner is already a carrier, the chance that the other will also be a carrier in a first-cousins marriage will be 1:16. Consequently, unless otherwise appropriate prevention measures are taken, the number of affected births will continue to grow.

In spite of the high prevalence of the Hb S and IVS-I-5 mutations, less common defects must be taken into consideration. For instance, in the region of Musandam, separated from the rest of Oman by the United Arab Emirates, three out of the four unrelated alleles studied carried the Hb Monroe mutation. This variant is associated with a β-thal phenotype (8) and at risk for β-TM (9). Hb Monroe was also found in Batinah (4.8%), a region close to Musandam and was also observed in the United Arab Emirates (10). Apparently, Hb Monroe is prevalent in communities of the west coastal area of the Arabian Gulf peninsula, but this needs to be confirmed by a larger study.

Since our recruitment strategy was focused on β-thal, our cohort can be considered as random (not selected for α-thal). The prevalence of α^+-thal mutations, known to be high in Saudi Arabians (43.3%) (11), is found to be even higher in the Omani population (58.3%). With no less than 22 homozygotes for the $-\alpha^{3.7}$ deletion and 25 carriers out of the 84 unrelated individuals, is probably the highest frequency observed in the Arabian peninsula. On the other hand, none of the α^0 defects associated with Hb H or Hb Bart's hydrops fetalis were found. The reason for the absence of the α^0 defects is probably the high consanguinity due to negative selection of such lethal defects in the high consanguineous setting.

CONCLUSIONS

Our analysis has shown the existence of few common mutations but also the presence of a number of less common defects. Considering the fact that the Omani population is a society consisting of well defined groups (tribes), the distribution of the β-thal determinants is bound to be different in different regions. Our cohort was collected mainly from the northern part of

Oman because it has been previously reported by Daar et al. (6) that β-thal mutations are prevalent in these regions. Nevertheless, we presume that β-thal defects might be present at lower frequencies in other regions as well.

These data are important for organizing prevention and management of severe forms of hemoglobinopathies in Oman. Given their high prevalence, young Omani subjects are advised to get screened for hemoglobinopathies at a premarital stage either for their choice of a partner or for getting informed in advance of the risk and of the available options: not to have children, adoption or use of donor gametes or prenatal diagnosis.

Prenatal diagnosis is available in Islamic countries such as Egypt, Tunisia and Iran, and medical abortion is allowed in these countries when the fetus is found to be affected with a severe hemoglobinopathy, in accordance with a Fatwa permitting medical abortion prior to 3 months of conception in case of a severely affected fetus (Council of the World Islamic League, 15-22/07/1410 Hijri/10-17 February 1990). Medical abortion for a severely affected fetus

remains a matter of debate/discussion in Oman and views of prominent muftis are essential. Development of prevention, control and management programs of hemoglobinopathies in Oman should aim to reduce the number of affected births and simultaneously must offer the best quality of life by medical care to those born affected with an anomaly.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES

1. Higgs DR, Weatherall DJ. The Haemoglobinopathies, Vol. 6(1). London: Bailliere's Clin. Haematol. 1993.
2. van Delft P, Lenters E, Bakker-Verweij M, de Korte M, Baylan U, Harteveld CL, Giordano PC. Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations. Int J Lab Hematol. 2009 31(5):484–495.
3. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16(3):1215.
4. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of α-thalassaemia deletions and α-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol. 2000;108:295–299.
5. Giordano PC, Maatman RG, Niessen RW, van Delft P, Harteveld CL. β Thalassemia IVS-I-5 (G→C) heterozygosity masked by the presence of Hb J-Meerut in a Dutch-Indian patient. Haematologica. 2006;91(12 Suppl):ECR56.
6. Daar S, Hussain HM, Merghoub T, Krishnamoorthy R. 1998. Spectrum of β-thalassemia mutations in Oman. Ann N Y Acad Sci. 1998;850:404–406.
7. Amato A, Grisanti P, Lerone M, Ponzini D, Di Biagio P, Cappabianca MP, Giordano PC. Prevention strategies for severe hemoglobinopathies in endemic and nonendemic immigration countries: the Latium example. Prenat Diagn. 2009;29(12)1171–1174.
8. Gonzalez-Redondo JM, Stoming TA, Kutlar F, Kutlar A, Hu H, Wilson JB, Huisman THJ. 1989. HB Monroe or α_2 $\beta_2$30(B12)Arg→Thr, a variant associated with β-thalassemia due to a G→C substitution adjacent to the donor splice site of the first intron. Hemoglobin. 1989;13(1):67–74.
9. Sweeting I, Serjeant BE, Serjeant GR, Kulozik AE, Vetter B. Hb S-Hb Monroe; a sickle cell-β-thalassemia syndrome. Hemoglobin. 1998;22(2):153–156.
10. El-Kalla S, Mathews AR. A significant *b*-thalassemia heterogeneity in the United Arab Emirates. Hemoglobin. 1997;21(3):237–247.
11. Ganeshaguru K, Acquaye JK, Samuel AP, Hassounah F, Agyeiobese S, Azrai LM, Sejeny SA, Omer A. Prevalence of thalassaemias in ethnic Saudi Arabians. Trop Geogr Med. 1987;39(3): 238–243.

CHAPTER 5

BROADER SPECTRUM OF β-THALASSEMIA MUTATIONS IN OMAN: REGIONAL DISTRIBUTION AND COMPARISON WITH NEIGHBOURING COUNTRIES

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Hemoglobin. 2015; 39(2):1-4

ABSTRACT

The objective of this study was to expand and study the molecular spectrum of β-thalassemia mutations in Oman by examining cases from 7 different regions and comparing the prevalence with neighbouring countries. A total of 446 cases of β-hemoglobinoapthy were obtained and analyzed to determine the frequency and distribution of the different β-alleles. The molecular spectrum of β-thalassemia in Oman revealed the presence of 32 different mutations of different origin and 11 alleles are reported for the first time in the Omani population. The wide heterogeneous spectrum of β-thalassemia mutations found can be associated with the history of trade and migration as well as the past domination from other countries. The presented data will facilitate the development of a comprehensive prevention strategy in Oman.

INTRODUCTION

Oman is a country at the south east corner of the Arabian Peninsula. It faces the northern east part of the Arabian Gulf, bordering United Arab Emirates and Saudi Arabia in the west, Yemen in the south and is separated from Iran by a narrow sea strait from the north east. β-thalassemia is an autosomal recessive hemoglobin disorder and one of the most common genetic disease in man. Carriers of the disease are found at high frequencies in the tropic and sub-tropic regions including Oman. Untreated patients with β- thalassemia major are likely to die early in infancy and burdening treatment is needed to delay premature death. Prevention options such as premarital screening and genetic counselling of couples at risk are offered at the national level in Oman. However, this is limited to adapting partner choice as prenatal diagnosis (PD) is not offered in the country. As termination of pregnancy is not permitted in Oman, couples at risk that marry, may either accept the risk or decide to seek for PD or pre-implantation genetic diagnosis (PGD) abroad. As for PD and PGD molecular analysis is needed, knowledge of the local spectrum of mutations is essential in order to have a reliable diagnosis. Previous studies have reported a provisional spectrum of the β thalassemia alleles in Oman (1, 2). We report here the broadest spectrum of β-thalassemia mutations found in the country by examining the largest cohort studied thus far.

MATERIALS AND METHODS

Our cohort consisted of 446 unrelated individuals of native Omani nationality attending the Ministry of Health Hospitals in the country. The subjects were either affected with β-thalassemia major (TM), or HbS/β-thal or carriers of β-thalassemia. Age ranged from 1 to 48 years; 49.3% were females and 51.7% males. Patients'/parents' consent form was obtained prior to testing. The provisional diagnoses were confirmed by complete blood counts (CBC) performed on a Cell Dyn 4000 automated blood cell counter (Abbott Diagnostics, Santa Clara, CA, USA) and by biochemical analysis including high performance liquid chromatography (HPLC) (Variant, Bio-Rad Laboratories, Hercules, CA, USA). Finally DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) and the β-globin gene was amplified and sequenced on an ABI PRISM™ 3730 Genetic Analyzer (Applied Biosystems).

RESULTS

Among the 446 cases (892 chromosomes), 273 were beta-thalassemia carriers, 102 sickle/β-thalassemia compound heterozygotes and 71 had TM. In total 32 different β-thalassemia mutations were identified among 517 mutated alleles. Eleven of the β-thal alleles are reported for the first time in Oman. These include the IVS-I-128 T>G, Hb La Desirade Cd129 TGC>TGT, the promoter mutations -88 C>A and -101 C>T, IVS-II-849A>G, Cd8 (-AA), the 5nt poly A deletion 3'(+108-3'(+112), IVS-I-6 T>C, Hb Iraq Halabja Cd10 GCC>GTC, the Cd22 GAA>TAA and the PolyA 3'(+113)A>G mutation (Table 5.1).

As expected from our previous data, the IVS-I-5 G>C was still the most frequent mutation in each region except for Dhofar. The IVS-I-5 G>C allele frequency was 43.6%, while 30 different mutations accounted for the remaining 56.4%. The Cd44(-C) and -71 C>T occurred both at

Table 5.1. The β- molecular spectrum observed in the 7 studied regions in Oman. Determinants observed for the first time among Omani are highlighted.

Mutation Allele	HGVS	Musandam	Batinah	Muscat	Dhahira	Dakhiliya	Sharqiya	Dhofar	Total
IVS-I-5 G>C	HBB:c.92+5G>C	30	42	120	5	14	15		226
Cd44 (-C)	HBB:c.135delC	1	10	12	2	1	15		41
5′ (-71) C>T	HBB:c.-121C>T			34	2	5			41
Cd121 GAA>CAA	HBB:c.364G>C	5	4	13	2	3			27
IVS-I-128 T>G	HBB:c.93-3T>G	2	1	14			1		18
Cd129 TGC>TGT	HBB:c.389C>T	1		12	2				15
Cd26 GAG>AAG	HBB:c.79G>A	1	4	6		1	2		14
Cd5 (-CT)	HBB:c.17_18delCT	4	4	3		2			13
IVS-I-3′ (-25bp del)	HBB:c.93-21_96del		6	7					13
Cd58 CCT>CAT	HBB:c.176C>A			6	5		2		13
IVS-II-1 G>A	HBB:c.315+1G>A	2	6	3		1			12
Cd39 CAG>TAG	HBB:c.118C>T		8	1		1	2		12
Cd29 GGC>GGT/Cd58 CCT>CGT	HBB:c.90C>T/HBB:c.176C>G							12	12
IVS-I-1 G>A	HBB:c.92+1G>A	4	5	1					10
5′ (-101) C>T	HBB:c.-151C>T			6	1				7
5′ (-88) C>A	HBB:c.-138C>A	3		3					6
Cd15 TGG>TAG	HBB:c.47G>A			3			2		5
Cd121 GAA>AAA	HBB:c.364G>A		1	4					5
Cd30 AGG>ACG	HBB:c.92G>C	3		1					4
Cd(8/9) +G	HBB:c.27_28insG	1		2					3
IVS-II-849 A>G	HBB:c.316-2A>G			3					3
Cd6 GAG>AAG	HBB:c.19G>A			3					3
Cd8 (-AA)	HBB:c.25_26delAA	1	1	1					3
3′(+108) - 3′(+112) 5nt del	HBB:c.+108_+112delAATAA			2					2
IVS-I-6 T>C	HBB:c.92+6T>C	1		1					2
Cd10 GCC>GTC	HBB:c.32C>T			1		1			2
Cd22 GAA>TAA	HBB:c.67G>T			1					1
Cd63/37 (- T)	HBB:c.112delT				1				1
3′(+113) A>G	HBB:c.+113A>G		1						1
Cd30 AGG>AAG	HBB:c.92G>A			1					1
IVS-I-110 G>A	HBB:c.93-21G>A						1		1
Total (no. of indep. alleles)		59	93	264	20	29	40	12	517

8% while HbD Cd121 G>C was found at 5%. The other mutations were IVS-I-128 T>G and Hb La Desirade C>T, occurring at 3.5% and 2.9% respectively. HbE Cd26 G>A was found at 2.7% while Cd5(-CT), IVS-I-3'(-25bp del) and Hb-Sheffield Cd 58 C>A were observed both at 2.5%. The 21 other mutations accounted for the remaining 18.6%.

In Muscat, 27 β-alleles were found. The distributions of other β-thal alleles are shown in (Table 5.1). The origin of each mutation is described in Table 2 along with frequencies observed in the 3 countries/regions neighbouring Oman; Southern Iran (Hormozgan), United Arab Emirates and Eastern Saudi Arabia. Yemen was not included as no molecular data on beta-thalassemia were available as yet.

DISCUSSION

Although previous reports did examine the molecular spectrum of β-globin gene mutations in Oman, they were somehow limited in sample size and ethnic composition (1, 2). In addition, our samples have been randomly collected from 7 different regions, expanding by three regions from our pervious study (1). Therefore the present survey gives a better picture regarding the β-thalassemia spectrum and the distribution in a country of multi-ethnic tribes.

When comparing with neighbouring countries, the spectrum of β-thalassemia reported in our native Omani cohort (32 mutations, n=446) is higher than that reported in Eastern Saudi Arabia (14 mutations, n=196) (3) and UAE (25 mutations, n=412) (4). The most common mutation reported in the Eastern Province of Saudi Arabia was the Cd39 C>T followed by the IVS-II-1(G>A), IVS-I-5(G>C), IVS-I-25 bp deletion, and IVS-I-6 (T>C) which is also found in United Arab Emirates and Oman with varying allele frequencies (3). Among UAE nationals, the most frequent mutation found was the IVS-I-5(G>C) followed by the IVS-I-3' 25 bp del allele (4). In the Yemenis (n=10) living in Saudi Arabia, only the IVS-I-110(G>A) and IVS- II-1(G>A) mutations were identified (5). Data from Yemen are only preliminary. Although overall 52 different β-thalassemia mutations have been reported from different parts of Iran (6), the most predominant mutations found by the Iranian studies were IVS-II-I (G>A) in the north and IVS-I-5 (G>C) in the south (7, 8). The Iranian region nearest to Oman is Hormozgan. The β-thalassemia molecular spectrum of Hormozgan (9) describing 19 different mutations in 155 β-thal cases was used in our comparative study (Table 5.2).

The genetic heterogeneity of the native population shows the presence of Mediterranean, Asian Indian, Kurdish, Iranian and Turkish mutations that reflects the historical background of Oman. The Asian-Indian substitution at IVS-I-5(G>C), is the most common mutation in the UAE, Hormozgan and Oman but it is the third frequent in Eastern Saudi Arabia. Unlike our previous study where the IVS-I-5 G>C mutation was reported to constitute about 73 % of all the mutations in the Omani population studied (1), the present results revealed that IVS-I-5(G>C) constitutes about 43% of the mutations while the remaining 57% of the population carried a heterogeneous number of different mutant alleles (Table 5.1). Certain mutations occur in low frequencies and are tribe specific, indicating a founder effect. Alternatively some common mutations may vary in frequency between tribes as a consequence of bottle-necks which may wipe out certain mutations originally present in the founder population on one hand and increase the frequency of other mutations on the other hand. In the neighborhood of Muscat, immigration and trade

Table 5.2. The β-mutation frequency observed in Oman in comparison to its neighbouring countries; Hormozgan (Iran) (13), Eastern Saudi Arabia (5) and UAE (12). The most common mutation in each country is depicted in bold. 12 mutations described in Omani but not in other nationalities are highlighted in gray. β-determinants observed in other countries but not observed in Oman were not included in the table.

		Oman	Hormozgan	East KSA	UAE
Mutation Allele	**Origin**	**n=446**	**n=155 (9)**	**n=196 (3)**	**n=412 (4)**
IVS-I-5 G>C	Asian Indian	**43.7**	**69**	13.3	**44.5**
Cd44 (-C)	Kurdish	7.9	2.5	0.5	0.7
5′ (-71) C>T	Omani	7.9			
Cd121 GAA>CAA	Indian/Pakistani	5.2			2.2
IVS-I-128 T>G	Punjapi	3.5			
Cd129 TGC>TGT	Black	2.9			
Cd26 GAG>AAG	Asian Indian	2.7			0.1
Cd5 (-CT)	Mediterranean	2.5	2	3.1	2.1
IVS-I-3′ (-25bp del)	Asian Indian	2.5	1	13	8.6
Cd58 CCT>CAT	British/Omani	2.5			
IVS-II-1 G>A	East Mediterranean	2.3	9.6	22.2	2.8
Cd39 CAG>TAG	West Mediterranean	2.3	2.4	**25**	2.2
Cd29 GGC>GGT/Cd58 CCT>CGT	Omani	2.3			
IVS-I-1 G>A	Mediterranean	1.8		3.8	
5′ (-101) C>T	Turkish	1.4			0.2
5′ (-88) C>A	Kurdish	1.2	0.34		1.1
Cd15 TGG>TAG	Asian Indian	1	0.34		0.9
Cd121 GAA>AAA	Arabian/African	1			
Cd30 AGG>ACG	African	0.8	0.7		2.1
Cd(8/9) +G	Asian Indian	0.6	0.34	1.5	3
IVS-II-849 A>G	Black	0.6			
Cd6 GAG>AAG	Black	0.6			
Cd8 (-AA)	Turkish	0.6	3.4	2.1	2.2
3′(+108) - 3′(+112) 5nt del	Arabian	0.4			
IVS-I-6 T>C	West Mediterranean	0.4	0.34	7.1	1.5
Cd10 GCC>GTC	Iraqi	0.4			
Cd22 GAA>TAA	Reunion Island	0.2			
Cd36/37 (- T)	Kurdish/Iranian	0.2	0.7	0.5	0.1
3′(+113) A>G	Kurdish	0.2	2		0.4
Cd30 AGG>AAG	Bulgarian	0.2			
IVS-I-110 G>A	East Mediterranean	0.2		2.6	

may have had a considerable contribution to the variety of Asian and African beta-thal alleles as demonstrated in Table 5.2. The broad range of mutations identified in our study suggests a large effect of gene flow attributable to historical migration patterns.

Accurate characterization of the mutation at the DNA level is necessary during premarital counselling. Therefore it is essential to describe the molecular basis of the mild β^+ thalassemia alleles such as -71 C>T, IVS-I-128 T>G and Hb La Desirade Cd129 C>T since these mutations may cause problems when risk assessment is done during premarital genetic counselling. This is because these cases when accompanied with HbS, show an almost equal ratio of Hb A and Hb S (50:50) resulting in an asymptomatic phenotype as observed in previously studied cases in Omani $\beta^S/\beta^{-71C>T}$ patients (10). Moreover, variants such as Hb Sheffield Cd 58 C>A which co-elutes in the same Hb A_2 window as HbE Cd 26 G>A on high performance liquid chromatography (11) can lead to a wrong interpretation and consequently mis-diagnosis. Differentiating between mild conditions such as HbD Cd121 G>C homozygotes and compound heterozygotes (HbD/β-thalassemia) is important if the other partner is a carrier of β-thalassemia trait as there is a 25% risk of having a child affected with a severe β-thalassemia major (12). Hemoglobin variants should always be genotyped to differentiate between homozygotes and compound heterozygotes with beta-thalassemia for effective genetic counselling.

The only available option for prevention of severe hemoglobinopathies in Oman is through early diagnosis and genetic counselling. Thus molecular investigations and genotype / phenotype correlation are essential to reveal the exact β-thalassemia mutations and to make a phenotype prediction when possible. Our molecular studies show that the β-thalassemia mutations present in Oman vary in severity ranging from very mild β^+ form to severe cases, taking all studies up to date into account, at least 35 determinants are present in the country (1,2,10,11).

In conclusion, each country and region has its own prevalence and spectrum of β-thalassemia defects with a handful of common mutations and several less frequent or rare ones. Therefore, extensive knowledge on the heterogeneity of β-thalassemia mutations is needed to offer genetic counselling as a service to each population and in the highly consanguineous Omani population in particular. Finally, novel mutations will continue to be identified as genetic analysis of β-thalassemia is performed at a national level.

ACKNOWLEDGEMENTS

The authors declare to have conducted this study according to local ethical regulations and to have no conflict of interest on the presented matters.

REFERENCES

1. Hassan SM, Hamza N, Jaffer Al-Lawatiya F, Jaffer Mohammed A, Harteveld CL, Rajab A and Giordano PC. Extended molecular spectrum of beta- and alpha-thalassemia in Oman. Hemoglobin 2010; 34(2): 127-34.
2. Daar S, Hussain HM, Merghoub T and Krishnamoorthy R. Spectrum of β-thalassemia mutations in Oman. Ann N Y Acad Sci 1998; 850: 404-406.
3. Al Sultan A, Phanasgaonkar S, Suliman A, Al Baqushi M, Nasrullah Z and Al Ali A. Spectrum of β-thalassemia mutations in the Eastern province of Saudi Arabia. Hemoglobin 2011; 35(2): 125-134.
4. Baysal E. Molecular basis of beta-thalassemia in the United Arab Emirates. Hemoglobin 2011; 35(5-6): 581-588.
5. Al Hazmi MA, Warsy AS and Al Swailem AR. The frequency of 14 beta-thalassemia mutations in the Arab population. Hemoglobin 1995; 19(6): 353-360.
6. Strauss BS. Genetic counseling for thalassemia in the Islamic Republic of Iran. Perspectives in Biology and Medicine 2009; 52(3): 364–376.
7. Najmabadi H, Karimi-Nejad R, Sahebjam S, Pourfarzad F, Teimourian S, Sahebjam F, Amirizadeh N and Karimi-Nejad MH. The beta-thalassemia mutation spectrum in the Iranian population. Hemoglobin 2001; 25(3): 285-296.
8. Miri-Moghaddam E, Zadeh-Vakili A, Rouhani Z, Naderi M, Eshghi P and Feizabad K. Molecular basis and prenatal diagnosis of β-thlassemia among Balouch population in Iran. Prenatal Diagnosis 2011; 31(8): 788-791.
9. Yavarian M, Harteveld CL, Batelaan D, Bernini LF and Giordano PC. Molecular spectrum of beta-thalassemia in the Iranian Province of Hormozgan. Hemoglobin. 2001; 25(1): 35-43.
10. Al Zadjali S, Wali Y, Al Lawatiya F, Gravell D, Al Kindi S, Al Falahi K, Krishnamoorthy R and Daar S. The β-globin promoter -71 C>T mutation is a β+ thalassemic allele. European Journal of Haematology 2011; 87(5): 457-460.
11. Al Zadjali S, Daar S, Al Kindi S, Gravell D, Al Haddabi H, Berbar T and Krishnamoorthy R. Hb Sheffield [β58(E2)ProàHis] in Oman: Potential pitfall in genetic counselling. Hemoglobin 2011; 35(2): 111-116.
12. Belhoul KM, Bakir ML and Abdulrahman M. Misdiagnosis of Hb D-Punjab/β-thalassemia is a potential pitfall in hemoglobinopathy screening programs: a case report. Hemoglobin 2013; 37(2): 119-123.

CHAPTER 6

HB LANSING AND A NEW β PROMOTER TRANSVERSION (- 52 G>T): AN ATTEMPT TO DEFINE THE PHENOTYPE OF TWO MUTATIONS FOUND IN THE OMANI POPULATION

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Hemoglobin. 2015;39(2):111- 4

ABSTRACT

We report two examples showing how problematic it can be to define the phenotype of new or rare globin genes mutations. We describe two mutations observed for the first time in Omani: The first has been found in the consanguineous parents of a deceased newborn with hepatomegaly, cardiomegaly and severe hemolytic anemia, putative homozygous for the rare Hb-Lansing (α2 cd87 CAC>CAG). The second is a novel β-globin gene promoter mutation (-52 G>T) observed in four independent patients. Two with borderline/elevated HbA_2, α-thalassemia and hypochromic red cell indices and two with HbS heterozygosis, alpha thalassemia and with HbA / HbS ratios possibly indicating a very mild β^+ thalassemia mutation.

INTRODUCTION

Stable and well expressed α globin chain variants have usually no clinical consequences. Those unstable or thalassemic, although nearly asymptomatic in the carriers, may present with a more severe phenotype in the homozygous state (1) or in association with α-thalassemia mutations, while those with abnormal oxygen affinity may lead to polycythemia, cyanosis, tissue hypoxia or respiratory distress (2).

Homozygosis for rare hemoglobin (Hb) variants is rarely observed and generally in cases of consanguinity while combinations with thalassemia or with common variants can be found more frequently in endemic areas. We present the case of a newborn with hepatomegaly, cardiomegaly and severe hemolytic anemia who died shortly after birth. Although examination of the propositus at the molecular level was not possible, due to the presence of a rare Hb variant in both consanguineous parents, we presume that this could be the first described case of homozygosis for Hb Lansing.

Screening couples for premarital prevention of Hemoglobinopathies is done in Oman by routine hematology and high-performance liquid chromatography (HPLC). The common Hb variants will then be putatively recognized while an elevated HbA_2 will diagnose the β-thalassemia carriers. Borderline HbA_2 measurements can, if disregarded, lead to the misdiagnosis of normal HbA_2 β-thalassemia traits and further investigation at the molecular level might be necessary to avoid mistakes and to check for unexpected mutations. In this report, we describe the characterization of a novel β^+ promoter determinant associated with borderline HbA_2 found in four independent cases and we report how the expression of the mutated allele has been assumed.

MATERIALS AND METHODS

The putative Hb Lansing homozygosity [α87(F8)His→Gln; CAC>CAG (*HBA2*: c.264C4G)]

After the death of a newborn, blood was collected in EDTA from the consanguineous parents. A complete blood count (CBC) was performed and was analyzed by high performance liquid chromatography using the VARIANT II_ (Bio-Rad Laboratories, Hercules, CA, USA), as previously described (3). Genomic DNA was extracted from whole blood, using the Qiagen kit as per the manufacturer instructions. Alpha-globin genotype was established by GAP-PCR for the seven most common alpha thalassemia deletions (4). The α_2- and α_1-globin genes were sequenced using an ABI Prism 3730 DNA sequencer (Applied Biosystems, Perkin Elmer Corporation, Foster City, CA, USA) as previously described (5). Due to blood transfusion directly after birth, no HPLC was performed on the new born, neither was material made available for further DNA studies. This was the first child born to the consanguineous couple.

The putative β+-thalassemia mutation [-52 (G>T)]

Blood was collected in EDTA during premarital screening from four independent Omani individuals. Hematological data were obtained from a reference Hematology laboratory. Due to borderline HbA_2 values in two cases and the presence of HbS (HBB: c.20A>T) in the other two cases, molecular investigation was undertaken using the same methodology described above.

RESULTS

Case one

The infant was born after 37 weeks of gestation. The parents (first cousins) originated from southern Oman. Covered with a thick meconium, the newborn, presented in respiratory distress with low oxygen saturation (SpO_2 < 50%). In spite of resuscitation attempts the newborn died shortly after birth. The clinical description was pulmonary hypertension, hypoxia, hepato- and cardiomegaly, multi-organ dysfunction and severe anemia. Due to urgent transfusion no blood was collected and no material was made available for further investigations.

Parent's analysis revealed normal hematology (Table 6.1) and unclear HPLC patterns with low HbA_2 levels (data not shown). DNA analysis revealed none of the common alpha thalassemia deletions but sequencing showed heterozygosis for Hb-Lansing [α2 (F8) His>Gln Cd87 CAC>CAG (HBA2:c.264C>G)] in both healthy parents.

Table 6.1. Hematological and molecular data of the parents heterozygous for Hb-Lansing (SpO_2 was not measured in the parents).

	Mother	Father
HbA%	84.9	84.3
HbA_2%	2.1	2.3
HbF%	0.4	0.6
α- genotype	$\alpha\alpha^T/\alpha\alpha$	$\alpha\alpha^T/\alpha\alpha$
Hb (g/dl)	14.3	15.9
RBC	4.9	5.4
MCV (fl)	87.1	87.5
MCH (pg)	29	29.3

Case two

All four cases, carriers or non-carriers of HbS, presented with low-normal Hb levels and microcytic hypochromic parameter which is not unusual in Oman due to the high frequency of α-thalassemia. The lowest Hb and MCV were measured in a male homozygous for the $-\alpha^{3.7}$ alpha thalassemia deletion with nevertheless a borderline HbA_2 of 3.6%. The second non-carrier of HbS was a female heterozygous $-\alpha^{3.7}$ with a border line MCV and a slightly elevated HbA_2 level of 3.9%.

The two HbS carriers, both females and carriers of the $-\alpha^{3.7}$ deletions, presented with an HbS expression higher than expected for their genotype combination. Their HbA_2 level was decisively elevated (4.1 and 5%) but unreliable because of the overlapping HbS1c.

DNA analysis of the *HBB* gene in the two independent non HbS carriers showed a normal sequence except for the heterozygous state for a G→T transversion at position −52 relative to the Cap site (Figure 6.1). In the other 2 subjects, carriers of HbS, DNA sequencing revealed the same -52 (G>T) transversion and confirmed the HbS mutations. Data are summarized in Table 6.2.

Table 6.2. The hematological and molecular profiles of 4 independent subjects all carriers of the -52 mutation.

β-genotype	age/ gender	HbA %	HbA_2 %	HbS %	HbF %	Hb (gm/dl)	MCV (fl)	MCH (pg)	α-thal genotype
β^{-52}/β^{A}	4y/M	84.1	**3.6**	_	1.9	10.8	58.8	18.6	-α/-α
β^{-52}/β^{A}	27y/F	86.6	**3.9**	_	0.6	12.4	82.6	24.5	-α/αα
β^{-52}/β^{S}	28y/F	45.5	**4.1**	**44.1**	2	ND	ND	ND	-α/αα
β^{-52}/β^{S}	20y/F	55.7	**5**	**38.6**	0.7	12.6	69.5	22.2	-α/αα

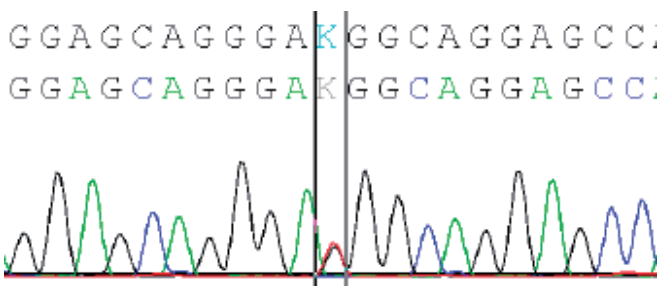

Figure 6.1. DNA sequence electropherogram of the β-globin promoter region showing heterozygosity for the -52 (G>T) nucleotide transversion.

DISCUSSION

Case one

This is the first report of a presumed Hb Lansing homozygosis. In spite of the lack of a molecular confirmation and of the fact that the condition of the newborn could be caused by other unknown congenital conditions (of which we have no knowledge or evidence), we presumed that the phenotype we have observed could be caused by homozygosis for Hb Lansing. If this is indeed the case and if the variant is relatively frequent in Oman, homozygosis could account for other cases of unexplained neonatal mortality in the country.

Hb Lansing, firstly reported by Sarikonda et al. as unstable but visible in carriers (6), was not measurable or visible in the mother and father of our propositus using routine HPLC (Figure 6.1).

In the original description Hb Lansing was observed in a 24-year-old asymptomatic Hispanic man (6) with SpO_2 of 88% and normal parameters (Hb =13.7 g/dl; HCT = 40.1%; reticulocyte count = 1.6%). These authors reported an abnormal fraction of 10-12% eluting "in front of HbA" on HPLC. Such an expression does not fit with our results and with the severe condition we have seen in our presumed homozygous and could be the honest report of a methemoglobin fraction which is regularly seen in front of HbA when running conserved samples on HPLC. More compatible with our observations seems to be the expression reported by Ishitsuka et al. in a 52-year-old Asian woman heterozygous for Hb-Lansing with a

low SpO_2 (83–86 %) with the following parameters: Hb = 13.3 g/dl; HCT = 40.2 %; MCV = 87.0 fl and reticulocyte count = 70,000/lL (7). These authors show in their Fig. 1b the electrophoretic separation of Hb Lansing in which a band of approximately 1% is visible on position F in a further normal separation.

The proximal histidine α87 residue (F8) anchoring the heme is critical for the molecule structure. Substitution of this residue is bound to cause instability and loss of function (8). Four other mutations have been reported at codon 87 resulting in other hemoglobin variants. The unstable Hemoglobin Iwata variant (His > Arg) was first identified in a healthy Japanese carrier with slight reticulocytosis (9). Hemoglobin M-Iwate (His > Tyr), a methemoglobin which decreases oxygen affinity and leads to cyanosis (10). Hemoglobin Auckland (His > Asp) which causes molecular instability and heme loss leading to mild hemolytic anemia (8). Finally, Hemoglobin Grifton (His > Pro) leading to microcytosis without clinical phenotype in the carrier (6).

In our case, Hb Lansing was not disturbing the hematological parameters of the consanguineous parents. However, since no other explanations have been found for the clinical symptoms of the newborn we could assume that the infant could have inherited 2 copies of the Hb Lansing mutation and that this genotype could be accounted for the severe intrauterine condition. If this is indeed the case, diagnose before conception and intrauterine transfusion could have kept the fetus in reasonably good conditions until birth. The question is how severe would have been the postnatal condition and our conjecture would be a severe transfusion dependent HbH disease like phenotype, similar to homozygosis for Hb Constant Spring (11). Mutations affecting the α_2 gene are generally more severe as they normally account for approximately 2/3 of α-globin chain synthesis (12). Low SpO_2 in healthy patients could be due to hemoglobin variants and care should be taken when low SpO_2 levels are detected (13).

Case two

Routine diagnosis of beta-thalassemia trait is based on microcytic parameters and elevated levels of HbA_2 measured using dedicated high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) devices. Although very precise, these machines are subject to some inevitable variability and cutoff values should never be taken for granted (14). While values above 4% are in general fairly diagnostic, "grey values" between 3.5 and 4% are not and need to be investigated further. Mutations in the promoter region of the beta globin, the evolutionary conserved sequences responsible for transcription regulation, have been reported to be associated with relatively mild forms of β-thalassemia (15, 16). These motifs includes the CACCC boxes at nt –105 to –101 and –90 to –86 from the cap site (17), the CCAAT box at –76 to –72, and the TATA box at –30 to –26.

A common mutation on the promoter region with HbA_2 values around 3.5% is the C>T transition at -101 (18). Another silent promoter mutation (-71 C>T) was recently reported in Omani (19) while another promoter region known as direct repeat element (DRE) has been suggested to play a role in β-globin transcriptional regulation and found to be an important regulatory element required for maximum transcription levels from the β-globin promoter in erythroid cells in mouse (20). The novel -52 G>T trasversion described in this paper lies within the conserved DRE and other mutations in *HBB* DRE surrounding associated with β-thalassemic

have been described by Li DZ et al (−50 G→A) (21) and Irenge et al. (−42 C→G) (22). To the best of our knowledge the present case is the third report of a mutation in the DRE region. The question rises is this mutation a silent polymorphism or a mild β+ thalassemia determinant?

Due to the presence of alpha thalassemia very common in Oman (23), this question cannot be easily answered looking at the hematological phenotype of the plain carriers with borderline HbA_2. Conversely, looking at the two cases compound heterozygous -52 and HbS we can observe the specific expression of the two alleles. Then, in the presence of $-\alpha^{3.7}$ heterozygosis the HbS expression should be around 30-35% while it is measured between 38 and 44% in our cases. The last measurement in particular could indicate that the -52 mutation is not on the HbS allele and that at least a 10% reduction in the expression of the -52 would probably cause a potential >50% HbS expression in absence of α-thalassemia. Based only on these calculations, the clinical impact of the -52 mutation seems to be very mild. On the other hand one needs to see the results of a genotype combination with a β° thalassemia mutation to exclude for 100% the risk of a (mild) thalassemia intermedia. Moreover, it is important to mention that Hb S-Oman (*HBB*: c.[20A>T;364G>A]) is a more severe sickling double mutation on the same β chain, which clinically manifests as a moderate hemolytic anemia in carriers with only 20.0% Hb S, and that the impact of the -52 mutation in combination with Hb S-Oman could be more significant than a plain -52/Hb S compound heterozygosity (24).

Take home message

Although rarely observed in homozygous state, rare variants may come together in the progeny of consanguineous partners and one can expect to see rare variants in homozygous form or in combination with common variants or β and α thalassemia more often in Oman than elsewhere. If Hb Lansing, would appear to be relatively frequent in Oman, and if indeed, the presumed severe phenotype associated with homozygosis is correct, the condition could account for other cases of unexplained neonatal mortality in the country.

Knowledge on the variant's clinical implications and predicting the underlying pathology is important for genetic counselling but not always uncomplicated and molecular analysis for couples presumed at risk becomes essential if unstable variants cannot be detected at the hematological level. As carriers of Hb Lansing are asymptomatic and practically undetectable on HPLC, one should consider adding SpO_2 to the premarital screening protocol in isolated communities with consanguineous traditions where Hb Lansing is known to occur.

Likewise, one can expect mild β^{+} thalassemia alleles with normal or slightly elevated HbA_2 levels such as -52 G>T to combine with severe β°-thalassemia mutations possibly causing intermediate conditions.

REFERENCES

1. Wajcman H, Traeger-Synodinos J, Papassotiriou I, Giordano PC, Harteveld CL, Baudin-Creuza V and Old J. Unstable and thalassemic α chain haemoglobin variants: a cause of HbH disease and thalassemia intermedia. Hemoglobin. 2008;32(4):327-349.
2. Abdulmalik O, Safo MK, Lerner NB, et al. Characterization of haemoglobin bassett (alpha94Asp-->Ala), a variant with very low oxygen affinity. Am J Hematol. 2004;77:268-276.
3. van Delft P, Lenters E, Bakker-Verweij M, de Korte M, Baylan U, Harteveld CL and Giordano PC. Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations. Int J Lab Hematol. 2009; 31, 484–495.
4. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of α-thalassaemia deletions and α-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol. 2000;108:295–299.
5. Harteveld CL, Yavarian M, Zorai A, Quakkelaar ED, van Delft P, Giordano PC. Molecular spectrum of alpha-thalassemia in the Iranian population of Hormozgan: three novel point mutation defects. Am J Hematol. 2003;74:99–103.
6. Sarikonda KV, Ribeiro RS, Herrick JL, Hoyer JD. Hemoglobin lansing: a novel hemoglobin variant causing falsely decreased oxygen saturation by pulse oximetry. Am J Hematol. 2009;84:541.
7. Ishitsuka K, Uchino J, Kato J, Ikuta M, Watanabe K, Matsunaga A and Tamura K. First reported case of hemoglobin lansing in Asia detected by false low oxygen saturation on pulse oximetry. Int J Hematol. 2012; 95:731–732.
8. Brennan SO, Matthews JR. Hb Auckland [alpha 87(F8) His—>Asn]: A new mutation of the proximal histidine identified by electrospray mass spectrometry. Hemoglobin 1997;21:393–403.
9. Ohba Y, Miyaji T, Hattori Y, et al. Unstable hemoglobins in Japan. Hemoglobin. 1980;4:307–312.
10. Mayne EE, Elder GE, Lappin TR, Ferguson LA. Hb M Iwate [alpha(2)87His—Tyr beta 2]: De novo mutation in an Irish family. Hemoglobin 1986; 10:205–208.
11. Schrier SL, Bunyaratvej A, Khuhapinant A, Fucharoen S, Aljurf M, Snyder LM, Keifer CR, Ma L, Mohandas N. The unusual pathobiology of Hemoglobin Constant Spring red blood cells. Blood. 1997; 89(5):1762–1769.
12. Orkin, SH and Goff SC. The Duplicated Human a-Globin Genes: Their Relative Expression as Measured by RNA Analysis. Cell. 1981;24(2):345–351.
13. Verhovsek M, Henderson MP, Cox G, Luo HY, Steinberg MH and Chui DH. Unexpectedly low pulse oximetry measurements associated with variant hemoglobins: a systematic review. Am J Hematol. 2011;85:882–5.
14. Giordano PC. Editorial: measurement of HbA_2. Int J Lab Hematol. 2012; 34(4): 335.
15. Li Q, Fang X, Olave I et al. Transcriptional potential of the γ-globin gene is dependent on the CACCC box in a developmental stage-specific manner. Nucleic Acids Res 2006; 34:3909–3916.
16. Marini MG, Asunis I, Porcu L, Salgo MG, Loi MG, Brucchietti A, et al. The distal b-globin CACCC box is required for maximal stimulation of the b-globin gene by EKLF. Br J Haematol 2004;127:114-117.
17. Gordon CT, Fox VJ, Najdovska S, et al. C/ EBPdelta and C/ EBPgamma bind the CCAAT-box in the human betaglobin promoter and modulate the activity of the CACCbox binding protein, EKLF. Biochim Biophys Acta 2005;1729:74–80.
18. Gonzalez-Redondo JM, Stoming TA, Kutlar A, Kutlar F, Lanclos KD, Howard EF, et al. A C>T substitution at nt -101 in a conserved DNA sequence of the promoter region of the b globin gene is associated with 'silent' b-thalassemia. Blood 1989;73:1705-11.
19. Shoaib Al Zadjali, Yasser Wali, Fatma Al Lawatiya, David Gravell, Salam AlKindi, Kareema Al Falahi, Rajagopal Krishnamoorthy and Shahina Daar. The b-globin promoter -71 C>T mutation is a b+ thalassemic allele. European Journal of Haematology. 2011: 87; 457–460.
20. Stuve LL and Myers RM. A directly repeated sequence in the beta-globin promoter regulates transcription in murine erythroleukemia cells. Mol Cell Biol; 1990;10:972–981.
21. Li DZ, Liao C, Xie XM, Zhou JY. A novel mutation of -50 (G-->A) in the direct repeat element of the beta-globin gene identified in a patient with severe beta-thalassemia. Ann Hematol. 2009;88(11):1149-1150.
22. Irenge LM, Heusterspreute M, Philippe M, Derclaye I, Robert A, Gala JL. Validation of a recombinant DNA construct (micro LCR and full-length beta-globin gene) for quantification of human beta-globin expression: application to mutations in the promoter, intronic, and 5'- and 3'-untranslated regions of the human beta-globin gene. Clin Chem. 2002; 48:1787–1791.
23. Hassan SM, Hamza N, Jaffer Al-Lawatiya F, Jaffer Mohammed A, Harteveld CL, Rajab A,Giordano PC. Extended molecular spectrum of beta- and alpha-thalassemia in Oman. Hemoglobin. 2010;34(2):127-34.

CHAPTER 7

MOLECULAR SPECTRUM OF α-GLOBIN GENES DEFECTS IN OMANI

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Hemoglobin, 2014;38(6):422-6

ABSTRACT

We describe the molecular characterization of α-globin gene defects in a cohort of 634 Omani patients. A total of 21 different α-gene mutations were found in 484 subjects. Overall, we identified 3 different large deletions, 3 small deletions, 11 point mutations (2 in the poly A tail of α_2 and 9 alpha-chain variants), 3 $\alpha\alpha\alpha^{anti\ 3.7}$ triplication, a 21nt duplication in the α_1 gene and 2 novel presumed polymorphisms in the alpha 3.7kbp hybrid gene namely; -5 C>T and + 46 C>A. Out of these defects, 15 have not been previously reported in the Omani population. This large heterogeneity of α-thalassemia observed in the Omani population could be expected in neighbouring Arab countries. The high frequency of α-thalassemia, solely or in association with β-globin gene defects, emphasize the necessity of adding α-thalassemia testing to pre-marital programs for accurate genetic counselling.

INTRODUCTION

Alpha-thalassemia, one of the commonest autosomal recessive diseases in man, results from the absence of expression of one or more of the four α-globin genes and can result in phenotypes ranging from asymptomatic to severe or lethal hemolytic anemia (1). The majority of the mutations causing α-thalassemia are deletions involving one or both α-genes on chromosome 16, leading to α^+ or α^0 defects respectively, being the so called rightward ($-\alpha^{3.7}$kb) deletion the most common worldwide (1). However, a growing number of non-deletion defects have been identified, being the α_2 polyadenylation signal mutation (HBA2:c.*94A>G: AATAAA>AATAAG) and the α_2 IVS-I 5-bp deletion (HBA2:c.95+2_95+6delTGAGG) the most common. These two mutations have already been reported in a study conducted in Oman, aimed to investigate the spectrum of α- thal mutations in HbH patients and in newborns showing the Hb-Bart's fraction (2). However, no studies have been conducted on Omanis to determine the prevalence of α-thal mutations in β-hemoglobinopathy patients as well as in individuals with microcytic hypochromic red cell indices (low MCV and low MCH). The present work gives a more extended picture of the prevalence and spectrum of α-gene mutations in Omanis. Characterization of co-existing α-globin gene defects is essential in prevention programs as well as in tailoring treatment strategy.

MATERIALS AND METHODS

Between 2010 and 2013, a cohort of 634 individuals was selected attending Ministry of Health Hospitals in Oman for hemoglobinopathy screening. Consent form was obtained from all patients through their direct clinicians. The median age was 22 years and the gender ratio was 58% males and 42% females. The cohort was subdivided into 7 categories (Table 7.1) based on their clinical and/or haematological profiles. Group 1 consisted of 487 patients with either β-thalassemia major, β-thalassemia minor, sickle cell trait or sickle cell disease. Group 2 counted 93 subjects with normal HPLC readings and hypochromic microcytic red cell indices. Group 3 consisted of 32 patients with normal HPLC and normal red cell indices. Group 4 consisted of 11 patients with un-known peaks on HPLC and normal β-globin gene sequence. Group 5 of 5 patients with un-explained anemia. Group 6 of 4 HbH patients and finally group 7 of only two patients with low HbA_2, normal δ-globin gene sequence and normal Ferritin values.

Blood samples were collected in EDTA, had complete blood count (CBC) and were analyzed on High Performance Liquid Chromatography (HPLC) using the Variant II (Bio-Rad Laboratories, USA) as previously described (3). Genomic DNA was extracted from whole blood, using the Qiagen kit as per the manufacturer instructions. Alpha-globin genotype of the 634 cases was established by gap-PCR for the most common 7 alpha thalassemia deletion defects (4). Groups 4-7 underwent direct sequencing of the α_2- and α_1-globin genes using an ABI Prism 3730 DNA sequencer (Applied Biosystems, Perkin Elmer Corporation, Foster City, CA, USA) as previously described (5). Groups 1 and 2 were sequenced following specific criteria (Figure 7.1).

Table 7.1. Subdivision of the 634 subjects based on their clinical and hematological data.

Group	no. of patients	category
1	487	β - thalassemia minor/major, SCT or SCD
2	93	Normal HPLC but with mycrocitic, hypochromic (MCV < 78fl; MCG < 26pg)
3	32	Normal HPLC, normal red cell indices (MCV > 78fl; MCH >26pg)
4	11	Un-known fraction on HPLC (Hb X) with a normal β - gene sequence
5	5	Un-explained anaemia, normal HPLC ,mycrocitic, hypochromic, not responding to iron
6	4	HbH peak on HPLC, anemic, mycrocitic, hypochromic
7	2	Low HbA_2 , normal δ - gene sequence, normal iron but microcitic, hypochromic
Total	634	

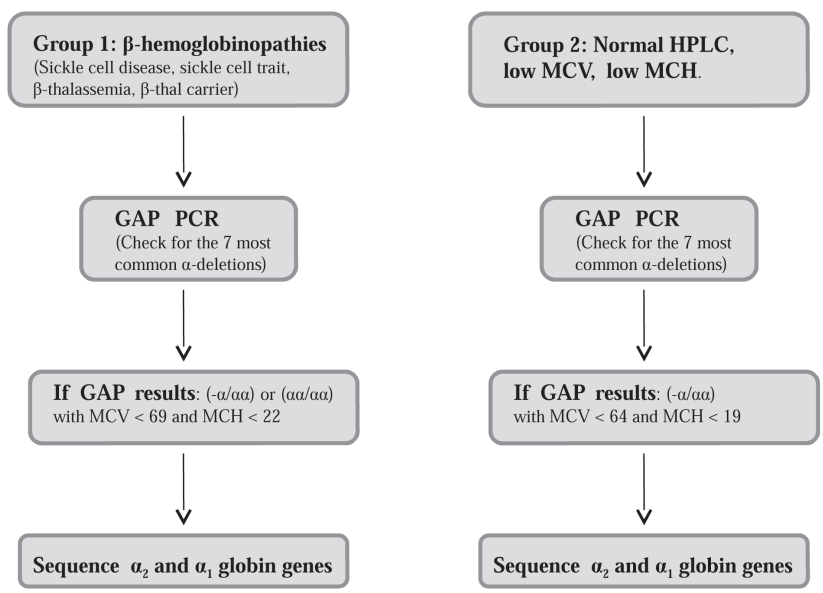

Figure 7.1. Criteria used to sequence α_2- and α_1-globin genes in groups 1 and 2.

RESULTS

In addition to the expected high prevalence of deletions such as ($-\alpha^{3.7}$) and ($-\alpha^{4.2}$) (6), we found in group 1 an un-expected number of α-mutations interacting with β-defects. A total of 10 defective alleles were found in this group. Three have been previously reported in the Omani population; the common (-5nt) deletion at the splice donor site of the α_2-globin gene, the α_2 cd19 (-G) (HBA2:c.56delG) mutation and the α_2 polyadenylation signal mutation +94 A>G. The other 7, are reported for the first time in the Omani population; the $\alpha\alpha\alpha^{anti\ 3.7}$ triplication,

α_1 21nt duplication (+GACCCGGTCAACTTCAAGG TG) between IVS-II-3 and IVS-II-4 in a beta-thalassemia carrier patient (MCV=75.3fl, MCH=18.9pg), the α_2 polyadenylation signal mutation (HBA2:c.*92A>G), Hb Le Lamentine Cd20 CAC>CAA (HBA2:c.63C>A) in HbS carrier with HbX=25.8%, Hb Tatras Cd7 AAG>AAC (HBA2:c.24G>C) in another HbS carrier with HbX=12.7%, Hb Al Ain Cd18 GGC>GAC (HBA2:c.56G>A) in a third HbS carrier with HbX=1.5%, and finally a novel mutation (+46 C>A) in the poly A tail of the alpha2/alpha1 ($-\alpha^{3.7}$) hybrid gene. The haematology of the latter mutation is summarized later in Table 4 as the same allele was also found among patients from group 5.

Moreover, among group 1, 78 individuals were carriers of HbS (SCT). Thirty were homozygous (-α/-α) (38.5%), 24 heterozygous (-α/αα) (30.8%), 3 heterozygous for α-chain variants mentioned above (3.8%) and 21 normal (αα/αα) (26.9%). HbS levels in SCT were inversely proportional to the associated number of alpha-thalassemia genes. Average levels were as follows; -α/-α (HbS % »26.6), -α/αα (HbS% » 32.5), αα/αα (HbS % »37.7) (Table 7.2).

Table 7.2. The effects of α-thalassemia on the average HbS% in Omani SCT individuals.

	Hb S (%)	MCV (fl)	MCH (pg)
(-α/-α) n=30	26.6	62.8	20.9
(-α/αα) n=24	32.5	72	23.9
(αα/αα) n=21	37.7	79	26.4

In group 2, besides high percentages of ($-\alpha^{3.7}$) and ($-\alpha^{4.2}$) deletions, we found a 12 year old female who presented with a normal clinical picture with low MCV = 64.2fl and MCH = 19pg, and was found to be heterozygous for the $-\alpha^{3.7}$ deletion and hemizygous for the α_2 Hb Dagestan variant Cd 60 AAG>GAG (HBA2:c.181A>G). This electrophoretically I-like variant is not associated with any clinical symptoms or haemoglobin instability in the carrier state.

In group 3, although patients had a normal haematological data, 43.75% and 6.25% of the patients were carrier of the ($-\alpha^{3.7}$) and ($-\alpha^{4.2}$) deletions respectively.

In group 4, we report the identification of 6 alpha-variant alleles that are reported for the first time in Omani. Two of these variants have been also observed in two HbS carriers from group 1. The variants detected were; the α_2 Hb Fontainebleau Cd21 GCT>CCT (HBA2:c.64G>C), α_2 Hb-Tatras Cd7 AAG>AAC, α_2 Hb Al-Ain Abu Dhabi Cd18 GGC>GAC, α_1 Hb Evanston Cd14 TGG>CGG (HBA1:c.43T>C), α_2 Hb Constant Spring Cd142 TAA>CAA (HBA2:c.427T>C) and the α_2 Hb J-Paris-I Cd12 GCC>GAC (HBA2:c.38C>A) (Table 7.3).

We found a new single nucleotide substitution in the 3' PolyA tail (+46 C>A) on the (α-3.7) alpha2 alpha1 hybrid gene in homozygous state ($-\alpha^{3.7\ (+46\ C>A)}$/ $-\alpha^{3.7\ (+46\ C>A)}$) in 4 anaemic patients from group 5. This allele was also observed in heterozygous state in 3 patients from group 1 ($-\alpha^{3.7\ (+46\ C>A)}$/αα). Among group 5, two patients had borderline ferritin values and were treated with iron without improvement (Table 7.4) suggesting that 3'(+46 C>A) might be a new candidate

Table 7.3. Hematological data of group 4 with Hb alpha chain variants.

Genotype	HGVS nomenclature	patient no.	HbX %	MCV (fl)	MCH (pg)
1. $\alpha_2^{Cd21\ GCT>CCT}\alpha_1/\alpha\alpha$	HBA2:c.64G>C	1.1	8.7	60.2	18.7
		1.2	5.8	82.2	26.5
		1.3	11.6	69.7	20.7
2. $-\alpha^{3.7}/\ \alpha_2^{Cd21\ GCT>CCT}\ \alpha_1$	HBA2:c.64G>C	2.1	9.8	57.2	16.5
		2.2	12.2	75	22.1
3. $\alpha_2^{Cd7\ AAG>AAC}\alpha_1/\alpha\alpha$	HBA2:c.24G>C	3.1	19.7	70.6	24.2
		3.2	12.7	73.3	24.2
4. $-\alpha^{3.7}/\ \alpha_2^{Cd18\ GGC>GAC}\ \alpha_1$	HBA2:c.56G>A	4.1	22.2	78.4	24.2
		4.2	30.5	78.5	25.3
5. $\alpha_2^{Cd18\ GGC>GAC}\ \alpha_1/\alpha\alpha$	HBA2:c.56G>A	5.1	12.9	88.9	28.7
6. $\alpha_2\alpha_1^{Cd14\ TGG>CGG}/\ \alpha\alpha$	HBA1:c.43T>C	6.1	0.6	77.9	23.6
7. $\alpha_2^{Cd142\ TAA>CAA}\ \alpha_1/\ \alpha_2^{Cd142\ TAA>CAA}\ \alpha_1$	HBA2:c.427T>C	7.1	4.6	78.3	22.4
8. $\alpha_2^{Cd12\ GCC>GAC}\ \alpha_1/\ \alpha_2^{-5nt}\ \alpha_1$	HBA2:c.38C>A/ HBA2:c.95+2_95+6delTGAGG	8.1	36.7	82.9	25.2

Table 7.4. Hematological findings of the 3' PolyA tail (+46 C>A) cases found in the alpha2/alpha1 3.7 hybrid gene. (HR) = Heterozygous.

alpha - genotype	patient no.	beta - genotype	Age	HbA_2 %	Hb (g/dl)	MCV (fl)	MCH (pg)	Ferritin	Phenotype
1. $-\alpha^{3.7\ (+46C>A)}/\ \alpha\alpha$	1.1	HBB:c.92+5G >C (HR)	2y	4.3	8.8	55.1	16.5	normal	mild anemia
	1.2	HBB:c.92+5G >C (HR)	1y	5.9	9.2	56.5	18.2	normal	mild anemia
	1.3	HBB:c.93-21_ 96del (HR)	7y	6.1	10.4	57.6	18.1	not done	mild anemia
2. $-\alpha^{3.7\ (+46\ C>A)}/\ -\alpha^{3.7\ (+46\ C>A)}$	2.1	normal	1y	3.5	8	76.1	23.4	not done	anemia and jaundice
	2.2	normal	15y	1.8	9.2	57.6	18.7	normal	anemia
	2.3	normal	39y	2.6	12.5	65.9	21	border line	anaemia not responding to Fe therapy
	2.4	normal	5y	2.3	7.6	47.6	13.6	border line	anaemia not responding to Fe therapy
3. $-\alpha^{3.7\ (-5\ C>T)}/\ -\alpha^{3.7}$	3.1	normal	56y	1.3	8.2	64.5	20.9	normal	hypochromic microcytic anemia

mutation. In addition, among group 5, we identified the -$\alpha^{3.7}$/ $\alpha_2\alpha_1^{Cd38/39(-ACC)}$ genotype in a patient with a mild anaemia (Hb=6.8, MCV=66.8 and MCH=22.5). We believe that this is the first study reporting the α_1 Hb Taybe Cd38/39 –ACC (HBA1:c.118_120delACC) deletion allele in Omani.

In group 6, 4 HbH genotypes were identified, three genotypes in a combination that has been previously reported in the Omani and one with the $-\alpha^{4.2}$/ $\alpha_2^{+94\ A>G}\alpha_1$ genotype which is observed for the first time in a patient from northern Oman (Khasab).

Among group 7, we characterized two single nucleotide substitutions, one in the 5' promoter (-5 C>T) in the alpha2/alpha1 hybrid gene which was found in heterozygous form in a patient with homozygous α-3.7kbp deletion (-$\alpha^{3.7\ (-5\ C>T)}$/-$\alpha^{3.7}$). The patient presented with hypochromic microcytic anaemia (MCV=64.6fl, MCH=20.9pg), low HbA_2 (1.3%) and normal ferritin value. The second, in a patient homozygous for Hb Icaria variant Cd142 TAA>AAA (HBA2:c.427T>A) with low HbA_2 (1.6%) and microcytic hypochromic (MCV=76.3fl, MCH=24pg). The first mutation could be a novel thalassemia defect down regulating the hybrid gene and the second is described for the first time in Oman.

Mutations found in all groups are summarized in Table 7.5.

Table 7.5. Summary of the spectrum of alpha-globin genotypes found in our cohort subdivided into 7 groups. (alleles marked with * might be new candidates).

	Genotype Mutation	HGVS allele nomenclature	no. of patients	genotype prevalence among the sub-group (%)
Group 1 (n = 487)	-$\alpha^{3.7}$/-$\alpha^{3.7}$		171	35.1
β-hemoglobinopathies	-$\alpha^{3.7}$/-αα		145	29.8
	-$\alpha^{3.7}$/-$\alpha^{4.2}$		10	2.1
	-$\alpha^{4.2}$/-αα		10	2.1
	ααα$^{anti\ 3.7}$ /αα		3	0.6
	-$\alpha^{3.7(+46\ C>A)*}$/ αα		3	0.6
	$\alpha_2^{-5nt}\alpha_1$/ αα	HBA2:c.95+2_95 +6delTGAGG	2	0.4
	$\alpha_2^{+92\ A>G}\alpha_1/\alpha_2^{+92\ A>G}\alpha_1$	HBA2:c.*92A>G	1	0.2
	$\alpha_2^{+92\ A>G}\alpha_1$/αα	HBA2:c.*92A>G	1	0.2
	$\alpha_2^{Cd19(-G)}\alpha_1$/αα	HBA2:c.56delG	1	0.2
	$\alpha_2^{Cd19(-G)}\alpha_1/\alpha_2^{Cd19(-G)}\alpha_1$	HBA2:c.56delG	1	0.2
	$\alpha_2^{+94\ A>G}\alpha_1$/ αα	HBA2:c.*94A>G	1	0.2
	$\alpha_2\alpha_1^{dup\ 21nt}$ / αα	HBA1:c.283_300+3dup	1	0.2
	-$\alpha^{3.7}/\alpha_2^{Cd20\ CAC>CAA}\alpha_1$	HBA2:c.63C>A	1	0.2
	$\alpha_2^{Cd7\ AAG>AAC}\alpha_1$/αα	HBA2:c.24G>C	1	0.2
	$\alpha_2^{Cd18\ GGC>GAC}\alpha_1$/αα	HBA2:c.56G>A	1	0.2
	-$\alpha^{4.2}/\alpha_2^{+92\ A>G}\alpha_1$	HBA2:c.*92A>G	1	0.2
	αα/αα		133	27.3

Table 7.5. Summary of the spectrum of alpha-globin genotypes found in our cohort subdivided into 7 groups. (alleles marked with * might be new candidates). (*Continued*)

	Genotype Mutation	HGVS allele nomenclature	no. of patients	genotype prevalence among the sub-group (%)
Group 2 (n = 93)	$-\alpha^{3.7}/-\alpha^{3.7}$		72	77.4
Normal HPLC,	$-\alpha^{3.7}/-\alpha\alpha$		17	18.3
hypochromic, mycrocytic	$-\alpha^{3.7}/-\alpha^{4.2}$		3	3.2
red cell indices	$-\alpha^{3.7}/\alpha_2^{Cd60\ AAG>GAG}\alpha_1$	HBA2:c.181A>G	1	1.1
Group 3 (n = 32)	$-\alpha^{3.7}/-\alpha\alpha$		14	43.75
Normal HPLC,	$-\alpha^{4.2}/-\alpha\alpha$		2	6.25
Normal red cell indices	$\alpha\alpha/\alpha\alpha$		16	50
Group 4 (n = 11)	$\alpha_2^{Cd21\ GCT>CCT}\alpha_1/\alpha\alpha$	HBA2:c.64G>C	3	27.2
Un-known peak on HPLC	$-\alpha^{3.7}/\alpha_2^{Cd21\ GCT>CCT}\alpha_1$	HBA2:c.64G>C	2	18.2
Normal β-globin gene seq	$-\alpha^{3.7}/\alpha_2^{Cd18\ GGC>GAC}\alpha_1$	HBA2:c.56G>A	2	18.2
	$\alpha_2^{Cd7\ AAG>AAC}\alpha_1/\alpha\alpha$	HBA2:c.24G>C	1	9.1
	$\alpha_2\alpha_1^{Cd14\ TGG>CGG}/\alpha\alpha$	HBA1:c.43T>C	1	9.1
	$\alpha_2^{Cd142\ TAA>CAA}\alpha_1/\alpha_2^{Cd142\ TAA>CAA}\alpha_1$	HBA2:c.427T>C	1	9.1
	$\alpha_2^{Cd12\ GCC>GAC}\alpha_1/\alpha_2^{-5nt}\alpha_1$	HBA2:c.38C>A/ HBA2:c.95 +2_95+6delTGAGG	1	9.1
Group 5 (n = 5)	$-\alpha^{3.7(+46\ C>A)*}/-\alpha^{3.7\ (+46\ C>A)*}$		4	80
un-explained anaemia	$-\alpha^{3.7}/\alpha_2\alpha_1^{Cd38/39\ (-ACC)}$	HBA1:c.118_120delACC	1	20
Group 6 (n = 4)	$\alpha_2^{+94\ A>G}\alpha_1/\alpha_2^{+94\ A>G}\alpha_1$	HBA2:c.*94A>G	1	25
HbH disease	$-\alpha^{4.2}/\alpha_2^{+94\ A>G}\alpha_1$	HBA2:c.*94A>G	1	25
	$-\alpha^{3.7}/\alpha_2^{+94\ A>G}\alpha_1$	HBA2:c.*94A>G	1	25
	$-\alpha^{3.7}/-\alpha^{MedI}$		1	25
Group 7 (n =2)	$-\alpha^{3.7\ (-5\ C>T)*}/-\alpha^{3.7}$		1	50
low HbA_2, normal δ and Fe	$\alpha_2^{Cd142\ TAA>AAA}\alpha_1/\alpha_2^{Cd142\ TAA>AAA}\alpha_1$	HBA2:c.427T>A	1	50

DISCUSSION

The broad range of α-thalassemia defects identified in the current study, demonstrate that the heterogenic pattern prevalent in Oman is similar to that reported in Saudi (7). This can be accounted to gene-flow within the nomadic populations of the Arab peninsula and to the past trade with other countries as well as to the past Portuguese domination in Oman. A total of 21 different α-gene defects have been reported in this study. Six defects have been previously identified in the Omani population (2,6,8). These include the most common $\alpha^{3.7}$, $\alpha^{4.2}$ and $--^{MEDI}$ large deletions, but also the less common Mediterranean donor site IVS-I (-5nt) deletion, the

polyadenylation signal site (+94 A>G) point mutation of the α2 globin gene affecting the RNA transcription termination (9) and the point deletion Cd19 (-G) which was first reported in the Iranian population, resulting in premature termination of the α-globin chain (5).

The majority of our cohort carried the large $\alpha^{3.7}$ deletion either in homozygous or heterozygous states followed by $\alpha^{4.2}$ and a minority consisted of α-small deletions or point mutations including Hb-variants. In one patient, a 21nt duplication was identified which most probably arose as a consequence of homologous crossing over between the two alpha-1 globin genes. Although the original splice donor site remains intact but having two sites might result in the mutant allele using most times the original one and sometimes the other one resulted from the duplication which is located in the intron creating an instable mRNA due to nonsense mediated decay. This might also explain why having this mutation appears very mild.

Among group 1, an important remark is made with respect to genetic counselling on one hand and the alpha spectrum on the other hand is when the group of beta-thalassemia carriers or sickle cell trait couples are counselled for a beta-thalassemia major or sickle cell disease in the offspring overlooking an important alpha-thalassemia mutation that can result in a severe HbH disease in the offspring in combination with an alpha zero-thalassemia carrier that can go 'unnoticed' by only looking at the beta-thalassemia parameters and not to the alpha-thalassemia mutation spectrum.

Alkindi et al studied 32 HbH Omani patients and found that the most common α-globin genotype was $\alpha_2^{+94\ A>G}\ \alpha_1/\ \alpha_2^{+94\ A>G}\ \alpha_1$ followed by $-\alpha^{3.7}/--^{MED}$ (2). We found the same two genotypes in group 6 (patients with HbH) along with another two genotype combinations; $-\alpha^{4.2}/\ \alpha_2^{+94\ A>G}\alpha_1$ and $-\alpha^{3.7}/\ \alpha_2^{+94\ A>G}\alpha_1$. The latter has been previously reported in an Omani family study (8).

It is not clear to which extent the homozygous mutation $-\alpha^{3.7(+46\ C>A)}/\ -\alpha^{3.7(+46\ C>A)}$ found in four patients in group 5, with haemolytic anaemia, might affect the phenotype by reducing the expression of the $-\alpha^{3.7}$ gene. If the same $-\alpha^{3.7(+46\ C>A)}$ allele reported by Alkindi's in 4 HbH patients with $-\alpha^{3.7(+46\ C>A)}/-\alpha^{3.7}$ genotype (2) would suggest a total expression failure, then our homozygous case should have been lethal hydrops foetalis, which was not the case. Hb H disease is the only intermediate form of α-thalassemia compatible with postnatal life and the clinical severity of the disease is associated with the type of mutation (1). The typical genotype of Hb H disease results from the loss of three functional genes due to large deletions. Less frequently, more severe combinations of α° deletion defects with point mutations such as Hb Constant Spring or other similar defects, while less severe HbH forms may arise from combinations with poly A signal mutations (10). However, a point mutation on the $-\alpha^{3.7}$ hybrid might down regulate or disrupt the allele expression causing severe forms. The risk of developing anaemia in case of homozygous + 46 C>A on the alpha2/alpha1 hybrid gene highlights the importance of screening for α-thalassemia defects by molecular analysis rather than by routine blood count alone which is based on detecting hypochromic microcytic red cell indices.

Although not always clear, we have provided additional evidence that besides cases with delta thalassemia or consistent iron deficiency, a reduction in HbA_2 level can be associated with α-thalassemia as shown for the novel -5C>T allele in the $-\alpha^{3.7(-5C>T)}/\ -\alpha^{3.7}$ genotype. Therefore, in patients originating from areas where haemoglobinopathies are common, it is necessary to perform molecular tests to clearly differentiate between iron deficiency and alpha- or delta-thalassemia for accurate diagnosis.

In conclusion, four α-thalassaemia categories were recognized in this cohort: the silent carrier, the alpha-thalassemia trait, the α -variant and the anaemic form of Hb H disease. Moreover, we have shown that 40% of the α-alleles were normal while 56% of the alpha thalassemia alleles studied were large α-thalassemia deletion and 4% non-large deletions/ point α-thalassemia mutations. The consistent occurrence of point mutations could represent a risk factor for severe haemolytic anaemia in combination with large deletions (11). The + 46 C>A on the alpha2/alpha1 hybrid gene might be a candidate mutation since it is associated with anaemia, however larger number of genotype/phenotype correlation studies should be carried out to confirm our findings. Identifying the α-globin gene spectrum is not only clinically important but also fundamental for a better genetic counselling.

ACKNOWLEDGEMENTS

The authors declare to have conducted this study according to local ethical regulations and to have no conflicts of interest on the presented matters.

REFERENCES

1. Hartevel CL. And Higgs DR. α-thalassemia. Orphanet J Rare Dis 2010; 5: 13.
2. Alkindi SS, AlZadjali S, Daar S, Sindhuvi E, Wali Y, Pathare AV, Venugopal S, Lapoumeroulie C, Srivastava A and Krishnamoorthy R. A stepwise α-thalassemia screening strategy in high-prevalence areas. European Journal of Haematology 2013; 91(2): 164-169.
3. van Delft P, Lenters E, Bakker-Verweij M, de Korte M, Baylan U, Harteveld CL and Giordano PC. Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations. Int J Lab Hematol 2009; 31, 484–495.
4. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of α-thalassaemia deletions and α-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol 2000; 108: 295–299.
5. Harteveld CL, Yavarian M, Zorai A, Quakkelaar ED, van Delft P, Giordano PC. Molecular spectrum of alpha-thalassemia in the Iranian population of Hormozgan: three novel point mutation defects. Am J Hematol 2003; 74: 99–103.
6. Hassan SM, Hamza N, Jaffer Al-Lawatiya F, Jaffer Mohammed A, Harteveld CL, Rajab A,Giordano PC. Extended molecular spectrum of beta- and alpha-thalassemia in Oman. Hemoglobin 2010; 34(2): 127-34.
7. Akhtar MS, Qaw F, Borgio F, Albuali W, Suliman A, Nasserullah Z, Al-Jarrash S and Ali A. Spectrum of α-thalassemia mutations in transfusion dependent β-thalassemia patients from the eastern province of Saudi Arabia. Hemoglobin 2013; 37(1): 65-73.
8. Wali Y, Al Zadjali S, Elshinawy M, Beshlawi I, Fawaz N, Al Kindi S, Rawas A, Alsinani S, Daar S and Krishnamoorthy R, Severity ranking of non-deletional alpha thalassemic alleles: insights from an Omani family study. European Journal of Haematology 2011; 86: 507-511.
9. Thein SL, Wallace RB, Pressley L, Clegg JB, Weatherall DJ, Higgs DR. The polyadenylation site mutation in the alpha-globin gene cluster. Blood 1988; 71: 313-319.
10. Fei YJ, Oner R, Bozkurt G, Gu LH, Altay C, Gurgey A, Fattoum S, Baysal E and Huisman THJ. Hb H Disease Caused by a Homozygosity for the AATAAA-»-AATAAG Mutation in the Polyadenylation Site of the a2-Globin Gene. Hematological Observations. Acta Haematol 1992; 88: 82-85.
11. Traeger-Synodinos J, Kanavakis E, Tzetis M, Kattamis A, Kattamis C. Characterization of nondeletion α-thalassemia mutations in the Greek population. Am J Hematol 1993; 44: 162–167.

CHAPTER 8

KNOWN AND NEW δ GENE MUTATIONS AND OTHER FACTORS INFLUENCING HBA2 MEASUREMENT IN THE OMANI POPULATION

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Hemoglobin. 2014;38(4):299-302

ABSTRACT

Although delta thalassemia is not categorised as a severe disease, it is essential to know the molecular spectrum of the delta gene mutations frequently occurring in specific areas in particular if these areas are characterized by a high rate of beta thalassemia such as Oman. This is because co-inherited delta globin gene defects can interfere with the basic diagnosis of β-thalassemia carrier when this is based upon the measurement of the HbA_2 only. For that, we have investigated 33 patients with low HbA_2 levels collected from different hospitals in Oman. Some cases had a second HbA_2 fraction, while others had only significantly lower HbA_2 levels. Among these patients, 20 did carry a δ-globin gene mutation, the rest were carrier of alpha thalassemia defects or could be iron depleted or both. In total, eight different known mutations and 2 novel delta determinants were found. The characterization of the δ-gene mutation spectrum will improve carrier diagnostics and genetic counseling in the Omani population screened for beta thalassemia.

INTRODUCTION

After the age of two, postnatal haemoglobin A (HbA) is the major haemoglobin component of the red cells. Besides HbA and in normal conditions, about (2.5-3.5%) of the haemoglobin content will consist of haemoglobin A_2 (Hb A_2) while traces of HbF (<1%) will present in adult life (1). Mutations that occur in the δ-globin gene (HBD, MIM# 142000) can affect the structure or the expression of the delta globin chain as it is the case for all other globin genes. Structural defects, if stable, will produce a second and usually visible Hb A_2 fraction (2). If unstable, the mutation will behave as a thalassemic defect and be undetectable using basic methods such as high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Thus, DNA analysis will be required to differentiate between low Hb A_2 due to iron deficiency, alpha-thalassemia or delta gene defects (3). If a person is heterozygous for a δ-globin gene defect, an abnormal Hb A_2 and/or a reduction in the Hb A_2 level will be measured. It is important to identify the presence of delta gene defects, particularly during first level beta thalassemia diagnostics (screening) for the identification of couples at risk of getting a child with a severe disease. This is because a delta defect can mask the presence of beta thalassemia trait. The co-existence of a delta gene defect will decrease the HbA_2 level of the beta thalassemia carrier to a normal range, and microcytosis could be attributed to alpha thalassemia which is very frequent in many countries and particularly in Oman (6). This could compromise the basic diagnosis of beta thalassemia trait during genetic counseling. For that, it is essential to be aware of the existence of delta gene defects for diagnostic purposes. In this study, we present the occurrence of common, rare and new delta gene mutations in a cohort of independent Omani patients.

MATERIAL AND METHODS

Out of a total of approximately 3,400 individuals, we have selected 33 independent cases attending our clinics for haemoglobinopathy screening. All cases were of Omani ethnicity. The age average was 31 and the gender was 60% females and 40% males. Samples were selected upon giving a low value of Hb A_2 (<1.9%) and/or showing second Hb A_2 fractions. Measurements were done using High Performance Liquid Chromatography (HPLC) on the Variant II (Bio-Rad Laboratories, USA) as previously described (4). DNA was extracted from whole blood, using the Qiagen kit as per the manufacturer instructions. Polymerase Chain reaction was performed as previously reported (5). The PCR products were sequenced using an ABI Prism 3730 DNA sequencer (Applied Biosystems, Perkin Elmer Corporation, Foster City, CA, USA). Iron status was not performed in all samples as it is not a mandatory test in Oman. Beta and alpha gene defects were examined at the molecular level as previously described (6).

RESULTS

Out of the 33 cases selected, 20 were found to either carry a known and/or a novel delta-globin gene mutation revealing a frequency of at least 60% in the selected group and of at least 0.6% in the random population. Eight different known mutations were observed and two novel ones.

The 2 novel mutations:

Cd147 TGA>TTA

This new mutation (HBD: c.443 G>T) resides in the stop codon of the delta gene and was found in one patient with 1.8% Hb A_2 (Figure 8.1a). The mutation results in an elongation of the transcript with 15 extra amino acids before reaching the new stop codon (TAG).

Cd110-Cd111 (+GT)

Another new delta-thalassemia mutation (HBD c.333-334 insGT) was found in a patient with 1.5% Hb A_2. The mutation involves an insertion of 2 nucleotides (+GT) between condon 110 and codon 111 (Figure 8.1b). The outcome of this insertion is a frameshift with a new stop codon (TAG), 102 amino acids beyond the insertion site. All data are summarized in Table 8.1.

Table 8.1. Summary of the delta-globin gene mutations found in 20 cases. Mutations marked with * are novel.

Sample #	HbA_2	HbX	HBD mutation	HBD HUGO nomenclature	α-genotype	Other mutations
1	2.1	1.5	Cd16 GGC>CGC	c.49G>C	αα/αα	
2	1.3	1.2	Cd16 GGC>CGC	c.49G>C	−α/−α	
3	1.5	1.4	Cd16 GGC>CGC	c.49G>C	−α/αα	
4	1.7	1.1	Cd16 GGC>CGC	c.49G>C	αα/αα	
5	1.5	1.0	Cd16 GGC>CGC	c.49G>C	−α/−α	
6	1.3		Cd116 CGC>CAC	c.350G>A	−α/αα	
7	1.6		Cd116 CGC>CAC	c.350G>A	αα/αα	
8	1.4		Cd116 CGC>CAC	c.350G>A	−α/αα	
9	1.7		Cd27 GCC>TCC	c.82G>T	−α/−α	
10	1.6		Cd27 GCC>TCC	c.82G>T	−α/αα	
11	0.6		Cd27 GCC>TCC/ IVS-I-128 G>C	c.82G>T/c.93-1 G>C	−α/αα	
12	1.2		Cd136 GGT>GAT	c.410G>A	−α/−α	
13	1.3		Cd136 GGT>GAT	c.410G>A	−α/−α	
14	1.9		– 68 C>T	c.-118C>T	−α/αα	
15	5.1	1.7	– 68 C>T	c.-118C>T	−α/−α	HBB:c.20A>T/ c.92+5G>C
16	1.3		Cd4 ACT>ATT	c.14C>T	−α/αα	
17	1.6		IVS-I-128 G>C	c.93-1 G>C	−α/αα	
18	4.4		Cd100 CCT>TCT	c.301 C>T	−α/−α	HBB:c.+108_ +112delAATAA
19	1.8		Cd147 TGA>TTA*	c.443 G>T	−α/−α	
20	1.5		Cd110-Cd111 (+GT)*	c.333-334 insGT	−α/αα	

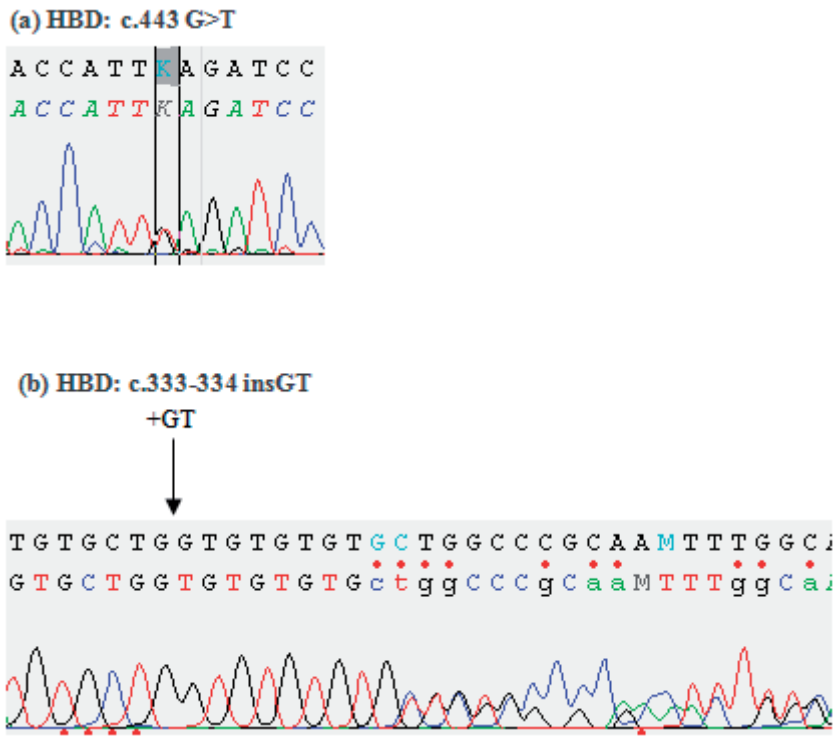

Figure 8.1. Sequence results of the 2 new delta-globin mutations found in this study.

Out of these 33 cases, 16 had the (-α/-α) genotype, 13 had the (-α/ αα) genotype and only 4 cases were normal for the alpha genes. In 13 cases, no association was found between low Hb A_2 levels and delta-globin mutations.

DISCUSSION

Delta-gene defect were found in 73.3% of the cases with low or abnormal Hb A_2 separation while in 13 out of 33 individuals, no association was found between the low Hb A_2 levels and mutations in the delta-globin gene. Iron deficiency and/or alpha thalassemia can cause a reduction in the normal Hb A_2 level while lower amount of total hemoglobin loaded on the HPLC column can also be accounted for artifacts (3).

Hb A2' or HbB2

Hb A_2' is stable and produces a second Hb A_2 peak and is mainly found in Africans (7). This mutation could have arrived to Oman by gene flow due to the past trading contact between Oman and Zanzibar. In one case, this delta variant was linked to codon 97 (HBD:c.294C>T) with a neutral change of the amino acids (His>His). This neutral polymorphism was previously described in Greek Cypriots (8).

Hb A2 – Coburg

The Hb A_2 Coburg peak cannot be detected on HPLC because it co-migrates in the tail of HbA (9). This variant has been described in Sicilian families in trans to a beta thalassemia allele, reducing the Hb A_2 level to normal (10). Due to the Arab domination in Sicily, the mutation could be of African origin.

Hb A2 – Yialousa

Hb A_2-Yialousa is the most frequent delta-globin mutation in the Mediterranean area, probably indicating a common South European origin (11). Being Hb A_2-Yialousa one of the common delta defects found in the Portuguese (12), the presence of the mutation in Omanies could be associated with the history of the Portuguese domination in Oman (1507 – 1650) (13).

Hb A2 – Babinga

HbA2-Babinga was primarily described in Babinga pygmies living in the Central African Republic and other African populations (14) and it could be compatible with the African ancestry of Omani tribes. A homology of this defect was also found in the beta globin gene (Hb Hope, β136 Gly>Asp) (15). We have found this mutation in 2 individuals from the northern part of the country. These patients were anemic, presenting with low Hb A_2 levels (1.2 and 1.3% respectively) and had a normal iron profile.

5'UTR (- 68 C>T)

The delta-thalassemia promoter defect (HBD c.-118C>T, -68 C>T), has no homology to the β-globin gene (5) but the CCAAT sequence residing in the β-globin gene promoter is considered to be a regulatory element, critical for the correct initiation and high level of transcription in the globin genes (16). Therefore, the δ (CCAA<u>C</u> to CCAA<u>T</u>) mutation can be considered responsible for the lower transcription level of the δ -globin gene.

Cd4 ACT>ATT

The nucleotide change C to T at the second position of codon 4 resulting in a Thr > Ile single amino acid substitution was first described in a Greek patient (8). The variant is unstable and behaves as a thalassemic defect with a low Hb A_2 value (1.3%) slightly lower than what was found in the Greek patient (1.4%) (8), possibly due to the coexisting heterozygous -$\alpha^{3.7}$ deletion in our patient.

(δ°): IVS-I-128 G>C

A 59-year-old male from Muscat, showed a very low Hb A_2 value (0.6%). Sequencing of the δ-globin gene revealed compound heterozygosis for two different mutations: The known A_2 Yialousa (HBD c.82G>T) and IVS-I-128 G>C (HBD: c.93-1 G>C). We believe that the IVS-I-128 mutation reduces or nearly abolish the efficiency of the 3' splicing site, leading to a deficiency in mRNA production. Compound heterozygosis for delta globin gene defects with very low Hb A_2 values observed in our patient have been reported in few cases. Amirian et al. reported a patient from Iran with two delta defects (HBD:c.92+5G>T and c.428C>A) with 0.6% Hb A_2 (17). We found the δ° IVS-I-128 mutation also solely in another patient with Hb A_2 value of 1.6%.

(δ⁺):Cd100 CCT>TCT

We have observed this recently reported delta variant (HBD c.301 C>T) with a Serine substituting a Proline in a single patient who was also a carriers of a beta thalassemia mutation (HBB:c.110_114del) This delta mutation was recently published by Colaco et al. as Hb A_2-Saurashtra (20) and was found in cis with (HBB:c.110_114del). This is the same beta-thalassemia mutation found in the present paper, indicating that the HBD:c.301C>T and HBB:c.110_114del mutations may also be in cis.

(δ°): Cd147 TGA>TTA

This novel δ-stop codon mutation (Cd147 TGA>TTA) results in an elongation of the transcript with 15 additional amino acids, stopping at the 16th codon (TAG). . The elongated chain is unsuitable for functional tetramer formation and is probably proteolysed.

(δ°): Cd110-Cd111 (+GT)

Finally, the last sample showed a novel insertion of two nucleotides between codon 110 and codon 111 in exon 3 of the delta globin gene. The frame shift results in an elongated sequence with a new stop codon (TAG) 102 amino acids further from the insertion site. This mutation could also be the result of a duplication event as the region is characterized by a nucleotide repeat of (GTGTGTGT).

CONCLUSIONS

We have shown that lower Hb A_2 levels are often associated with δ-globin gene defects that may compromise screening for β-thalassemia trait when the diagnosis is based on the Hb A_2 level solely. Moreover, low levels of Hb A_2 can also be due to iron deficiency and/or alpha thalassemia due to preferential binding of the erythroid elements (3). The latter was observed in the 13 samples that had a normal delta- gene sequence. Hb A_2 levels can be moderately lowered in patient with iron deficiency due to the preferential binding of the heme to the beta and alpha chains rather than to delta chains (18).

Our results show that δ gene mutations are present in Oman at a considerable frequency and that attention should be paid during haemoglobinpathy screening to not miss beta thalassemia carriers. Double Hb A_2 fractions must be summed up to calculate the real Hb A_2 level. Samples with low Hb A_2 and microcytosis should always be checked for iron depletion before checking the alpha genotype and the δ and β globin genes sequences. Eventually, loading a more concentrated sample on HPLC is advisable when an unstable δ-globin gene variant is suspected (19). It should also be noted that in δβ-thalassemia deletions, the level of Hb F is usually raised while the level of Hb A_2 will remain normal. Only in solely δ-thalassemia cases, the Hb F level will stay normal (17).

ACKNOWLEDGEMENTS

The authors declare to have conducted this study according to local ethical regulations and to have no conflicts of interest on the presented matters.

REFERENCES

1. Mosca A, Paleari R, Ivaldi G, Galanello R and Giordano PC. The role of haemoglobin A2 testing in the diagnosis of thalassaemias and related haemoglobinopathies. J Clin Pathol 2009;62:13–17.
2. Phylipsen M, Gallivan MVE., Arkesteijn SGJ, Harteveld CL, Giordano PC. Occurrence of common and rare δ-globin gene defects in two multiethnic populations: thirteen new mutations and the significance of d-globin gene defects in β-thalassemia diagnostics. Int. Jnl. Lab. Hem. 2010; 33(1), 85-91.
3. Giordano PC. The effect of iron deficiency anemia on the levels of hemoglobin subtypes: possible consequences for clinical diagnosis. Clin Lab Haematol 2003;25:203.
4. van Delft P, Lenters E, Bakker-Verweij M, de Korte M, Baylan U, Harteveld CL and Giordano PC. Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations. Int J Lab Hematol 2009; 31, 484–495.
5. Marelle J. Bouva, Cornelis L. Harteveld, Peter van Delft and Piero C. Giordano. Known and new delta globin gene mutations and their diagnostic significance. Haematologica 2006; 91:129-132.
6. Hassan SM, Hamza N, Al-Lawatiya F, Jaffer Mohammed A, Harteveld CL, Rajab A,Giordano PC. Extended molecular spectrum of beta- and alpha-thalassemia in Oman. Hemoglobin. 2010;34(2):127-34.
7. Jones RT and Brimhall B. Structural characterization of two d chain variants. J Biol Chem 1967:242:5141-5.
8. Trifillis P, Kyrri A, Kalogirou E, Kokkofitou A, Ioannou P, Schwartz E and Surrey S. Analysis of delta-globin gene mutations in Greek Cypriots. Blood 1993 82: 1647-1651.
9. Giambona A, Passarello C, Ruggeri G, Renda D, Teresi P, Anzà M, and Maggio A. Analysis of δ-globin gene alleles in the Sicilian population: identification of five new mutations. Haematologica 2006;91:1681-1684.
10. Sharma RS, Williams L, Wilson JB and Huisman TH. Hemoglobin-A2-Coburg or alpha2delta2116Arg leads to His (G18). Biochim Biophys Acta 1975;393(2):379-82.
11. Angioletti M, Lacerra G, Gaudiano C, Mastrolonardo G, Pagano L, Mastrullo L, Masciandaro S, Carestia C. Epidemiology of the delta globin allele in Southern Italy shows complex molecular, genetic, and phenotypic features. Hum Mutat 2002;20:358–67.
12. Morgado A, Picanc I, Gomes S, Miranda A, Coucel M, Seuanes F, Seixas MT, Roma L and Faustino P. Mutational spectrum of delta-globin gene in the Portuguese Population. European Journal of Haematology, 2007; 79:422–428.
13. Miles, Samuel Barrett; Robin Bidwell (1997). The Countries and Tribes of the Persian Gulf. Garnet & Ithaca Press.
14. Jong WW and Bernini LF. Haemoglobin Babinga (delta 136 glycine-aspartic acid): a new delta chain variant. Nature. 1968; 219(161), 1360-2.
15. Rahbar S, Nozari G, Asmerom Y, Martin PA, Yeh CH, Lee TD. Association of Hb Hope [beta 136(H14)Gly---Asp] and alpha-thalassemia-2 (3.7 Kb deletion) causing severe microcytic anemia. Hemoglobin. 1992;16(5):421-5.
16. Antoniou M and Grosveld F. beta-globin dominant control region interacts differently with distal and proximal promoter elements. Genes Dev. 1990 4: 1007-1013.
17. Amirian A, Jafarinejad M, Kordafshari AR, Mosayyebzadeh M, Karimipoor M and Zeinali S. Identification of a novel δ-globin gene mutation in an Iranian family Hemoglobin, 2010; 34(6):594–598.
18. Galanello R, Ruggeri R, Addis M, et al. Haemoglobin A2 in iron-deficient beta-thalassaemia heterozygotes. Haemoglobin 1981;5:613–8.
19. Chi-Chiu So, Amy Y.Y. Chan, Hong-Yuan Luo, Madeleine Verhovsek, David H.K. Chui, Siu-Cheung Ling, and Li-Chong Chan. Hb A2 Hong Kong – A novel δ-globin variant in a Chiense family masks the diagnosis of β-thalassemia trait. Hemoglobin. 2011; 35(2):162–165.
20. Colaco S, Trivedi, A Colah RB, Ghosh K and Nadkarni AH. Masking of a β-thalassemia determinant by a novel δ-globin gene defect [Hb A_2-Saurashtra or δ100(G2)Pro→Ser; *HBD*: c.301CT] in *Cis*. , Nov 7 2013, PMID 24200152.

CHAPTER 9

HAPLOTYPES, SUB-HAPLOTYPES AND GEOGRAPHICAL DISTRIBUTION IN OMANI PATIENTS WITH SICKLE CELL DISEASE

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Thalassemia Reports. 2015; 5(4739): 6-11

ABSTRACT

Introduction

Despite the fact that patients homozygous for the sickle cell disease (SCD) mutation have an identical genotype, the severity of the disease can be extremely variable. The HbS mutation has been described on five different haplotypes with different clinical expression. Identifying the genotypes, haplotypes and sub-haplotypes of the β gene cluster in Oman needs to be studied in more details to establish a correlation between the genotype/haplotype and phenotype diversity observed in SCD patients for prognostic purposes, accurate diagnosis and thus planning for the best tailored treatment.

Methods

We have investigated 125 HbS homozygotes from different parts of Oman and determined their haplotypes and sub-haplotypes and correlated this to the hematological and clinical expression.

Results

We have found 11 haplotype combinations differently distributed in the country. The Asian/Asian HbS haplotype was the most predominant (37.6%) and was associated with a milder disease. The Benin/Benin came second (20.0%) and was associated with a more severe condition. A new haplotype, in combination with Asian, which we called Asian/OmanI was the third most common (11.2%), CAR/CAR (10.4%) and CAR/OmanI were fourth (10.4%) and CAR/Asian fifth (6.4%). Other haplotype combinations were found at a lower frequency (4%). In patients with CAR/OmanI haplotype, 3 different sub-haplotypes were found. As expected, the correlation between haplotypes, sub-haplotypes and disease severity was mainly associated with HbF expression.

Conclusion

Our study on haplotype/phenotype correlation has shown which major haplotypes occur in the different regions of Oman. Furthermore, neither the haplotype or sub-haplotype nor the HbF alone appeared to be fully associable with the variable clinical phenotypes. External factors do occur and are associated with the expression of the disease.

INTRODUCTION

Sickle cell disease (SCD) is one of the most common autosomal recessive disorder in human and was first described by Herrick in 1910 (1). The disease is caused by a single nucleotide transversion at codon 6 GAG>GTG (HBB:c.20A>T) (NM_000518.4) of the beta globin gene resulting into the commonest haemoglobin variant worldwide (HbS) characterized by the single Glu®Val amino acid substitution at position β6 (2). Despite the fact that all patients homozygous for the HbS allele have an identical genotype, the severity of the disease can be extremely variable among affected subjects (3). The disease may manifest with full blow severity, with chronic and acute infarctions in organs and tissues causing excruciating pain episodes (crisis), brain infarctions, splenic infarction, massive hemolytic events and acute chest syndrome with risk of premature death. Other cases however, may present with milder symptoms and the variability is mainly associated with the haplotype, sub-haplotype, alpha thalassemia (4) and the presence of fetal hemoglobin (HbF) ($\alpha 2\gamma 2$) (8) which may be attributed to the coinheritance of Xmn-I polymorphism. This marker is important for early prevention or reduction of morbidity in SCD patients treated with hydroxyurea therapy (10) or to decide whether or not bone marrow transplantation should be considered (11).

The HbS mutation has been described on five distinct haplotypes based on the presence or absence of the 5 different restriction enzyme sites in the beta-globin gene cluster located on the 5' and two restriction enzyme sites on the 3' sides of the beta gene. These haplotypes are known as Benin, Bantu or Central African Republic (CAR), Senegal, Cameroon and Asian (5). The first four are African haplotypes, named after their origin and ethnic group (6) while the last was described in Central India and Saudi Arabia (7). It has been previously reported that the CAR haplotype is usually associated with a more severe disease when compared with the intermediate phenotype of the Benin haplotype and to the milder conditions associated with the Senegal and Asian haplotypes (9). Therefore, analysis of the polymorphic sites of the β genes cluster is of genetic, anthropologic and clinical interest, and it can also be used to predict the prognosis of the disease and to plan a tailored treatment.

The work reported here involves the investigation of a serial of polymorphic sites (SNP's) within the beta globin gene cluster to identify the HbS haplotype of Omani patients. For this, we have selected 125 Omani SCD patients with homozygous HbS conditions and compared their haplotype with haematological and clinical data. Moreover, to look if there are any sub-haplotypes within each known haplotype that might be associated with the clinical differences seen in patients with the same HbS haplotype, extra SNP's, in addition to the common ones have been studied. Looking for correlations with the clinical phenotypes, we have also charted the distribution of the beta gene haplotypes in different regions of the country and characterized the different haplotypes and sub-haplotypes using advanced molecular technologies

MATERIALS & METHODS

Subjects

We have collected, with signed consent of patients and families, EDTA blood samples from a cohort of 125 SCD patients, whether admitted or following up in one of the Ministry of Health Hospitals in Oman. Gender distribution was; 84 males (45%) and 103 females (55%). The age of the subjects was on average 36 years.

Strategy

Cation-exchange high performance liquid chromatography (HPLC) was performed on all samples on either D-10 (short and extended programs) device and/or Variant II (Bio-Rad Laboratories, Hercules, CA, USA) to measure the rate of HbF/HbS (12) in absence or before blood transfusion. DNA was extracted using the Qiagen kit according to the manufacturer instruction as previously described (13). 47 SNP's, covering 13 sites in the β-globin gene cluster that are known to be variable and informative were analysed (Figure 9.1). This was carried out by an asymmetric PCR (31) and analysed by melting curve analysis (MCA) (14). The common haplotyping of the β-globin gene cluster was determined according to the presence (+) or absence (-) of a composition of single nucleotide polymorphisms (SNP's) corresponding to the traditional five 5' known polymorphic restriction endonuclease sites (F1, F2 (SNP 2), F3 (SNP 2), F4 and F5) (31). The remaining 42 SNP's were used to look for sub-haplotypes to find out if there are nucleotide variations within similar haplotypes that might be associated with the phenotypical differences observed. F1 contains 1 SNP in the 5' region of the ε-gene. The preGframe in the 5' region of the Gγ-gene contains 5 SNPs that are linked and only occur in a limited number of combinations. The promoter regions of the γ-genes; promGγ and promAγ contain 10 SNPs each, among which are the non-deletional HPFH point mutations. Also among the Gγ promoter is the Xmn-I site which is known to cause continued expression of HbF during adult life in case of erythropoietic stress. F2, in intron 2 of the Gγ-gene, and F3, in intron 2 of the Aγ-gene both contain 4 SNPs. F4 contains 1 SNP in the pseudo β-gene. F5 contains 1 SNP in the 3' region of the pseudo β-gene. F6 contains 1 SNP in the 3' region of the δ-gene. The voBRsa1 fragment in the 5' region of the β-gene contains 5 SNPs. This fragment also contains a very polymorphic $(AT)_xT_y$ repeat located after SNP 1 that includes SNP 2. The β-frame in the β-gene contains 3 linked SNPs that only occur in a limited number of combinations. The naBHpa1 fragment in the 3' region of the β-gene contains 1 SNP. F7 in the 3' region of the β-gene contains 1 SNP.

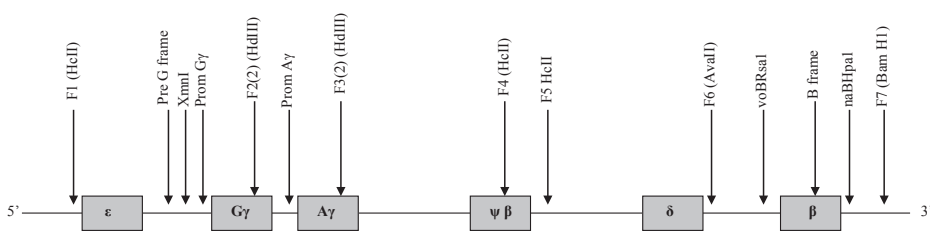

Figure 9.1. Schematic representation of the β-globin gene cluster. The arrows indicate the locations of the 13 different regions that contain the 47 SNPs, including the 5 sites screened for the traditional haplotypes. In addition, the XmnI site has also been indicated in the figure.

Genotyping procedures

Genotypes of PCR products were determined using the Light Scanner, (Idaho Technologies Inc. USA) after performing asymmetric PCR with LCGreen fluorescent DNA dye and unlabelled

oligonucleotide probes (31). The PCR was performed asymmetrically so that the strand complementary to the probe is produced in excess, allowing probe annealing at the SNP site. The primers were designed to yield a product not larger than 200bp as it is the optimal length for accurate scanning and genotyping with MCA. A fluorescent dye (LC Green plus) that emits light in the presence of double stranded DNA was added to the reaction and as the temperature increases, the fluorescence decreases as the dsDNA melts out; this produces a characteristic melting curve (15). Unlabelled oligonucleotide probes were designed and used to genotype targeted sequence variations. These probes increases specificity of the melting reaction as it decreases the size of the product that is melted. Therefore, the probe is designed to anneal to either the wild type or the mutant allele; the characteristic melting curves identify the genotype of each sample (15).

The shape of the PCR amplicon melting curve reveals the presence or absence of the SNP in comparison to the wild type sequence, allowing clear recognition and genotyping (14). The haplotype/sub-haplotype is then drawn from the obtained genotypes. In case of homozygosity for all markers, the haplotype can be defined. In the case of a single SNP difference, two distinct haplotypes can be defined. In case of two or more SNP differences, the most likely/frequent haplotype in the population is defined. SNP's that were doubtful were sequenced by Sanger sequencing for confirmation.

Phenotype Classification

In order to define disease severity, a number of well-defined clinical parameters were analyzed: hemoglobin level, frequency of transfusion which is based on episodes of acute hemolysis (e.g pulmonary hypertension, jaundice, gallstones, splenic crisis), number of annual hospitalization, frequency of painful crises, splenectomy (indicated by acute splenic sequestration and chronic hypersplenism), acute chest syndrome (ACS), body pain (e.g abdomen, chest, bones, joints, episode of dactylitis) and records of any major organ damage such as heart and liver (Table 9.1).

The severity of disease expression was then correlated with the haplotype and hematological parameter readings of HbF and HbS.

Table 9.1. Classification criteria for the 125 homozygous HbS/S patients to assess SCD severity into mild, intermediate and severe.

Clinical classification of SCD patients (n = 125)	Mild n = 46	Intermediate n = 31	Severe n = 48
Haemoglobin level	↑ 9.1	↓ 8.5	↓ 8
Transfusions/haemapheresis	Not required	Occasional	Frequent
Hospitalizations per year	1 - 2	2 - 4	> 5
Crisis frequency	↓ 3 per year	↑ 3 per year	↑ 6 per year
Splenectomy	Yes	Yes	Yes
Acute Chest Syndrome (ACS)	No	No	Yes
Body Pain	mild	moderate	intense
Severe organ damage	No	No	Yes

RESULTS

Determination of Genotypes and Haplotypes

A total of 125 selected patients with SCD that were found to be homozygous for HbS were studied for their β-gene cluster haplotype. The 250 chromosomes from the 125 patients with identical homozygous HbS/S genotype showed 11 different haplotype combinations that were defined by melting curve analysis. The homozygous Asian haplotype was the most predominant (37.6%). The second most prevalent was the homozygous Benin haplotype (20.0%). The remaining haplotypes were distributed as follow: compound heterozygous Asian/OmanI (11.2%), homozygous CAR (10.4%), compound heterozygous CAR/OmanI (10.4%), CAR/Asian (6.4%), homozygous OmanI (0.8%), compound heterozygous of Senegal/OmanI (0.8%), Benin/OmanII (0.8%), Benin/OmanIII (0.8%) and finally Asian/OmanIV (0.8%). Data are summarized in Table 9.2.

Clinical severity

The phenotypes of the patients were classified into mild, intermediate and severe based on the described criteria (Table 9.1). Data were obtained from patient's medical records anonymously provided by the doctor. Among the Asian/Asian haplotype, mostly were presented with a mild disease and none with a severe form. Conversely, Benin/Benin ranged from severe to intermediate while none presented with the mild form. Among the Asian/OmanI haplotype, the percentage was equal between mild and intermediate. The majority of homozygous CAR and compound heterozygous CAR/OmanI beta cluster had a severe clinical profile. Finally, the phenotype of Asian/CAR haplotype ranged from mild to intermediate and even severe cases. Data are summarized in Table 9.2.

Hematological data

The Hb F value correlates with the different haplotypes, giving a direct indication of the disease severity. The average hemoglobin values, HbF and HbS percentage of each haplotype are summarized in Table 9.3. The Asian haplotype, being associated with a mild phenotype, presented with the highest expression of HbF% and the lowest HbS% when compared to the other haplotypes. Patients with Asian/OmanI haplotype had a mild to intermediate phenotype. This can be attributed to the elevated expression of HbF% (Table 3). Conversely, the homozygous CAR and compound heterozygous CAR/OmanI haplotypes had the most severe clinical picture with the lowest Hb F values (Table 3).

Table 9.3. Summary of hematological data (average) for each haplotype

	Asian/Asian	Benin/Benin	Asian/OmanI	CAR/CAR	CAR/OmanI	CAR/Asian
Hb g/dl	9.5 ±1.3	9.3 ±1.7	10.3 ±1.9	7.5 ±1.1	8.7 ±1.2	9.1 ±1.4
HbF %	18.2% ±5.9	6% ±3.1	14.2% ±5.5	5.9% ±4.9	5% ±3.5	12.6% ±5.4
HbS %	72.3% ±6.9	80.3% ±5.7	75.2% ±7.6	80.7% ±7.7	78.5% ±9.1	78.3% ±7.9

Table 9.2. Summary of the genotypes from which the 11 haplotypes were determined in the Omani HbS/S homozygous patients using the five 5′ traditional sites. The Xmn-I genotype and percentages of mild, intermediate and severe cases within each haplotype is also indicated.

	HcII (5′ ε)	Xmn-I	Hd III (Gγ)	Hd III (Aγ)	Hc II (ψβ)	Hc II (3′ψβ)	Ava II (β)	Bam HI (3′β)				
Haplotype	F1 (A: +/C: -)	(C: -/T: +)	F2(2) (G: - /T: +)	F3(2) (G: - / T: +)	F4 (G: -/ A: +)	F5 (G:- / A: +)	F6 (G: -/C:+)	F7 (C,T:-/ A:+)	Total	Mild%	Intermediate%	Severe%
Asian/Asian	AA	TT	TT	GG	AA	AA	GG	C,T/C,T	47	78,7	21,3	0
Benin/Benin	CC	CC	GG	GG	GG	AA	GG	AA	25	0	36	64
Asian/Oman I	AC	TC	TT	GG	GA	AA	GG	A/C,T	14	50	50	0
CAR/CAR	CC	CC	TT	GG	GG	GG	GG	AA	13	0	7,7	92,3
CAR/Oman I	CC	CC	TT	GG	GG	GA	GG	AA	13	0	25	75
CAR/Asian	AC	TC	TT	GG	GA	GA	GG	A/C,T	8	12,5	37,5	50
Oman I/Oman I	CC	CC	TT	GG	GG	AA	GG	AA	1	0	0	100
Senegal/OmanI	CC	TC	TT	GG	GA	AA	GG	AA	1	0	0	100
Asian/Oman II	AA	TC	TG	GG	GA	GA	CG	C,T/C,T	1	0	0	100
Benin/Oman III	AC	CC	TG	GG	GG	GA	CG	AA	1	0	0	100
Benin/Oman IV	CC	CC	GG	GG	GG	GA	GG	AA	1	100	0	0

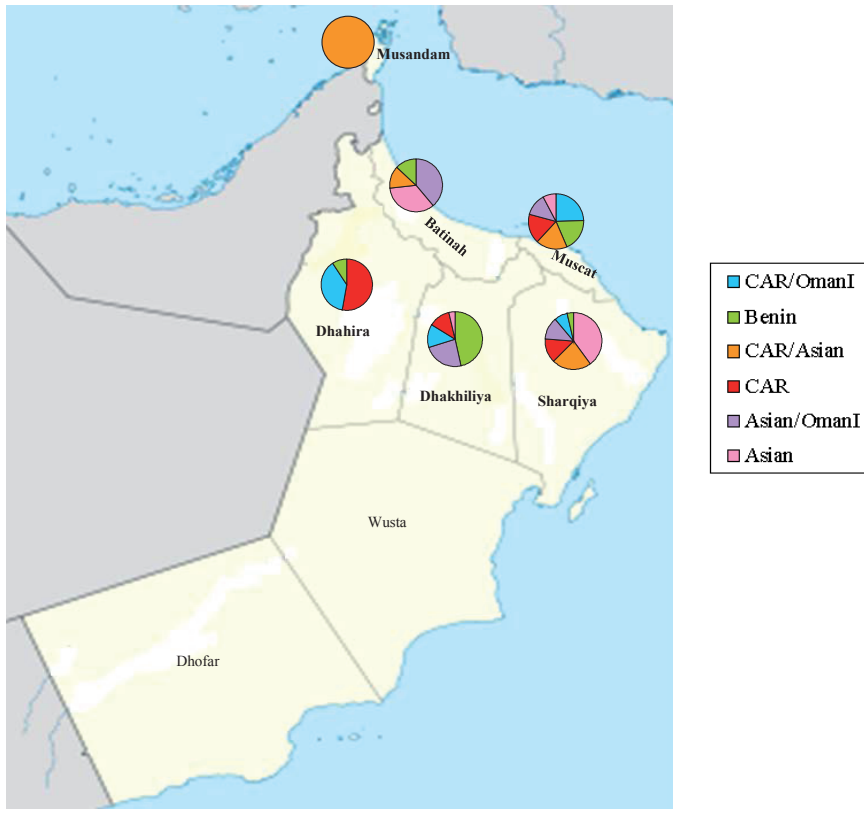

Figure 9.2. The geographical distribution of the β globin gene cluster haplotypes within Oman.

Geographical distribution

The overall distribution of each haplotype in our SCD patients in the different regions of Oman is presented in percentages in Table 9.4. The occurrence frequency of each haplotype in a particular region is depicted in Figure 9.2. In Musandam, CAR/Asian is the only haplotype found. In Batinah, Asian/OmanI is the most abundant. In Muscat, CAR/OmanI is found at the highest frequency and in Sharqiya, the Asian haplotype was the most prevalent. In Dhakhiliya, Benin was the major haplotype found and finally in Dhahira, the CAR haplotype was the most prominent. Data are summarized in Figure 9.2.

Sub-haplotypes

Based on additional SNP's, a subdivision could be made from the original five HbS haplotypes. Trying to find a molecular explanation for the different phenotypes seen within similar basic haplotypes, sub-haplotypes were determined by looking at a total of 42 SNP's in all the125 homozygous HbS patients. Out of the 42 SNP's, only 15 SNP's were found modifying the 11 identified haplotypes. However, no sub-haplotypes were found to be associated with a specific haplotype except for the CAR/OmanI that showed nucleotide variations at the G-γ (SNP1) (SNP position: 5232979-5232984)

Table 9.4. The frequency distribution of the 6 most common haplotypes in 6 different regions in Oman.

Region\ Haplotype	Asian/Asian	Benin/Benin	Asian/OmanI	CAR/CAR	CAR/OmanI	CAR/Asian
Musandam	–	–	–	–	–	12,50%
Batina	31,90%	12%	35,70%	–	–	12,50%
Muscat	21,30%	52%	35,70%	46,20%	66,70%	50%
Sharqiya	44,70%	4%	14,30%	15,30%	8,30%	25%
Dhakhiliya	2,10%	28%	14,30%	7,70%	8,30%	–
Dhahira	–	4%	–	23,10%	16,70%	–
Total %	100	100	100	100	100	100

located in the G-gamma promoter region from which three different sub-haplotypes were defined (a,b and c) (Table 9.5). This site might be associated with the different clinical expression observed in CAR/OmanI patients. The patient with sub haplotype (CAR/OmanI - c) presented with a very severe clinical manifestations and no improvement was seen even after doubling the dosage of hydroxyurea. This patient appears to be homozygous for the wild type 6 nucleotides (CTTTAA) at the G-gamma promoter. On the other hand, another patient with sub haplotype (CAR/OmanI - b) was homozygous for the deletional mutation of the 6 nucleotides at the same position and had a milder clinical presentation and was not taking hydroxyurea consistently as the patient was feeling better after a short period of being on the drug. The remaining patients with sub-haplotypes (CAR/OmanI – a) had the compound heterozygous composition of CTTAA/6nt del at the G-gamma promoter region, needed higher than the average dose of hydroxyurea in order to observe a reduction of the severe phenotype. Although more case studies are needed to confirm our hypothesis, our data allow us to assume that carrying the 6 nucleotide deletion might be beneficial to CAR/OmanI patients for better response to hydroxyurea.

Table 9.5. The different genotypes observed at the G-gamma promoter (SNP1) among CAR/OmanI patients, suggesting the existence of three different sub-haplotypes (a,b and c).

CAR/OmanI sub-haplotype	Gγ(1)	No. of patients	Response to HU
a	CTTTAA/6nt del	11	Mild
b	6nt del/6nt del	1	Positive
c	CTTTAA/CTTTAA	1	None

Hydroxyurea (preliminary data)

The clinical symptoms in severely affected patients that were put on hydroxyurea therapy improved in general but at different levels. As mentioned above, only in patients with CAR/OmanI haplotype, the drug dosage had to be doubled to see some improvements in the treated subjects. However, the different effect of hydroxyurea therapy in correlation with the haplotypes requires further analysis and results will be presented in another study.

DISCUSSION

Haplotypes distribution and disease severity

The Omani populations are known for their high incidence of hemoglobinopathies, including alpha and beta-thalassemia as well as sickle cell disease (13). As mentioned above, patients with SCD present with a variable clinical picture ranging from severe to very mild forms, where haplotypes have been found to be associated with the severity of the disease (20, 21). In Daar et al, a study conducted in Oman in 2000 on 52 HbS/S individuals, it was found that the Benin/Benin haplotype was the most prevalent and twice more frequent than the Asian/Asian (16). In the present study however, the Asian/Asian haplotype was the most prominent while Benin/Benin was the second in rank (Table 9.2). The reason for the higher percentages of Benin haplotype in Daar et al, might be due to a selection among patients attending the Sultan Qaboos University Hospital which are mainly from the Dhakhiliya region and based on our findings, the Benin haplotype has been observed to be present at a high rate in this region (Figure 9.2). Although the effect of these haplotypes on the phenotypes is clearly correlating with the HbF expression, disease severity remains variable within the same haplotypes and more molecular and external factors need to be taken into consideration. The high frequency of alpha-thalassemia reported in the Omani population (13) is another modulating factor influencing the clinical outcome of the disease. The effect of alpha thalassemia on the clinical expression of SCD is under evaluation in our cohort and will be presented in another paper.

Gene flow

The distribution of the HbS haplotypes in Oman is explained by the historical migrations from Zanzibar and India. The presence of the Asian haplotype can be attributed to ancient migrations and to centuries of trade with India and Pakistan. Contacts with East Africa, Zanzibar and Mombasa, explain the presence of the Benin and CAR haplotypes (16). Muscat, being the capital, had the widest diversity of different haplotypes. This reflects more recent migrations of "native people" from the interior to the capital seeking for jobs and better lives. The Asian haplotype was highest in Sharqiya and Batinah and these regions are known to have SCD patients with a mild clinical profile in comparison to Dhakhiliya and Dhahira regions in which they have a more severe manifestation of the disease and this could be explained by the Benin and CAR haplotypes in these regions respectively (Figure 9.2). Only one haplotype combination was found among patients from Musandam (CAR/Asian) and this can be due to the isolation of this region from the rest of the country by mountains and the UAE. SCD is absent in Wusta and Dhofar due to low levels of malaria in the past in these two regions (17). The 4 identified haplotypes which we have referred to as OmanI, II, III and IV are expected in an admixed population such as Oman and might have been derived differently by recombinant events (32). OmanI could have probably been derived from CAR with a mutation at F5. It is also possible to say that it is a result from a recombination event between CAR and Cameroon haplotypes but this is less likely as Cameroon haplotype was not found in the Omani population. Oman II could be a result of a recombinant event between Benin and CAR while OmanIII could be the outcome of a recombination between Asian and CAR haplotypes. Oman IV could be a derivative of OmanII with a mutation at F1. However, these are just assumptions and further studies are eventually required to track back the origin of each haplotype.

Early diagnosis, haplotype, HbF and prognosis

Identifying the disease at an early stage and defining genotype and haplotype allows clinicians to predict to some extent the prognosis and to plan a tailored treatment. Early prediction of the clinical expression will help in preventing or reducing acute painful episodes (crisis) in this cohort, which is the most common traumatic experience in SCD (18), and acute chest syndrome (ACS) which is the most common cause of death in Omani SCD patients (19).

The Asian haplotype was associated with highest HbF levels, fewer hospitalizations and painful episodes and patients did not develop acute chest syndrome although vaso-occlusive events did occur. Our study also confirms that the CAR haplotype whether homozygous or combined heterozygous is associated with lowest HbF level and the highest incidence of organ damage and renal failure as reported elsewhere (22, 23).

Carriers of the HbS gene on the Asian haplotype on one chromosome and OmanI haplotype on the other presented in our cohort high HbF levels (average 14.2%) and a milder clinical course than other compound heterozygous haplotypes. Bakioglu al. (25) reported mild SCD cases of Asian/Benin haplotype with high levels of Hb F (average 22.2%). This shows that carrying the HbS mutation on an Asian haplotype on one chromosome could still contribute to elevating the HbF expression.

Higher concentrations of HbF in the cell lead to lower concentrations of HbS (24), better oxygenation and less clinical severity. A potential threshold of 20% HbF has been suggested to effectively prevent recurrent vasoocclusive episodes (26). This is true in most cases, however, in another study, some patients with HbF levels near 20% had a devastating disease manifestation (18). The same observation was seen in one of the patients in our cohort, with the Asian/CAR haplotype. Despite the high HbF (19.7%), the patient is frequently admitted to the hospital with vaso-occlusive crisis, dactilitis and severe abdominal pain. This patient has no iron overload and is heterozygous for the α-3.7 deletion. This finding imply that other circumstantial or genetic factors or external transacting determinants such as blood viscosity, elevated PCV, vascular adherence, acidosis and dehydration as well as patient's life style and diet might contend with the beneficial effect of the high HbF level. Our findings support the conclusion by Acquaye et al. (27) and Seltzer et al. (28) that fetal hemoglobin levels are very important but not the only parameters that mitigate the severity of the disease and we are at the moment inquiring which other factors could be associated with the severity of this case.

Sub-haplotype

The sub-haplotype study of 42 SNP's on the 11 haplotype combinations observed in our 125 SCD patients revealed that 15 different positions differentiate the 11 identified haplotypes. No sub-haplotypes were determined except in patients with the CAR/OmanI haplotype, which revealed variation at the G-gamma promoter SNP-1 giving 3 different sub-haplotypes (Table 9.5; a, b and c). One patient with sub-haplotype-c who presented with homozygosity for the wild type 6 nucleotide sequence (CTTTAA) at the G-gamma promoter, a very severe phenotype and not responding to high doses of Hyrdoxyurea treatment. This element could be the cause of low HbF expression and of non-response to Hydroxyurea and this hypothesis seems to be sustained by the fact that another patient with sub-haplotype-b with a homozygous mutational

deletion of the 6 nucleotides at the same position became better as soon as being on the drug. The presence or absence of the 6nt sequence in at the G-gamma promoter in the CAR/OmanI patients could be associated with the differences in clinical presentation and with response to hydroxyurea therapy, probably an element is in linkage to the 6nt deletion and is responsible for the positive response to the drug.

CONCLUSION

Our study on haplotype/phenotype correlation has shown the existence of at least 11 different haplotype combinations in Oman. These are differently distributed among the six main regions of the country. Sub-haplotype was only observed in CAR/OmanI combination and could be associated with the clinical differences observed in patients with the same haplotype. Identifying haplotypes and sub-haplotypes in early life may allow a better prognosis and a more accurate risk predictions and a better tailored therapy, to match disease-related risks and to facilitate planning of clinical trials to prevent the development of severe complications later in life. Nevertheless, we have shown that when the phenotypes are classified into; mild, intermediate and severe, neither the haplotype or the HbF alone appeared to be fully associable with the clinical phenotypes as also been observed by Alexander et al (29). External and/or modifying, or epistatic factors, which potentially modulate the phenotype of SCD do occur and more efforts should be done trying to chart them. The implementation of primary prevention with simple cost effective interventions for SCD are essential (30) and are likely to lead to lower incidence, lower costs for public health and improved survival rate of SCD patients in Oman.

ACKNOWLEDGEMENTS

The authors declare to have conducted this study according to local ethical regulations and to have no conflicts of interest on the presented matters.

REFERENCES

1. Herrick JB. Peculiar elongated and sickle-shape red blood corpuscles in a case of severe anemia. Arch Intern Med 1910; 6: 517-21.
2. Ronald L. Nagel, Shahina Daar, Jose R. Romero, Sandra M. Suzuka, David Gravell, Eric Bouhassira, Robert S. Schwartz, Mary E. Fabry and Rajagopal Krishnamoorthy. HbS-Oman Heterozygote: A New Dominant Sickle Syndrome. Blood. 1998; 92: 4375-4382.
3. Inati A, Taher A, Bou Alawi W, Koussa S, Kaspar H, Shbaklo H, Zalloua PA. b-Globin gene cluster haplotypes and HbF levels are not the only modulators of sickle cell disease in Lebanon. Eur J Haematol 2003; 70: 79–83.
4. Giordano PC, Huisman W, Harteveld CL. Iron depletion: an ameliorating factor for sickle cell disease? ISRN Hematol. 2011;2011:473152.
5. Kamel K. Heterogeneity of sickle cell anaemia in Arabs: review of cases with various amounts of fetal haemoglobin. J Med Genet 1979;16:428–430.
6. Pagnier J, Mears JG, Dunda-Belkhodja O, Schaefer-Rego KE, Beldjord C, Nagel RL, Labie D: Evidence for the multicentric origin of the sickle cell hemoglobin gene in Africa. Proc Natl Acad Sci USA 1984; 81: 1771–1773.
7. Nagel RL, Fleming AF: Genetic epidemiology of the beta s gene. Baillieres Clin Haematol 1992; 5: 331–365.
8. Kulozik AE, Thein SL, Kar BC, Wainscoat JS, Serjeant GR, Weatherall DJ. Raised Hb F levels in sickle cell disease are caused by a determinant linked to the beta globin gene cluster. Prog Clin Biol Res 1987;251:427–439.
9. Padmos MA, Roberts GT, Sackey K, Kulozik A, Bail S, Morris JS, Serjeant BE, Serjeant GR: Two different

forms of homozygous sickle cell disease occur in Saudi Arabia. Br J Haematol 1991; 79: 93–98.

10. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. N Engl J Med 1995;332: 1317-22.
11. Walters MC, Patience M, Leisenring W, et al. Bone marrow transplantation for sickle cell disease. N Engl J Med 1996;335:369-76.
12. Van Delft P, Lenters E, Bakker-Verweij M, de Korte M, Baylan U, Harteveld CL, Giordano PC. Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations. Int J Lab Hematol. 2009 Oct;31(5):484-95.
13. Hassan SM, Hamza N, Jaffer Al-Lawatiya F, Jaffer Mohammed A, Harteveld CL, Rajab A,Giordano PC. Extended molecular spectrum of beta- and alpha-thalassemia in Oman. Hemoglobin. 2010 Jan;34(2):127-34.
14. Jesse Montgomery, Carl T Wittwer, Robert Palais & Luming Zhou. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. Nature Protocoles. 2007; Vol.2 (1): 59-66.
15. Montgomery J, Wittwer CT, Palais R, Zhou L. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. Nat Protoc 2007; 2(1):59-66.
16. Shahina Daar, H. Mohamed Hussain,David Gravell, Ronald L. Nagel and Rajagopal Krishnamoorthy. Genetic Epidemiology of HbS in Oman: Multicentric Origin for the bS Gene. American Journal of Hematology. 2000; 64:39–46.
17. Rajab A, Patton MA. Major factors determining the frequencies of hemoglobinopathies in Oman. Am J Med Genet (Letter). 1997;71: 240–242
18. M. H. Steinberg. Predicting clinical severity in sickle cell anaemia. British Journal of Haematology. 2005; 129: 465–481.
19. Lamk Al Lamki. Deaths from Sickle Cell Disease in Intensive Care Units. Sultan Qaboos Univ Med J. 2012 May; 12(2): 133–136.
20. Steinberg MH, Hsu H, Nagel RL, Milner PF, Adams JG, Benjamin L, Fryd S, Gillette P, Gilman J, Josifovska O, Hellman-Erlingsson S, Safaya S, Huey L, Rieder RF. Gender and haplotype effects upon hematological manifestations of adult sickle cell anaemia: effects of haplotype in sickle cell anaemia. Am J Hematol 1995;48:175–181.
21. Nagel RL, Fabry ME, Pagnier J, Zohoun I, Wajcman H, Baudin V, Labie D. Hematologically and genetically distinct forms of sickle cell anemia in Africa: the Senegal type and the Benin type. New Eng J Med 1985; 312:880–884.
22. Bakanay, S.M., Dainer, E., Clair, B., Adekile, A., Daitch, L., Wells, L., Holley, L., Smith, D. & Kutlar, A. Mortality in sickle cell patients on hydroxyurea therapy. Blood. 2005; 105: 545–547.
23. Nagel, R.L. & Steinberg, M.H. Genetics of the bS gene: origins, epidemiology, and epistasis. In: Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management (ed. by M. H. Steinberg, B. G. Forget, D. R. Higgs & R. L. Nagel). 2001; 711–755. Cambridge University Press, Cambridge.
24. Bailey K, Morris JS, Thomas P, Serjeant GR. Fetal haemoglobin and early manifestations of homozygous sickle cell disease. Arch Dis Child 1992;67:517–520.
25. Bakioglu I, Hattori Y, Kutlar A, Mathew C, Huisman THJ. Five adults with mild sickle cell anaemia share a bS chromosome with the same haplotype. Am J Hematol 1985;20:297–300.
26. Powars DR, Weiss JN, Chan LS, Schroeder WA. Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia? Blood. 1984; 63:921-926.
27. Acquaye JK, Omer A, Ganeshaguru K, Sejeny SA, Hoffbrand AV. Non-benign sickle cell anaemia in western Saudi Arabia. Br J Haematol 1985;60:99–108.
28. Seltzer WK, Abshire TC, Lane PA, Roloff JS, Githens JH. Molecular genetic studies in black families with sickle cell anemia and unusually high levels of fetal hemoglobin. Hemoglobin 1992;16:363–377.
29. Alexander, N., Higgs, D., Dover, G. & Serjeant, G.R. Are there clinical phenotypes of homozygous sickle cell disease? British Journal of Haematology. 2004: 126, 606–611.
30. Giordano PC. Prospective and retrospective primary prevention of hemoglobinopathies in multiethnic societies. Clin Biochem. 2009;42(18):1757-66.
31. Marion Phylipsen, Supawadee Yamsri, Emmely E. Treffers, Diahann T. S. L. Jansen, Warsha A. Kanhai, Elles M. J. Boon, Piero C. Giordano, Supan Fucharoen, Egbert Bakker and Cornelis L. Harteveld. Non-invasive prenatal diagnosis of beta-thalassemia and sickle-cell disease using pyrophosphorolysis-activated polymerization and melting curve analysis. Prenatal Diagnosis 2012, 32, 578–587.
32. Zago MA, Silva WA, Gualandro S, Yokomizu IK, Araujo AG, Tavela MH, Gerard N, Krishnamoorthy and Elion J. Rearrangements of the beta-globin gene cluster in apparently typical betaS haplotypes. Haematologica 2001; 86:142-145.

CHAPTER 10

ASSOCIATION OF XMNI (-158 γ^G) POLYMORPHISM AND RESPONSE TO HYDROXYUREA IN OMANI S/S AND S/β PATIENTS

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Genetic Genome Research, 2014, 1:1
ISSN: 2378-3648

ABSTRACT

Objective

To describe the effect of hydroxyurea (HU) treatment in Omani sickle cell disease (SCD) patients with different beta-globin gene cluster haplotypes.

Materials and Methods

A total of 52 cases treated with HU were enrolled in this study. Response to the drug was compared between patients with and without the XmnI polymorphism in the different beta-globin gene cluster haplotypes. We have classified our cohort into three categories: good responders to HU for those patients who had no crises and no hospitalization after 6 months of treatment; partial responders for those who had a reduction in the number of crises after the same period and non responders for those that remained clinically unchanged even after doubling the HU prescription.

Results

Most patients homozygous or heterozygous for the Xmn I polymorphism (T/T or T/C) had higher levels of HbF prior treatment than those having the CC genotype and were classified under good or partial responders.

Conclusions

Being the Xmn-I polymorphism associated with the haplotypes frequent in Oman and acting as enhancer of the already elevated HbF expression, HU treatment can be prospectively applied to predict responsiveness and treatment can be given to those with low HbF expression for beneficial lowering of cellular adhesion. HU treatment can ameliorate the clinical phenotype of the large majority of Omani patients with SCD.

INTRODUCTION

The mutation responsible for sickle cell disease (SCD) is the GAG > GTG transversion at codon 6 of the beta globin gene, resulting into a Glu>Val amino acid substitution and in the change of the wild type HbA tetramer into the commonest abnormal Hb variant (HbS). The condition is recessive and carriers of HbS are in general asymptomatic while the mutation in homozygous or compound heterozygous form in combination with β-thalassemia is in most of the cases a severe condition. Although HbS is the causal allele, the combination of HbS with other common alleles (HbC, HbE, HbD) and a number of less common ones, may also cause the disease with large phenotype variability (1).

Milder forms may be caused by the combination of HbS with a less severe β^+-thalassemia allele with residual HbA expression or with δβ-thalassemia deletion because of the characteristic elevated HbF expression. The ameliorating role of HbF in SCD and β-thalassemia and the association of high HbF with specific genotypes / haplotypes have been known for a long time. In spite of strenuous efforts, no effective cure associated with a permanent increase of HbF expression in post-natal life has been found so far while different drugs have been tested that can temporarily increase the HbF level with acceptable collateral effects. Among these drugs the most successful thus far is hydroxyurea (HU) a medication that may reduce the severe symptoms in different ways in SCD patients (2). A better practical knowledge on these differences may allow an early prognosis for severe patients that are likely to respond to HU and for others that are not responding or respond in a different way, allowing early planning for an alternative treatment for non-responders such as bone marrow transplantation which, if successful, could be an alternative curative solution (3).

Response to HU has been shown to be largely associated with the presence of the C>T polymorphism at -158 Xmn-I site (HBG2:c.-53-158C>T) upstream of the Gγ globin gene and it is thus far the most studied nucleotide change to have a significant association to drug response. This particular polymorphism acts as an enhancer of HbF expression during erythropoietic stress, resulting in a beneficial effect in SCD patients (4). The frequency of Xmn-I polymorphism in SCD patients has not yet been investigated in the Omani population. Therefore we have studied the association of the XmnI polymorphism and the response to hydroxyurea treatment in HbS/S and S/β-thalassemia patients in Oman and we have further investigated if the HbS haplotype is accountable for a more differentiated response to HU in patients with identical Xmn-I genotype.

MATERIALS AND METHODS

A total of 52 SCD patients attending the Ministry of Health Hospitals in Oman (between January – June 2012) and started the treatment with HU were randomly enrolled. These patients were then followed for the subsequent 6 months afterwards. Gender distribution was; 52% males and 48% females. The age range of the subjects was 23- 32 years. At the laboratory level, high performance liquid chromatography (HPLC) was performed on all samples, prior treatment, either on D-10 (short and extended programs) device and/or the Variant II (Bio-Rad Laboratories, Hercules, CA, USA). DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia,

CA, USA). The XmnI (–158 (C>T) polymorphism of the Gγ gene promoter) (rs 7842144, HBG2:c.-53-158C>T) and the haplotype of the HbS/S beta globin cluster were defined by melting curve analysis (LightScanner HR96, Idaho Technology, Inc) as previously described (5). The β globin gene sequencing was performed on an ABI PRISM® 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) and the α-cluster genotype was obtained by GAP-PCR as previously described (6). The initial hydroxyurea dosage was 500 mg (capsule). If no improvement was observed after 3 months the dosage was increased to 1000 mg. Classification of responders was as follow: Good responders: Patients became clinically asymptomatic 6 months after the therapy (no crises, no hospitalization, no definitive pain). Partial responders: Significant decrease is noted in number of vaso-occlusive crises and hospitalizations (<3/year) and experienced much milder pain. Non responders: No clinical improvement despite dosage increase after 6 months.

RESULTS

DNA analysis revealed 40 cases homozygous for HbS/S and 12 S/β-thalassemia patients. Of these 52 patients, 22 were either homozygous or heterozygous for the Xmn I (C>T) polymorphism at position -158 site at the G-γ promoter. Of the patients homo- or heterozygous for the -158 C>T, 18 (82%) showed good response to HU, 3 (13.6%) partial response and 1 (4.5%) showed no response. In those without Xmn I polymorphism (n=30), only 1 (3.3%) showed good response, 24 (80%) showed partial response and 5 (16.7%) showed no response. On average, good responders became asymptomatic after 6 months of being on HU, partial responders had significant decrease in complications within the same time span while non-responders had no significant reduction after 6 month even after doubling the HU dosage. Among the 40 HbS/S patients, 15 that had at least one copy of the T allele, were all good responders while among those 21 that became better (partial responders) only two had at least one copy of the T allele. Of the remaining 4 patients that did not respond, even after double prescription, none had the T allele.

Prior to treatment, HbS/S patients with the genotype T/T or T/C had on average an elevated expression of HbF% (15.2%) and (13.5%) respectively. Patients having the genotype C/C had a lower HbF level (5.6%).

None of the S/β-thal patients, had the TT genotype, however, HbF levels prior treatment were found to be on average high in S/β individuals bearing the TC genotype (11.3%) and lower in those with the CC genotype (7.3%). Among the S/β-thal group, we found 4 patients that were good responders to HU, 6 became better in terms of severity while 2 did not respond to the drug. Data are summarised in Table 10.1. One of the non responders was found to have the Xmn I T/C polymorphism and the β IVS-I-1 G>A mutation while the other had the C/C polymorphism and the β Cd36/37 (-T) mutation. Data are summarized in Table 10.2. Over all, the clinical symptoms improved in most patients and the best response was associated with the presence of the Xmn I polymorphism.

Haplotype analysis of the HbS/S is summarised in Table 10.3. The 4 non responders all had the CAR/Oman haplotype. The Oman haplotype differs from CAR by a mutation at SNP position 49994 (4). Moreover, the presence of the T allele at the XmnI polymorphism was linked to the Asian haplotype. Our findings show that the presence of Xmn I polymorphism in Omani SCD population is a predictor of response to HU.

Table 10.1. Association between improvement of disease after HU treatment and the presence of the polymorphic site (C>T) at position -158 Xmn I at the G-γ promoter region in the S/S (i) and S/β (ii) cohorts.

(i)

S/S patients	T/T (%) (n=11)	T/C (%) (n=6)	C/C (%) (n=23)
Good responders	91% (n=10)	83.3% (n=5)	-
Partial responders	9% (n=1)	16.7% (n=1)	82.6% (n=19)
Non responders	-	-	17.4% (n=4)

(ii)

S/β patients	T/C (%) (n=5)	C/C (%) (n=7)
Good responders	60% (n=3)	14.3% (n=1)
Partial responders	20% (n=1)	71.4% (n=5)
Non responders	20% (n=1)	14.3% (n=1)

Table 10.2. Genotypes of the HbS/β compound heterozygote patients treated with HU.

β genotype	XmnI genotype	response to HU
Cd6 GAG>GTG/IVS-I-5 G>C (n=7)	T/C (n=2)	Excellent (n=2)
HBB:c.20A>T/ HBB:c.92+5G>C		Partial (n=0)
		None (n=0)
	C/C (n=5)	Excellent (n=1)
		Partial (n=4)
		None (n=0)
Cd6 GAG>GTG/Cd121 GAA>CAA (n=2)	T/C (n=1)	Excellent (n=1)
HBB:c.20A>T/ HBB:c.364G>C		Partial (n=0)
		None (n=0)
	C/C (n=1)	Excellent (n=0)
		Partial (n=1)
		None (n=0)
Cd6 GAG>GTG/Cd44 (-C) (n=1)	T/C (n=1)	Excellent (n=0)
HBB:c.20A>T/ HBB:c.135delC		Partial (n=1)
		None (n=0)
Cd6 GAG>GTG/IVS-I-1 G>A (n=1)	T/C (n=1)	Excellent (n=0)
HBB:c.20A>T/ HBB:c.92+1G>A		Partial (n=0)
		none (n=1)
Cd6 GAG>GTG/Cd37(-T) (n=1)	C/C (n=1)	Excellent (n=0)
HBB:c.20A>T/ HBB:c.112delT		Partial (n=1)
		none (n=1)

Table 10.3. Summary of the HbS/S haplotypes in patients treated with HU.

Haplotype	XmnI genotype	response to HU
Asian/Asian (n=11)	T/T	Good (n=10)
		Partial (n=1)
		None (n=0)
Asian/CAR (n=1)	T/C	Good (n=1)
		Partial (n=0)
		None (n=0)
Asian/Oman (n=4)	T/C	Good (n=3)
		Partial (n=1)
		None (n=0)
Senegal/Oman (n=1)	T/C	Good (n=1)
		Partial (n=0)
		None (n=0)
Benin/Benin (n=9)	C/C	Good (n=0)
		Partial (n=9)
		None (n=0)
CAR/CAR (n=3)	C/C	Good (n=0)
		Partial (n=3)
		None (n=0)
CAR/Oman (n=11)	C/C	Good (n=0)
		Partial (n=7)
		None (n=4)

DISCUSSION

Bakanay et al. reported the highest incidence of organ damage and the poorest response to HU in SCD patients with the XmnI C/C genotype (7). Likewise, our patients bearing the C/C allele at the XmnI site, had a poorer response to the drug than those carrying the T allele.

In patients with the genotype TT, high HbF levels and the best response to HU were measured as previously reported by other authors (8) with significant reduction in both anaemia and the frequency of vaso-occlusive events (9). Studies on HU treatment for β-thalassemia have produced contradictory results. While Karimi et al. found no relationship between XmnI polymorphism and HU clinical response in their patients (10), Yavarian et al. (4) found that the C>T polymorphism at position -158 of the Gγ promoter was the most significant parameter correlating with HU response in β-thalassemia patients. In our study as many as 91% of the HbS/S patients with the T/T genotype fully responded to HU therapy while none of the patients with the C/C polymorphism were classified under 'good responders', confirming that this polymorphism is highly correlated with a positive response to HU treatment in the Omani SCD patients as well.

In Oman, HU is only used for SCD but not for β-thalassemia major and thus far a restricted inclusion criterion have been used to decide if SCD patients were eligible to be given HU or not. These include some severe symptoms noted by the clinician, such as frequent hospitalization (>4-5/year) with recurrent episodes of acute chest syndrome, vaso-occlusive events, severe pain crisis and severe, un-subsiding body and limp pain that last for days. The daily dose given is the recommended 500mg and it is only increased to reach a maximum of 1000mg when no improvement is seen with the 500mg dose. Although Charache et al, proved the effectiveness and safety of hydroxyurea usage and the improved outcomes (11), HU is not widely accepted by Omani patients and their families, due to the negative perception toward this treatment and the fears of birth defects, infertility, malignancy and concerns on long-term risks. Non-compliance may be found in patients because of anxiety and minor but disturbing side effects such as nausea or when a pregnancy is perceived. More studies have proven that HU (20 mg/kg/d) is a safe therapy even for very young children with SCD (starting at 9 months of age) and that the cure is in general associated with significantly lower rates of recurrent episodes of pain, dactylitis, acute chest syndrome and hospitalization (12). However, response prediction to HU treatment is not always straightforward. Also in our study some patients having the T/C XmnI polymorphism, did not improve with the standard 500mg dose which had to be doubled to observe some improvement. The few patients that did not show improvement after treatment with HU even after increasing the dose were all carries of the compound heterozygous haplotype (CAR/Oman). Our findings suggest that the C/T polymorphism at the XmnI site, although in most cases associated with good response, is neither a guarantee nor the only determinant that can ameliorate disease severity in SCD patients treated with HU and that other factors either haplotype and/or sub-haplotype related or associated with other regulatory elements might be involved. Therefore, increasing the dosage of HU to the maximal tolerated dose might be necessary for having some clinical response in SCD patients with African haplotypes (13) whereas SCD children with Asian haplotype treated with a dose as low as 10 mg/kg/day have shown some good clinical response (14). Moreover, it has been proved that HU a nitric oxide (NO) provider has an anti-adherence effect that may prevent cells to get stuck to the capillary walls and herewith improving the rheology of the blood during the critical passage of the deoxygenated HbS cells (15).

Alpha thalassemia has been proposed as a factor possibly associated with good response to HU therapy in β-thalassemia intermedia in addition to the presence of Xmn I polymorphism (16). While another study reported that co-inheritance of α-thalassemia in SCD patients may reduce the clinical response to HU therapy (17). Although we may imagine that α-thalassemia may partially restore the balance in β-thalassemia, we believe that this mechanism has little to do with the response mechanisms of HU in SCD. As a matter of fact when we correlated the presence or absence of α-thalassemia (very common in our cohort) to the HU response (HbS/β-thal included) we find no association between coexisting alpha deletions and good response to HU (data not shown).

In conclusion, being the Xmn-I polymorphism associated with haplotypes frequent in Oman and acting as enhancer of the already elevated HbF expression, HU treatment can be prospectively applied to predictably responsive patients and be tested in those with low HbF

expression for beneficial lowering of cellular adhesion. HU treatment can ameliorate the clinical phenotype of the large majority of Omani patients with SCD.

ACKNOWLEDGEMENTS

The authors declare to have conducted this study according to local ethical regulations and to have no conflicts of interest on the presented matters.

REFERENCES

1. Giordano PC. Strategies for basic laboratory diagnostics of the hemoglobinopathies in multi-ethnic societies: interpretation of results and pitfalls. Int J Lab Hematol. 2013; 35(5):465-479.
2. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. N Engl J Med 1995;332: 1317-22.
3. Walters MC, Patience M, Leisenring W, et al. Bone marrow transplantation for sickle cell disease. N Engl J Med 1996;335:369-76.
4. Yavarian, M., Karimi, M., Harteveld, C.L. and Giordano P.C. Response to hydroxyurea treatment in Iranian transfusion-dependent β-thalassemia patients. Haematologica. 2004;89:1172-1178.
5. Phylipsen M, Yamsri S, Treffers EE, Jansen DTSL, Kanhai WA, Boon EMJ, Giordano PC, Fucharoen S, Bakker E and Harteveld CL. Non-invasive prenatal diagnosis of beta-thalassemia and sickle-cell disease using pyrophosphorolysis-activated polymerization and melting curve analysis. Prenatal Diagnosis 2012, 32, 578–587.
6. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of α-thalassaemia deletions and α-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol. 2000;108:295–299.
7. Bakanay SM, Dainer E, Clair B, Adekile A, Daitch L, Wells L, Holley L, Smith D and Kutlar A. Mortality in sickle cell patients on hydroxyurea therapy. Blood, 2005; 105, 545–547.
8. Ware RE and Aygun B. Advances in the use of hydroxyurea. American Society of Hematology. Educ Prog 2009:62–9.
9. Steinberg MH. Predicting clinical severity in sickle cell anaemia. British Journal of Haematology, 2005; 129, 465–481.
10. Karimi M, Haghpanah S, Farhadi A and Yavarian M. Genotype-phenotype relationship of patients with β-thalassemia taking hydroxyurea: a 13-year experience in Iran. Int J Hematol (2012) 95:51–56.
11. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, McMahon RP and Bonds DR. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. New England Journal of Medicine. 1995;332, 1317–1322.
12. Thornburg CD, Files BA, Luo Z, Miller ST, Kalpatthi R, Iyer R, Seaman P, Lebensburger J, Alvarez O, Thompson B, Ware RE, Wang WC and BABY HUG Investigators. Impact of hydroxyurea on clinical events in the BABY HUG trial. Blood, 2012; 120, 4304–4310.
13. Steinberg MH, McCarthy WF, Castro O, Ballas SK, Armstrong FD, Smith W, et al. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia and MSH Patients' Follow-Up. The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: A 17.5 year follow-up. Am J Hematol. 2010;85:403-408.
14. Coleman E and Inusa B. Sickle cell anaemia: targeting the role of fetal haemoglobin in therapy. Clinical Pediatrics (Phila) 2007;46:386-91.
15. Gladwin MT, Shelhamer JH, Ognibene FP, Pease-Fye ME, Nichols JS, Link B, Patel DB, Jankowski MA, Pannell LK, Schechter AN and Rodgers GP. Nitric oxide donor properties of hydroxyurea in patients with sickle cell disease. Br J Haematol. 2002;116(2):436-444.
16. Panigrahi I, Dixit A, Arora S, Kabra M, Mahapatra M, Choudhry VP, et al. Do alpha deletions influence hydroxyurea response in thalassemia intermedia? Hematology. 2005;10:61–3.
17. Vasavda N, Woodley C, Allman M, et al. Effects of co-existing α-thalassaemia in sickle cell disease on hydroxycarbamide therapy and circulating nucleic acids. Br J Haematol. 2011;157(2):249–252.

CHAPTER 11

SICKLE CELL ANEMIA AND α-THALASSEMIA: A MODULATING FACTOR IN HOMOZYGOUS HBS/S PATIENTS IN OMAN

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Europ J of Med Gen. 2014; 57(11-12):603-606

ABSTRACT

We report the general phenotype severity and the hematological presentation in a cohort of 125 sickle cell anemia (SCA) patients with identical homozygous HbS/S genotype and categorized by identical β^S haplotype, both with and without alpha thalassemia. No clear general phenotype correlation was found when patients were compared regardless of the haplotype but overall, patients with homozygous alpha thalassemia (α−/α−) had the highest Hb, HCT, RBC and the lowest MCV, MCH and MCHC levels. When patients with identical haplotype were compared, the mildest hematological and clinical conditions were observed in patients of the Asian/Asian haplotype, also known as Arab-Indian haplotype, and carriers of α-thalassemia, suggesting an additional ameliorating effect of alpha thalassemia. In conclusion, our results show that alpha thalassemia improves the hematological conditions but amelioration of the general disease severity is only noticed when compared in cohorts of the same haplotype.

INTRODUCTION

Sickle cell anemia (SCA) is in general a severe condition caused by different genotype combinations of which HbS homozygosis is the most common. The pathophysiology of SCA is complex and involves HbS polymerization in hypoxic conditions in the post-capillary veins, erythrocyte sickling, chronic and acute vaso-occlusive events, hemolysis and progressive organ and tissue damage at variable levels (1). Although it is known that both environmental and genetic factors may contribute to this variability (2), patients with the same HbS/S genotype often display very different phenotypes in which the clinical manifestations may range from very severe to milder or can even in some cases be almost asymptomatic and be diagnosed accidentally (3,4). The clinical and hematological severity of SCA can be influenced by a number of factors among which the main one is the level of fetal hemoglobin (HbF) in postnatal life which is related to the beta globin gene haplotype (5). The role of co-inherited alpha thalassemia influencing or not the phenotype of SCA has been long debated (6).

The alpha-globin chains that are needed to form sufficient Hb tetramers are coded by two alpha genes located on the short arm of chromosome 16. The two α-globin genes, alpha 2 and alpha 1, are separated by less than 4 kb and code for identical alpha globin chains.

Alpha thalassemia occurs at a high prevalence in Oman (7) with -α3.7 kb deletion being the commonest (8).

The influence of alpha-thalassemia on SCA has been reported to ameliorate the hematological and clinical manifestation of the disease in several populations (9). Alpha-thalassemia lowers the mean cell volume (MCV) and the mean cell hemoglobin (MCH), and both these changes might be expected to be beneficial to patients with sickle-cell disease improving rheology and reducing the concentration of the Hb molecules in the red cells (2). However, to study the effect of alpha thalassemia on the severity of the disease one needs to compare cohorts of patients with identical genotype (HbS/S mutation) and haplotype (beta globin cluster).

Both alpha thalassemia and SCA are frequent in Oman (7) and the HbS mutation is present on severe and mild haplotypes. Therefore, in order to establish if alpha thalassemia has any effect on the severity of the disease, we have studied the hematological and general clinical phenotype of 125 patients, all HbS/S homozygous, with or without alpha thalassemia and categorized in specific and different haplotypes. We premise therefore that our study is not meant to go deeply into clinical details. Our goal is to build a bridge between the clinician and the geneticists, comparing the general clinical phenotype with the genetic background of the patients.

MATERIALS AND METHODS

Blood samples were collected in EDTA at the Ministry of Health Hospitals in Oman. Hematological and clinical data were obtained from routine hematology and from patient's medical history documented by the treating physician. We subdivided the phenotypes as mild, intermediate and severe by the occurrence of symptoms such as: acute chest syndrome, stroke and the presence of avascular necrosis (absence = mild, presence = severe); number of painful crisis per year (≤3 = mild, ≥6 = severe) and blood transfusion (2/year≤ = mild, 4/year≥ = severe).

The cohort consisted of individuals that had been previously diagnosed with SCA. The diagnosis was confirmed on HPLC (Variant II, Bio-Rad Laboratories, Hercules, CA, USA) (10) and molecular characterization of the genotypes and haplotypes was performed at Leiden University Hemoglobinopathies center. A group of 125 individuals confirmed homozygous HbS/S were enrolled in this study. The median age was 24 years. DNA extraction was done using the commercial Qiagen kit as per the manufacturer instruction (8). The beta genotype was confirmed by direct Sanger DNA sequencing. The haplotype of the β-globin gene cluster was determined by melting curve analysis as previously reported (11) and genotyping errors were ruled out by random sample sequencing. We found a Central African Republic (CAR) derivative haplotype in some patients and named it the Oman haplotype. This haplotype differs from CAR by a single variation at HcII RFLP G>A (SNP F5, position rs968857 5260458) (11). Five patients were not included in the haplotype grouping analysis because each had a single unique haplotype with no comparable cases. Alpha-globin genotype was established by GAP-PCR for the most common 7 alpha thalassemia deletion defects (12) for all the samples while the alpha globin genes were sequenced for selected samples. To asses if presence or absence of alpha thalassemia has an effect on disease expression, clinical and hematological comparison of the patient's history was made between genetically equivalent cohorts with and without alpha thalassemia.

RESULTS

α-thalassemia's frequency in the different cohorts

The gene frequency of α-thalassemia among HbS/S patient was confirmed to be very high. Homozygosis or compound heterozygosis (-α/-α), was found in 55 patients (44%). Specifically 54 had the ($-\alpha^{3.7}/-\alpha^{3.7}$) and 1 had the ($-\alpha^{3.7}/-\alpha^{4.2}$) combination while 42 patients (33.6%) had the heterozygous genotypes ($-\alpha^{3.7}/\alpha\alpha$) and 28 individuals (22.4%) had a normal alpha globin genotypes (αα/αα). No alpha° deletions or point mutations were found in the studied samples.

When HbS/S patients classified as mild, intermediate and severe, based upon their disease history, were compared with the presence or absence of α-thalassemia regardless the haplotype, no clear correlation was found.

Effect of α-thalassemia in HbS/S patients of specific haplotypes

HbS/S patients with Asian/Asian haplotype (also known as Arab-Indian) had a mild presentation in 82% and 87% of the cases with (-α/-α) and (-α/αα) thalassemia respectively. This in contrast with 66% of the cases without alpha thalassemia. Similarly, an intermediate state, was twice more frequent in absence of alpha thalassemia. None of the Asian/Asian presented with a severe condition (Table 11.1a).

In the smaller cohorts of HbS/S patients with Asian/Oman haplotype, the milder condition was present in 75% of the patients with (-α/-α) while none had the mild condition in absence of alpha thalassemia and none of them were severe (Table 11.1b).

In the few HbS/S patients with Asian/CAR haplotype and (-α/-α) all three conditions (mild, intermediate and severe) were observed but the severe phenotype was 3 time higher in absence of alpha thalassemia (Table 11.1c).

Table 11.1. Association between alpha-thalassemia, different β-cluster haplotypes and clinical severity.

(a)Asian/Asian haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=20)	82.4%	17.6%	-
- α/αα (n=9)	87.5%	12.5%	-
αα/ αα (n=18)	66.7%	33.3%	-

(b)Asian/Oman haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=5)	75%	25%	-
- α/αα (n=7)	57.1%	42.9%	-
αα/ αα (n=2)	-	100%	-

(c)Asian/CAR haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=3)	33.4%	33.3%	33.3%
- α/αα (n=3)	-	66.7%	33.3%
αα/ αα (n=2)	-	-	100%

(d)Benin/Benin haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=16)	-	25%	75%
- α/αα (n=8)	-	57.1%	42.9%
αα/ αα (n=1)	-	-	100%

(e)CAR/Oman haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=5)	-	40%	60%
- α/αα (n=6)	-	16.7%	83.3%
αα/ αα (n=1)	-	-	100%

(f)CAR/CAR haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=4)	-	-	100%
- α/αα (n=5)	-	20%	80%
αα/ αα (n=4)	-	-	100%

In spite of the limited number of cases found on other haplotypes, the same pattern seems to appear in the Benin/Benin and in the CAR/Oman cohorts (Table 11.1d and 11.1e) while no association seems to be present in the severe CAR/CAR cohort (Table 11.1f).

Effect of α-thalassemia in grouped HbS/S patients of mild and severe haplotypes

HbS/S patients with Asian/Asian and Asian/Oman were grouped under mild haplotype while those with Benin/Benin, CAR/Oman and CAR/CAR under severe haplotype. Asian/CAR was not included in the 2 groups as it had an intermediate phenotype. Patients grouped as mild haplotype had a mild presentation in cases with (-α/-α) and (-α/αα) thalassemia than cases without alpha thalassemia (Table 11.2a). Among the severe haplotype, all patients without alpha thalassemia were presented with a severer phenotype (Table 11.2b).

Table 11.2. Association between alpha-thalassemia, mild and severe β-cluster haplotypes and clinical severity.

(a) Mild haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=25)	77.3%	22.7%	-
- α/αα (n=16)	73.3%	26.7%	-
αα/ αα (n=20)	60%	40%	-

(b) Severe haplotype

Genotype	Mild	Intermediate	Severe
-α/- α (n=25)	-	24%	76%
- α/αα (n=19)	-	31.6%	68.4%
αα/ αα (n=6)	-	-	100%

Effect of α –thalassemia on the hematological parameters of HbS/S patients

The presence of alpha-thalassaemia homozygosis (-α/-α) resulted in significantly higher mean hemoglobin (Hb) levels, hematocrit (HCT), red blood cells counts (RBC) but lower levels of fetal hemoglobin (HbF), mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) than the group with a normal alpha genotype (αα/αα). Patients with heterozygous alpha complement (–α/αα) showed intermediate mean hematological values. The overall distributions of hematologic parameters in Hb S/S patients with three different alpha-globin genotypes are summarized in Table 11.3.

Table 11.3. The effects of the various α-thalassemia genotypes on the mean hematological parameters in HbS/S patients regardless of the haplotype.

	Hb F(%)	Hb (g/dl)	HCT (%)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
-α/-α (n=55)	10.4	9.7	29.2	4.2	69.3	22.9	33.2
-α/αα (n=42)	10.8	9.1	26.7	3.3	79.9	27.3	34.2
αα/αα (n=28)	15.9	9.0	25.8	3.1	84.8	29.8	35.1

Influence of α-thalassemia on hematological parameters in HbS/S patients of different haplotypes

Alpha-thalassemia increased the mean Hb, HCT, RBC and lowerd HbF, MCV, MCH and MCHC in patients with Asian/Asian, CAR/Oman and CAR/CAR haplotypes (Table 11.4 a, e and f). In patients with Asian/Oman and Asian/CAR haplotypes the HbF level was however found increased (Table 11.4 b and c). In HbS/S patients of the severe Benin/Benin haplotype only a decreased MCV and MCH were observed. Data are summarized in Table 11.4.

Table 11.4. Effects of various α-thalassemia genotypes on the average hematological parameters in HbS/S patients based on their haplotype:

(a)Asian/Asian haplotype

	Hb F(%)	Hb (g/dl)	HCT (%)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
-α/- α (n=20)	13.5	9.6	29.1	4.1	69.9	23.1	33.1
- α/αα (n=9)	18.4	9.6	27.8	3.7	74.9	25.7	34.3
αα/ αα (n=18)	20.4	9.2	26.2	3.1	83.9	29.6	35.2

(b)Asian/Oman haplotype

	Hb F(%)	Hb (g/dl)	HCT (%)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
-α/- α (n=5)	9.7	11.2	33.5	5.0	66.6	22.3	33.4
- α/αα (n=7)	17.3	10.6	31.8	3.9	79.8	26.7	33.5
αα/ αα (n=2)	9.2	8.4	23.5	2.8	82.1	29.7	35.6

(c)Asian/CAR haplotype

	Hb F(%)	Hb (g/dl)	HCT (%)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
-α/- α (n=3)	12.7	10.1	29.9	4.1	74.5	25.2	33.7
- α/αα (n=3)	14.1	8.1	23.9	3.1	77.7	26.5	34.0
αα/ αα (n=2)	10.4	9.5	26.6	2.9	90.8	32.4	35.7

Table 11.4. Effects of various α-thalassemia genotypes on the average hematological parameters in HbS/S patients based on their haplotype (*Continued*):

(d)Benin/Benin haplotype

	Hb F(%)	Hb (g/dl)	HCT (%)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
-α/- α (n=16)	5.9	9.3	28.5	4.0	69.2	22.7	32.9
- α/αα (n=8)	3.6	8.7	26.1	3.4	76.2	25.5	33.4
αα/ αα (n=1)	4.2	11.0	34.6	4.3	80.1	25.6	32.0

(e)CAR/Oman haplotype

	Hb F(%)	Hb (g/dl)	HCT (%)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
-α/- α (n=5)	4.7	9.3	28.5	3.9	72	23.3	32.6
- α/αα (n=6)	5.3	8.3	23.7	2.8	84.9	29.3	34.6
αα/ αα (n=1)	5.0	9.1	25.8	2.9	87.9	31.0	35.3

(f)CAR/CAR haplotype

	Hb F(%)	Hb (g/dl)	HCT (%)	RBC (x10^{12}/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)
-α/- α (n=4)	4.6	9.2	27.4	3.9	68.7	23.1	33.6
- α/αα (n=5)	5.8	7.5	21.6	2.5	86.2	30.1	34.9
αα/ αα (n=4)	7.5	7.6	22.1	2.4	89.3	30.8	34.5

DISCUSSION

When performing correlation studies between genotype and phenotype it is important to compare cohorts that are not only phenotypically similar but genotypically identical in order to reduce genetic variables to a minimum. Therefore we have selected groups with identical genotypes and haplotypes with and without alpha thalassemia. Although external and accidental factors cannot be avoided, we do believe that our cohorts are as comparable as possible. As mentioned in the introduction, our study is not meant to be a detailed clinical report but rather a correlation study based upon the occurrence of general symptoms that may indicate mild, intermediate or severe conditions.

The frequency of alpha thalassemia in the present cohort (72%) was higher than the (58.3%) measured by our self in a previous study (8) and by AlKindi et al (48.5%) (7). This is probably due to a bias deriving by the selection of homozygous HbS/S patients with a much higher chance of being the progeny of consanguineous parents.

Previous and present studies

In a similar study reported by Mukherjee et al., all SCA patients from Western India with homozygous α-thalassemia had a mild phenotype (13). The clinical presentation of our total cohort with homozygous alpha-thalassemia ranged from mild (38.2%), to intermediate (21.8%) and to severe (40%) cases. Mukherjee et al. studied patients of the Asian/Asian haplotype

with high HbF levels (13, 14) and our data on the 47 patients with Asian/Asian haplotype shows that among cases with (-α/-α), 82.4 % were mild and 17.6% intermediate while no severe cases were present. Considering the variability of the definition "intermediate" our results are quite compatible with the observations of the Indian study and with the conclusions of other authors reporting milder conditions among Saudis and Kuwaitis carrying the Asian haplotype with α -thalassaemia, when compared with Asian haplotypes without α-thalassaemia (15). The same correlation was also observed in patients with Asian/Oman haplotype and (-α/-α) in which 75% had a mild disease and 25% were intermediate. On the other hand, our results also show that alpha thalassemia, although ameliorating the hematological parameters, is of little effect in reducing the symptoms of the HbS/S homozygous with the severe CAR and Benin haplotypes.

Hematological data and HbF

Alpha thalassemia is believed to improve the survival of the erythrocytes in SCA resulting in a milder form of anaemia due to decreased hemolysis (16). How relative can be the effect of alpha thalassemia in SCA is shown by many contrasting reports (17). In our study, the presence of alpha-thalassemia in Hb S/S homozygotes resulted in significantly higher mean Hb and HCT levels as well as higher RBC counts and these three parameters indicate a better RBC survival as a consequence of lower hemolysis. In addition, patients with homozygous alpha-thalassemia had on average lower levels of fetal Hb (HbF) and a lower MCHC than patients with a normal alpha globin genotype (αα/αα).

In our study the reduction of HbF expression was particularly relevant in patients with the mild "high HbF" (Asian) haplotypes and (-α/-α) alpha thalassemia. Conversely, in patients with severe "low HbF" haplotypes (CAR and Benin) the HbF remained either unchanged or increased in the presence of alpha thalassemia while little changes were observed in these patients also at the RBC level. Other studies have shown that coexistence of α-thalassemia enhances the levels of HbF associated with a specific haplotype such as the Senegal (18) and Benin haplotypes (19).

Higgs et al. (20) reported a decrease in the level of HbF in SCA patients with homozygous alpha-thalassemia while Embury et al. (21) reported an increase in HbF levels (9).

Higgs et al. (20) also observed that (-α/-α) individuals had the lowest cell volume and hemoglobin content per cell (MCHC) which reduce the polymerization risk of the Hb molecules in the smaller and less dense cells. In addition, low MCV in (-α/-α) generates a relatively larger cellular surface compared to (αα/αα) which might give the cells the property of increased membrane redundancy, providing a larger reserve of internal volume per given amount of polymer and thus giving protection against the deleterious consequences of membrane stretching during deoxygenation (21). We do believe that smaller cells (low MCV) might just be faster, passing the risk area of the post-capillary veins as this seems to be the same rheological advantage observed in mild SCA phenotypes with microcytic hypochromic parameters due to iron deficiency (12).

In conclusion the lower MCV, MCH and MCHC associated with alpha-thalassemia should diminish the amount of intravascular sickling while the decreased intra-erythrocytic hemoglobin S concentration associated with α-thalassemia should diminish polymerization (sickling) and herewith the degree of chronic hemolytic anemia typical of SCA (17). However, other factors (both genetic and environmental) are involved making the interpretation of phenotype/genotype association studies more complex.

CONCLUSION

Although some of the haplotype cohorts are small, it seems evident from the present study that alpha-thalassaemia can modulate the hematological picture of HbS/S but not clearly the overall severity manifestation in patients of all haplotypes. However, when the cohort was subdivided into larger groups of similar haplotypes, differences in the mild and severe forms became more evident. In addition, variability in clinical outcomes of sickle cell disease is not modulated by genetic factors alone, but also by environmental factors and life style.

REFERENCES

1. Ware RE and Aygun B. Advances in the use of hydroxyurea. American Society of Hematology. Educ Prog. 2009:62–69.
2. Steinberg MH. Predicting clinical severity in sickle cell anaemia. Br J Haematol. 2005; 129(4): 465-481.
3. Serjeant GR. Geography and the clinical picture of sickle cell disease. An overview. Ann NY Acad Sci. 1989;565:109-119.
4. Giordano PC, Huisman W and Harteveld CL. Iron depletion: an ameliorating factor for sickle cell disease? ISRN Hematol. 2011;2011:473152.
5. Kulozik AE, Wainscoat JS, Serjeant GR et al. Geographical survey of beta S-globin gene haplotypes: evidence for an independent Asian origin of the sickle-cell mutation. Am J Hum Genet. 1986;39:239-244.
6. Steinberg MH. Genetic etiologies for phenotypic diversity in sickle cell anemia. Scientific World Journal. 2009; 18(9): 46-67.
7. Alkindi S, Al Zadjali S, Al Madhani A, Daar S, Al Haddabi H, Al Abri Q, Gravell D, Berbar T, Pravin S, Pathare A, Krishnamoorthy R. Forecasting hemoglobinopathy burden through neonatal screening in Omani neonates. Hemoglobin. 2010; 34(2): 135-144.
8. Hassan SM, Hamza N, Jaffer Al-Lawatiya F, Jaffer Mohammed A, Harteveld CL, Rajab A,Giordano PC. Extended molecular spectrum of beta- and alpha-thalassemia in Oman. Hemoglobin. 2010;34(2):127-34.
9. El-Hazmi MAF. Clinical manifestation and laboratory findings of sickle cell anaemia in association with α–thalassaemia in Saudi Arabia. Acta haemat. 1985;74:155-160.
10. Van Delft P, Lenters E, Bakker-Verweij M, de Korte M, Baylan U, Harteveld CL, Giordano PC. Evaluating five dedicated automatic devices for haemoglobinopathy diagnostics in multi-ethnic populations. Int J Lab Hematol. 2009;31(5):484-495.
11. Phylipsen M, Yamsri S, Treffers E.E, Jansen D.T.S.L, Kanhai W.A, Boon E.M.J, Giordano P.C, Fucharoen S, Bakker E and Harteveld C.L. Non-invasive prenatal diagnosis of beta-thalassemia and sickle-cell disease using pyrophosphorolysis-activated polymerization and melting curve analysis. Prenatal Diagnosis 2012;32:578–587.
12. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of α-thalassaemia deletions and α-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol. 2000;108:295–299.
13. Mukherjee MB, Surve R, Tamankar A, Gangakhedkar RR, Ghosh K, Lu CY, Krishnamoorthy R, Colah R, Mohanty D. The influence of alpha-thalassaemia on the haematological & clinical expression of sickle cell disease in western India. Indian J Med Res. 1998;107:178-181.
14. Mukherjee MB, Lu CY, Ducrocq R, Gangakhedkar RR, Colah RB, Kadam MD, Mohanty D, Nagel RL and Krishnamoorthy R. Effect of a-Thalassemia on Sickle-Cell Anemia Linked to the Arab-Indian Haplotype in India. American Journal of Hematology. 1997;55:104–109.
15. Adekile AD and Haider MZ. Morbidity, bs haplotype and a-globin gene patterns among sickle cell anemia patients in Kuwait. Acta Haematologica. 1996;96:150–154.
16. de Ceulaer D, Higgs DR, Weatherall DJ, Hayes RJ, Serjeant BE, Serjeant GR. α-thalasemia reduces the haemolytic rate in homozygous sickle cell disease. N Engl J Med. 1983;309:189.
17. Ballas SK. Effect of alpha-globin genotype on the pathophysiology of sickle cell disease. Pediatr Pathol Mol Med. 2001; 20(2): 107-121.
18. Schroeder WA, Powars DR, Kay LM, Chan LS, Huynh V, Shelton JB and Shelton JR. Beta-cluster haplotypes, α-gene status and hematologic data from SS, SC, and S-β-thalassemia patients in Southern California. Hemoglobin. 1989;13:325-353.
19. Powars DR, Chan L, Schroeder WA. The influence of fetal hemoglobin on the clinical expression of sickle cell anemia. Ann NY Acad Sci 1989;565:262-178.

20. Higgs DR, Aldridge BE, Lamb J, Clegg JB, Weatherall DJ, Hayes RJ, Grandison Y, Lowrie Y, Mason KP, Serjeant BE, Serjeant GR. The interaction of alpha-thalassaemia and homozygous sickle cell disease. New engl. J. med. 1982;306:1441-1446.

21. Embury SH, Backer K, Glader BE: Monovalent cation changes in sickle erythrocytes: A direct reflection of a-globin gene number. J Lab Clin Med. 1985;106:75.

CHAPTER 12

MOLECULAR DIAGNOSTICS OF THE *HBB* GENE IN AN OMANI COHORT USING BENCH-TOP DNA ION TORRENT PGM TECHNOLOGY

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Blood Cells, Molecules and Diseases. 2014;53:133–137

ABSTRACT

Hemoglobinopathies, such as sickle cell disease (SCD) and beta thalassemia major (TM), are severe diseases and the most common autosomal recessive condition worldwide and in particular in Oman. Early screening and diagnosis of carriers is the key for primary prevention. Once a country-wide population screening program is mandated by law, a sequencing technology that can rapidly confirm or identify disease-causing mutations for a large number of patients in a short period of time will be necessary.

While Sanger sequencing is the standard protocol for molecular diagnosis, next generation sequencing starts to become available to reference laboratories. Using the Ion Torrent PGM sequencer, we have analysed a cohort of 297 unrelated Omani cases and reliably identified mutations in the beta globin (*HBB*) gene. Our model study has shown that ion torrent PGM can rapidly sequence such a small gene in a large number of samples using a barcoded uni-directional or bi-directional sequence methodology, reducing cost, workload and providing accurate diagnosis. Based on our results we believe that the Ion Torrent PGM sequencing platform, able to analyse hundreds of patients simultaneously for a single disease gene can be a valid molecular screening alternative to ABI sequencing in the diagnosis of Hemoglobinopathies and other genetic disorders in the near future.

INTRODUCTION

Severe Hemoglobinopathies (HBP), such as sickle cell disease (SCD) and thalassemia major (TM) are the most common recessive disorders in Oman and β-thalassemia and sickle cell disease carriers are widely present in the country. A national premarital screening program for the detection of carriers for primary prevention of β-thalassemia major and sickle cell disease is available but not mandatory by law. Following carrier detection at the haematological and biochemical level, a high-throughput screening approach is needed to facilitate a population targeted molecular analysis program for HBP (or any other common genetic disorder) for rapid confirmation. New DNA sequencing technologies and platforms are continuously being updated to accommodate the fast growth of science and research trying to improve molecular methods and techniques that can lead to fast and reliable molecular diagnosis. Next-generation sequencing technologies have been offering reliable approaches to rapid DNA genotyping. These newly evolved technologies have demonstrated advantages over Sanger sequencing by generating megabases to gigabases of data, allowing direct detection of sequence variants (1) in a very short period of time. If these new sequencing platforms are to be used routinely for diagnosis purposes, a number of factors such as sample scalability and cost should be addressed. Ion Torrent's Personal Genome Machine (PGM) (Guilford, CT, USA; now owned by Life Technologies, Carlsbad, CA, USA), is one of the options available (2) since late 2010 (3) and capable of generating 100 Mb of sequence data on a 314 chip, within several hours of machine run time (4). Sequence data are obtained by directly sensing the ions produced by template-directed DNA polymerase synthesis. The ion chip contains ion-sensitive, field-effect transistor-based sensors in 1.2 million wells on a 314 chip, which can measure independent sequencing reactions (3). DNA from different patients can be evaluated on the same sequencing chip using the barcoding methodology (5), allowing simultaneous sequencing for hundreds of patients.

To test the feasibility of using the Ion Torrent PGM for clinical variant analysis, we assessed its performance to detect variants in the beta globin gene (*HBB*) responsible for beta thalassemia and sickle cell disease. Most next generation sequencing studies involve investigating regions of interest of a large number of exons (up to 50,000 exons), from ten to thousands of genes on few patients (6). Instead, we have used the same platform to screen a large cohort of patients of 297 patients simultaneously for a relatively small single gene (*HBB*). Ion Torrent's PGM proved to be a valuable tool in identifying variants in high throughput analysis. It is suitable for large scale diagnostic screening for such a common disease gene, responsible for sickle cell disease and beta thalassemia in Oman.

METHODS

Sample collection and experimental design

We performed ion torrent PGM sequencing on 297 unrelated individuals. These samples were preselected based on the traditional hematological tests, Cell Blood Count (CBC) and High Performance Liquid Chromatography (HPLC) patterns. HPLC detects HbS carriers based on the appearance of an abnromal fraction and a beta-thalassemia carrier by an elevated

HbA_2 fraction. Patients suspected to be carriers of beta thalassemia (n=137), beta thalassemia major (n=15) and sickle cell disease (n=132) as well as normal controls (n=13) were all involved in the molecular study. A signed consent form of each patient has been provided by their designated clinician. DNA was isolated from peripheral blood using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). To cover the common mutations, including the

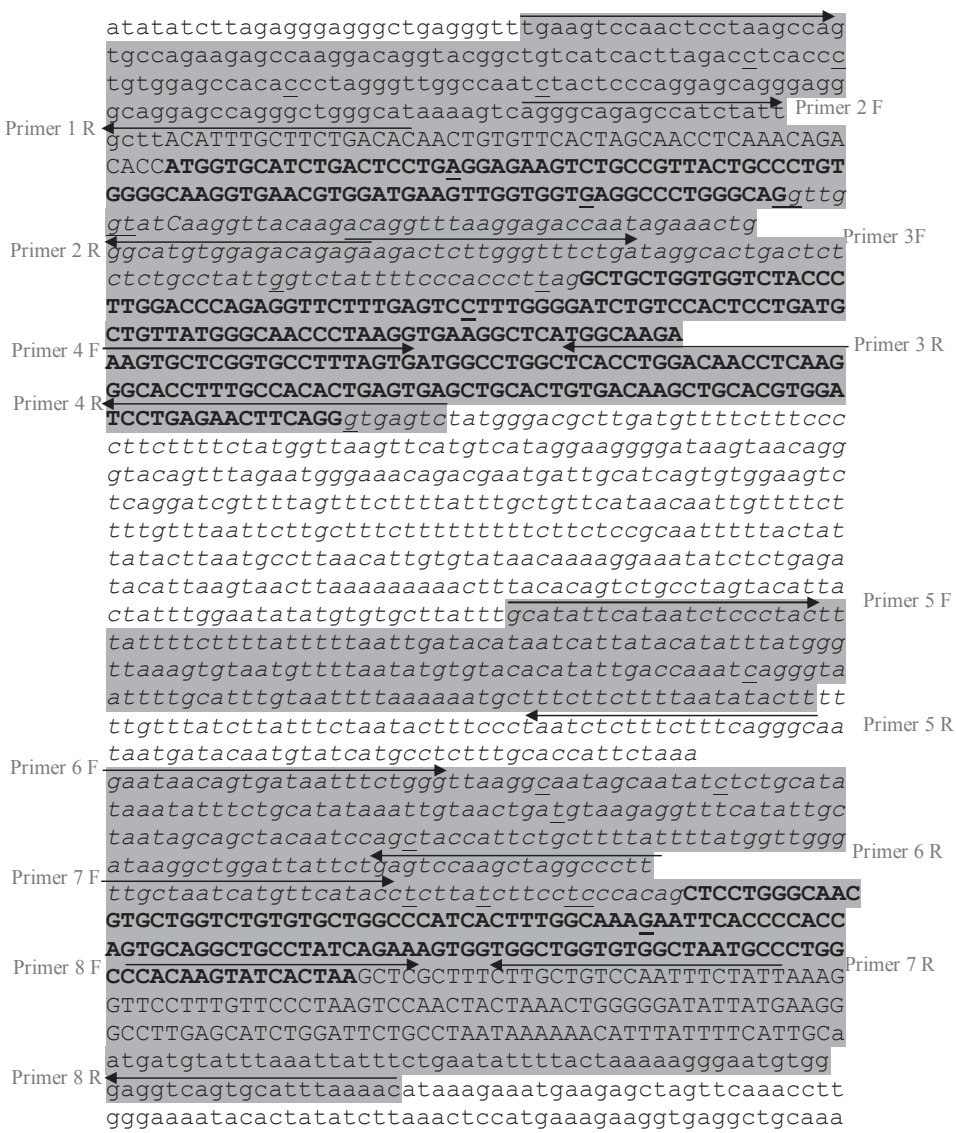

Figure 12.1. A schematic presentation of the beta globin gene (*HBB*). Exons are indicated in bold and introns in italics. The positions of the PCR primers are indicated as arrows above the sequence and the PCR products are highlighted. The frequently occurring mutations in the Omani population are underlined.

full coding regions, in the *HBB* gene, 8 separate PCR primer pairs were designed using the Primer3 oligonucleotide design tool (7). The primers sequences and the product size for each amplicon are listed in Table 12.1. The location of the primers within the *HBB* gene is depicted in Figure 12.1 covering the mutation spectrum present in the Omani population (11) that are underlined in the figure.

Ten different barcode primers consisting of 6 nucleotides were designed. For each fragment, the 5' end of the forward primer was tagged with M13 and the 10 barcodes giving 10 different primer sequences, whereas the 3' end of the reverse primer contained the P1-adapter sequence. Furthermore, the A-adapter - M13 primer was also tagged with the same 10 barcodes to enable indexing of a large number of patients using a relatively limited set of only 10 different barcodes (Figure 12.2); thus 10 barcoded target forward primers were designed to be combined with 10 barcoded A- adapter primers for a total of 100 (10x10) barcode combinations to perform

Table 12.1. Primer sequence of each fragment including the product size and the optimum annealing temperature.

Fragment	Primer	Fragment size	Tm
1	**Fw**: TGAAGTCCAACTCCTAAGCCA		
	Rv: GTGTCAGAAGCAAATGTAAGC	189 bp	55 °C
2	**Fw:** AGGGCAGAGCCATCTATT		
	Rv: TCTCTGTCTCCACATGCC	230 bp	55 °C
3	**Fw**: GAAGACTCTTGGGTTTCTGA		
	Rv: CTTGAGGTTGTCCAGGTGA	229 bp	56 °C
4	**Fw**: AAGTGCTCGGTGCCTTTAGTG		
	Rv: AGAAGGGGAAAGAAAACATCAAG	155 bp	55 °C
5	**Fw**: GCATATTCATAATCTCCCTAC		
	Rv: GCCCTGAAAGAAAGAGATTA	221 bp	55 °C
6	**Fw**: GAATAACAGTGATAATTTCTGGG		
	Rv: AAAGGGCCTAGCTTGGACTC	188 bp	55 °C
7	**Fw**: TGCTAATCATGTTCATACC		
	Rv: AATAGAAATTGGACAGCAAG	186 bp	53 °C
8	**Fw**: CCACAAGTATCACTAAGCTC		
	Rv: GTTTTAAATGCACTGACCTC	218 bp	53 °C

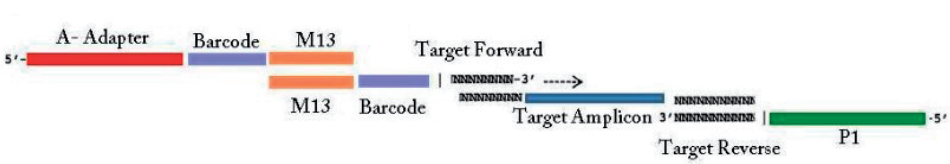

Figure 12.2. Schematic representation of the PCR showing the dual barcode design.

Table 12.2. List of the 10 barcode sequences used in this study model. The same 10 barcodes were tagged to the A-adapter-M13 primer and to the forward primer, giving (10x10) 100 different combination sequences for tagging/identification of 100 samples per run.

Barcode no.	Barcode sequence
1	GGTAAC
2	GAGAAC
3	GGATTC
4	AAGATC
5	AGGAAC
6	AAGTTC
7	TGATTC
8	GATAAC
9	CGGAAC
10	CCGAAC

analysis on 100 patients in a single run. The sequences of the barcodes are listed in Table 12.2. To ensure sequencing the total length of the amplified fragment, amplicon sizes were restricted to 230bp (including primers).

PCR conditions and library preparation

After PCR optimizations, amplifications were performed in a GeneAmp® PCR System 9700 (Applied Biosystems) in a total volume of 8µl PCR mix consisting of 1x PCR buffer, 1.8 mM $MgCl_2$, 200 µM dNTPs, 300 nM P1-adapter reverse primer, 300 nM A-adapter forward primer, 50 nM M13-target forward primer, 0.4 U FastStart High-Fidelity enzyme blend (5 U/µl, Roche Applied Science) and 10ng genomic DNA. Thermal cycling parameters were as follows: initial denaturation for 10 minutes at 95°C, followed by 35 cycles of 20s at 95°C, 30 s annealing at different temperatures (Table 12.1) and 40s at 72°C. Final extension was performed for 5 minutes at 72°C. The success rate of the amplifications was checked by adding 1µl of 20x EvaGreen® fluorescent dye (Biotium) to the wells of the PCR plate. By performing a melting analysis in the LC480 (Roche), the PCR yield of individual reactions could be visualised. PCR reactions deriving from 100 barcode-combinations were pooled according to their fluorescent levels after melting analysis. Size selection was done by isolating the correct band after electrophoresis on a 1.5% agarose gel. All fragments gave a single band except fragment 3 which had double bands. The correct band was cut out and the product was purified using the MinElute Gel Extraction Kit (Qiagen). The remaining pools were purified using Agencourt AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions. The concentration and integrity of the library was determined by measuring the sample with a Bioanalyzer on a High Sensitivity DNA chip Agilent Technologies. The final library concentration that was used for emulsion PCR was set at 25pM.

Emulsion PCR, sequencing and data analysis

Emulsion PCR was performed using the Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies) and the percentage of ion sphere enrichment was checked with a Qubit fluorometer (Life Technologies). Sequencing was done by using the Ion PGM Sequencing 200 Kit. Mutation analysis was done on patient-specific files using the NextGEN software version 2.3 (Softgenetics). Sanger sequencing was performed on few samples that were found to have a normal gene sequence in the carriers and one mutation in the homozygotes by ion torrent analysis.

RESULTS

Using our design, we were able to achieve overall very good coverage of the targeted regions. The coverage output for the first 100 patients is shown in Figure 12.3. Nearly identical coverage results were obtained from the other two runs. A subset of the base pair changes detected (Table12.3) were observed in all sequences of fragment 3 in controls and patients and therefore were excluded as artefacts. Seven patients that were suspected to carry one or two β-thalassemia mutation, revealed a normal sequence in the carriers and one mutation in the compound heterozygous diseased cases. These cases were re-sequenced by Sanger sequencing using a PCR with different primers and it confirmed the presence of one mutation in the *HBB* gene in the heterozygotes and two mutations in the compound heterozygotes. Four cases were found to carry the HbE mutation (Cd26 GAG>AAG), 2 in carriers and 2 in association with another β-mutation, and 3 cases to have the IVS-I-128 T>G mutation, 1 in a carrier and 2 in association with another β-mutation. To investigate why the Ion Torrent PGM sequencer did not detect these 2 mutations, another Ion Torrent run was performed on the same 7 samples for the 2 fragments that carried the mutation identified by Sanger sequence; fragment 2 and fragment 3. This was performed using a bi-directional method with a reverse barcode primer in addition to the forward barcode primer in order rule out strand-specific effects. The HbE mutation was detected by bi-directional sequencing but ambiguous results still remained in the 3 cases. Further investigation showed that the reason for these ambiguous results was that the primers used were not *HBB* specific and that the *HBD* gene was co-amplified generating a mixed sequence. The mutations found in the *HBB* gene are summarised in (Table 12.4).

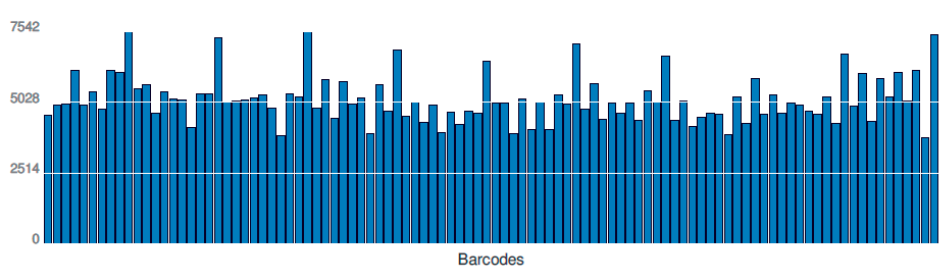

Figure 12.3. Coverage output of the evenly distributed sequence covering the first 100 samples.

Table 12.3. Summary of the artefacts obtained by the Ion Torrent sequence software due to the co-amplification of *HBB* and *HBD* fragments, showing the corresponding position in the *HBB* and *HBD* genes. These artefacts reside within fragment 3 of the *HBB* gene.

Artefact given by the Ion Torrent softaware in the HBB gene	Corresponding *HBB* position and nucleotide	Corresponding *HBD* position and nucleotide
c.[93-30G>C]	IVS-I-101, C	IVS-I-101, G
c.[93-28C>G]	IVS-I-103, G	IVS-I-103, C
c.93-24_93-23delAT	IVS-I-107 - 108, AT	IVS-I-107 - 108, TG (AT is deleted)
c.[93-19A>C]	IVS-I-112,T	IVS-I-112,T
c.[93-16T>C]	IVS-I-115,A	IVS-I-115,T
c.[93-9G>A]	IVS-I-122, C	IVS-I-122, C
c.[93-3A>G]	IVS-I-128, T	IVS-I-128, G
c.[93C>T]	Cd30, G	Cd30, A
c.[94G>A]	Cd31, C	Cd31, T
c.[96C>T]	Cd31, G	Cd31, A
c.[151T>A]	Cd50, A	Cd50, T

Table 12.4. List of mutations found in the Omani population by the ion torrent PGM sequencer. (HO = Homozygous, HR =Heterozygous).

Genotype	β-mutation HGVS name	no. of patients
Cd6 GAG>GTG (HO)	HBB:c.20A>T	85
IVS-I-5 G>C (HR)	HBB:c.92+5G>C	80
Cd6 GAG>GTG/IVS-I-5 G>C	HBB:c.20A>T/HBB:c.92+5G>C	26
Cd121 GAA>CAA (HR)	HBB:c.364G>C	9
Cd44 (-C) (HR)	HBB:c.135delC	9
IVS-I-1 G>A (HR)	HBB:c.92+1G>A	6
IVS-II-1 G>A (HR)	HBB:c.315+1G>A	6
IVS-I-3' (-25bp del) (HR)	HBB:c.93-21_96del	5
Cd39 CAG>TAG (HR)	HBB:c.118C>T	5
IVS-I-5 G>C (HO)	HBB:c.92+5G>C	4
Cd6 GAG>GTG/Cd44 (-C)	HBB:c.20A>T/HBB:c.135delC	4
Cd6 GAG>GTG/Cd121 GAA>CAA	HBB:c.20A>T/HBB:c.364G>C	4
Cd6 GAG>GTG/IVS-I-1 G>A	HBB:c.20A>T/HBB:c.92+1G>A	3
Cd6 GAG>GTG/Cd39 CAG>TAG	HBB:c.20A>T/HBB:c.118C>T	3
Cd5 (-CT) (HO)	HBB:c.17_18delCT	3
Cd30 AGG>ACG (HR)	HBB:c.92G>C	3
Cd26 GAG>AAG (HR)	HBB:c.79G>A	2
Cd44 (-C) (HO)	HBB:c.135delC	2

Table 12.4. List of mutations found in the Omani population by the ion torrent PGM sequencer. (HO = Homozygous, HR =Heterozygous). (*Continued*)

Genotype	β-mutation HGVS name	no. of patients
Cd6 GAG>GTG/IVS-II-1 G>A	HBB:c.20A>T/HBB:c.315+1G>A	2
Cd5 (-CT) (HR)	HBB:c.17_18delCT	2
Cd8 (-AA) (HR)	HBB:c.25_26delAA	2
5′ (-88) C>A (HR)	HBB:c.-138C>A	2
Cd6 GAG>GTG/Cd26 GAG>AAG	HBB:c.20A>T/HBB:c.79G>A	1
IVS-II-1 G>A (HO)	HBB:c.315+1G>A	1
IVS-I-5 G>C/IVS-I-3′ (-25bp del)	HBB:c.92+5G>C/HBB:c.93-21_96del	1
IVS-I-128 T>G (HR)	HBB:c.93-3T>G	1
Cd5 (-CT)/3′(+113) A>G	HBB:c.17_18delCT/HBB:c.+113A>G	1
3′(+108) - 3′(+112) 5nt del (HR)	HBB:c.+108_+112delAATAA	1
Cd26 GAG>AAG/IVS-I-3′ (-25bp del)	HBB:c.79G>A/HBB:c.93-21_96del	1
Cd129 TGC>TGT (HR)	HBB:c.389C>T	1
Cd22 GAA>TAA (HR)	HBB:c.67G>T	1
IVS-I-5 G>C/IVS-I-128 T>G	HBB:c.92+5G>C/HBB:c.93-3T>G	1
IVS-I-6 T>C (HR)	HBB:c.92+6T>C	1
Cd6 GAG>GTG/Cd121 GAA>AAA	HBB:c.20A>T/HBB:c.364G>A	1
Cd6 GAG>GTG/Cd6 GAG>AAG	HBB:c.20A>T/HBB:c.19G>A	1
Cd6 GAG>GTG/Cd5 (-CT)	HBB:c.20A>T/HBB:c.17_18delCT	1
Cd6 GAG>GTG/IVS-I-128 T>G	HBB:c.20A>T/HBB:c.93-3T>G	1
Cd121 GAA>CAA/IVS-I-1 G>A	HBB:c.364G>C/HBB:c.92+1G>A	1
Cd(8/9) +G (HR)	HBB:c.27_28insG	1
Normal controls		13
Total		297

DISCUSSION

The purpose of this study was to investigate if Ion Torrent PGM can be a suitable diagnostic method to sequence large number of samples for a small sized gene (e.g *HBB*) for a country wide Hemoglobinopathy screening program. Samples were preselected to contain 1 or 2 mutations in the beta globin gene by haematological and HPLC screening. Our primer design almost covered the complete *HBB* mutation spectrum worldwide except for a region in intron 2 as mutations in this region are very rare in the general population, at least not present in the Arabian area and not in the studied Omani population.

The reason for the observed artefacts in our samples and the discrepancy obtained in few cases was due to the co-amplification of the *HBD* gene in fragment 3. Existing sequence homology co-amplification is not always easily prevented. However, it should not cause a problem when data analysis is performed carefully and when necessary, Sanger sequencing

can be used as a confirmatory test. Moreover, although we have shown that a uni-directional method, by using a single barcode primer in the forward direction, is sufficient to give a unique sequence for each sample, it is always recommended to use bi-directional sequencing to discriminate between strand-specific artefacts and real mutations.

Based upon our results, we believe that Ion Torrent PGM could be a convenient option for diagnostic screening. One advantage of the PGM is its speed (8) and its ability to prepare a low-cost library with many different sample tags, allowing large number of samples to be pooled together and thus dividing sequencing costs among that many samples, generating sufficient data in a short period of time and avoiding lengthy hours of waiting to run samples in several batches. Moreover, PGM requires much less maintenance when compared to Sanger sequencing (4). Another advantage is that it uses a sequencing strategy where hydrogen ions (H^+) are detected (8), thus, no lasers, cameras or fluorescent dyes are needed, making it a cost-reasonable machine to purchase. Also, ion torrent's sequencing chips match the common manufacturing standards used for commercial microchips, meaning that it can be used for other technologies at equal low cost (9). Moreover, Ion Torrent requires as little as 10ng/ul of DNA to generate accurate results for challenging samples (10) whereas the traditional method of Sanger sequencing requires higher amount of DNA. Alternatively buccal swaps may be used instead of blood in a large scale screening programs. Although we have not tested the use of buccal swap DNA on Ion Torrent, we do believe that it should not be a problem for rapid population screenings. On the other hand, using buccal swap means having the DNA sample to be directly sequenced without haematological tests (CBC, HPLC) may save time and costs but will not provide with supportive data.

We have shown that the Ion Torrent PGM can be used to sequence relatively small genes (e.g *HBB*) of a large number of specimens rather than its traditional practice of sequencing highly complex genes on a small number of patients. However, before introducing Ion Torrent PGM sequence as a new diagnostic tool, few technical parameters should be focused at. First, manual PCR plate preparation raises chances of error; secondly, preparation of PCR plates is a lengthy procedure when multiple fragments are to be looked at. However, this could be avoided by the possibility of performing a multiplex PCR, amplifying the 8 fragments in a single PCR reaction in our case, thus, having 8x less PCR work. The reason why we amplified the fragments separately in this study was to ensure even coverage of every fragment since we used for the first time 10 x 10 combined barcodes to sequence 100 patients per run to have a robust and controllable amplification. To make PGM more feasible, more barcodes can be used to run as many patients (up to 400 samples) per chip. The reason why we have used a uni-directional sequencing in the first place was to reduce the number of primers and herewith the costs. However, when strand-specific sequence artefacts are expected, it is optimal to sequence bi-directionally. Economically speaking, cost will always remain an issue to see how worthy the new instrument can be in routine molecular analysis. However, increasing the sample size from 100 to 400 per run will reduce the costs per sample considerably and as the price per base continues to drop, PGM will soon be applicable routinely.

In conclusion, phenotypes cannot be predicted, explained or treated without determining the genotype. Our results show that the Ion Torrent Personal Genome apparatus may allow

genome sequencing in specialized reference labs and could be used within the screening program in Oman. With the new technology of PGM, multiple DNA sequence reads can be produced in a single run, generating sufficient data to solve the clinical and epidemiologic problems in a short time. Technically, the key to success would be to choose a methodological approach that avoids massive uneven PCR efficiencies and biased quantification and thus sequencing errors. A limitation of the ion torrent is the errors that arise from homo-polymeric stretches (4) that are being under- or over- called. However, *HBB* is very suitable to be analyzed by Ion Torrent PGM as it does not have homopolymers interfering with proper base-calling. Considering all that, Ion torrent could be a suitable technology for a national population screening program in Oman or elsewhere once DNA testing for HBP (or other diseases) becomes mandatory prior to marriage, promoting a quick tool of identification (diagnosis) and thus early prevention.

ACKNOWLEDGEMENTS

The authors declare to have conducted this study according to local ethical regulations and to have no conflicts of interest on the presented matters. Acknowledges goes to the Sardinian Regional Government for the financial support of P.O.R. Sadegna F.S.E. Operational Programme of Autonomous of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective I.3, Line of Activity I.3.1.

REFERENCES

1. Metzker ML. Sequencing technologies-the next generation. Nat Rev Genet 2010;11: 31-46.
2. Pourmand N, Karhanek M, Persson HH, et al. Direct electrical detection of DNA synthesis. Proc Natl Acad Sci USA 2006;103: 6466-6470.
3. Rothberg JM, Hinz W, Rearick TM, et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature. 2011;475:348-352.
4. Elliott AM, Radecki J, Moghis B, Li X, and Kammesheid At. Rapid Detection of the ACMG/ACOG-Recommended 23 *CFTR* Disease-Causing Mutations Using Ion Torrent Semiconductor Sequencing. Journal of Biomolecular Techniques. 2012; 23:24–30.
5. Knapp M, Stiller M and Meyer M. Generating barcoded libraries for multiplex high-throughput sequencing. Methods Mol Biol, 2012;840:155-170.
6. Dahl F, Stenberg J, Fredriksson S, Welch K, Zhang M, Nilsson M, Bicknell D, Bodmer WF, Davis RW and Ji H. Multigene amplification and massively parallel sequencing for cancer mutation discovery. Proc Natl Acad Sci. 2007;104:9387-9392.
7. Rozen S and Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000 132: 365-386.
8. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J and Pallen MJ. Performance comparison of benchtop high-throughput sequencing platforms. Nature biotechnology. 2012;30(5): 434-439.
9. Bolotin DA. Next generation sequencing for TCR repertoire profiling: platform-specific features and correction algorithms. Eur J Immunol. 2012; 42(11): 3073-3083.
10. Duhaime MB, Deng L, Poulos BT and Sullivan MB. Towards quantitative metagenomics of wild viruses and other ultra-low concentration DNA samples: a rigorous assessment and optimization of the linker amplification method. Society for Applied Microbiology and Blackwell Publishing. Environmental Microbiology. 2012;14(9): 2526-2537.
11. Hassan SM, Hamza N, Al-Lawatiya F, Jaffer AM, Harteveld CL, Rajab A,Giordano PC. Extended molecular spectrum of beta- and alpha-thalassemia in Oman. Hemoglobin. 2010;34(2):127-34.

CHAPTER 13

PRIMARY PREVENTION OF HEMOGLOBINOPATHIES BY PRENATAL DIAGNOSIS AND SELECTIVE PREGNANCY TERMINATION IN A MUSLIM COUNTRY: OMAN

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Thalassemia Reports. 2014;4(4171),19-21

ABSTRACT

Hemoglobiopathies (HBP) are the most common genetic disorder in Oman and are in need of prevention programs due to the high incidence of beta-thalassemia major and sickle cell disease. Prenatal diagnosis (PD) and selective pregnancy termination is shown to be the most effective prevention tool for the control of HBP. However, PD is not available in Oman thus far because abortion is subject to religious, cultural and ethical issues. We have examined the attitude of a number of Omani HBP carrier couples towards prenatal diagnosis and selective abortion. We have interviewed 35 couples at risk visiting the main pre-marital clinic in Muscat between Jan 2011 and Jan 2012. Couples were interviewed using a pre-structured questionnaire. The majority would have accepted prenatal diagnosis (94%) if the service would be available in the country but pregnancy termination was greatly influenced by religious values.

INTRODUCTION

Severe hemoglobinoapthies (HBP), such as β-Thalassemia major (TM) and sickle cell disease (SCD), are endemic in Oman. These diseases cause a huge suffering to patients and families and create an enormous healthcare burden to the country. Around ~6% of the population are carriers of sickle cell whereas about ~3% are carriers of beta-thalassemia (1,2).

The diseases can be prevented by screening carriers before marriage, before reproduction or in early pregnancy and by offering genetic counselling. If the couples are already married, the most usual preventative option is prenatal diagnosis (PD) which is offered to couples at risk in many countries (3,4). In order to implement PD in Oman, one should know if these interventions would be accepted by the population and if pregnancy termination, in case of a severely affected foetus, would be accepted since selective abortion on medical indication is restricted in Oman because of religious, legal, ethical and social implications. Omanis are Muslims with a high rate of consanguineous marriages and couples at risk for HBP are therefore quite common. If these couples are to seek for PD, they are forced to do it abroad.

Different Muslim countries and institutions have different opinions towards medical abortion. Most prohibit pregnancy termination at any time unless the mother's life is at threat. Others allow it only within the first 120 days of gestation if the foetus is severely affected (5). In some Muslim Arab countries such as Tunisia (6) and Egypt (7), PD and interruption of pregnancy is well accepted and currently offered to couples at risk to prevent severely affected progeny.

Premarital screening for β-thalassemia is subsidised and funded by the Omani government but it is not mandated by law as it is the case with neighbouring Muslim countries such as UAE and Iran. The national prevention program in Oman involves; carrier detections, molecular diagnostics in case of un-clear haematological findings, and genetic counselling. The prevention option thus far is not to marry if both partners are at risk. This study evaluates the implementation of HBP prevention in Oman in view of carrier couples towards PD and pregnancy termination.

MATERIAL AND METHODS

Data were obtained from the primary pre-marital clinic in Muscat. Detection of carriers was confirmed by haematological tests. A questionnaire was designed (Appendix) and couples in which both partners were carriers were surveyed. The survey questionnaire included sex, age, degree of consanguinity, risk acceptance and opinion concerning prenatal diagnosis and medical abortion in case of an affected foetus. The concept of prenatal diagnosis was explained to all participants. The median age of participants was 25 years old and all participants were of Omani nationality and Muslim.

RESULTS

From 420 couples visiting the main pre-marital clinic in Muscat between January 2011 to January 2012, 38 couples were found in which both partners were carriers of either beta-thalassemia trait or sickle cell trait. Three carrier couples did not want to undergo the survey, so 35 couples were interviewed and it was found that 40% of the couples were first cousins. Twenty five

couples (71%) underwent premarital testing and genetic counselling prior to marriage while the remaining 29% visited the premarital clinic after getting married and after getting an affected child. Out of the 25 couples who received counselling prior to marriage, 10 couples still decided to continue with the marriage while 15 broke the engagement and went to seek for a "non-carrier" partner. Out of the 10 couples who got married, 6 couples accepted the risk of getting an affected child while 4 couples did not and decided not to get any children. From the carrier couples who were counselled after getting an affected child, 20% travelled abroad for PD. The remaining decided not to get pregnant again. Out of the 35 couples, 33 couples would have undergone prenatal diagnosis if the service was available in the country but out of these 33 couples, 10 would not undergo selective abortion even if it was religiously allowed. Results are summarised in Table 13.1.

Table 13.1. Summary of the Omani couple views towards PD and pregnancy termination of affected foetus.

no. of couples interviewed	**35**
no. of consanguineous	14
no. in which both couples are carriers	35
no. of couples counselled before marriage	25
no. of couples counselled after marriage	10
no. of couples who would go for PD	33
no. of couples who would abort if religiously allowed	25

We further surveyed 20 selected couples in which only one partner was found to be a carrier; 10 male carriers engaged to normal female partners and 10 female carriers engaged to normal male partners to see if there is any bias between the different genders in marrying a carrier partner. Among the first group, 4 out of 10 were first cousins and one couple broke the engagement after finding out the female was a carrier. Among the second group, 2 out of 10 were first cousins and all couples continued with their marriages.

DISCUSSION

Prenatal diagnosis (PD) is a highly sensitive issue in Oman. In order to establish PD service in the country, awareness and knowledge should be clearly addressed to the targeted group and religious authority for a further discussion on the issue of medical abortion in case of a severely affected foetus. Based on our survey, the majority of the informed couples accepted to undergo PD if the service would be available in Oman but only if termination of pregnancy would be approved by the country's Mufti.

The majority of the participants were unaware of the Fatwa approved in some Arab-Muslim countries allowing selective abortion within the first trimester in case of an affected foetus based upon Islamic jurisprudence (Council of the World Islamic League, 15-22/07/1410 Hijri/ 10-17 February 1990). However, this Fatwa is not accepted by all Muslim leaders/scholars.

Participant's attitude was greatly influenced by Oman's Mufti's opinion and country's policy. Although it might seem reasonable, the relation between PD and abortion is not absolute. As a matter of fact, in countries offering PD, couples getting a positive PD result may still decide to keep the affected foetus. On the other hand, because of the 1-2% chance of losing a foetus due to procedure risk, PD is discouraged if the couples are not planning to interrupt pregnancy in case of a severely affected foetus. Therefore the abortion debate should not be an issue for health care leaders to discourage the implementation of a national PD service. PD can help the couple to decide whether or not to interrupt the pregnancy abroad or can prepare them if they decide to keep an affected child so they are mentally and psychologically ready on how to cope with such a situation. PD would be a useful tool for clinicians as well in order to plan a properly tailored treatment at an early stage.

Although the majority of our enquired couples were in favour of PD, accepting abortion was tightly bound to religious rules. This emphasise the critical role of religion in decision making in the Omani population who are known for their strict Islamic practice in the Arabian Gulf. The same impact of religious convictions on accepting PD has also been previously described in Saudies (8).

In a number of Muslim countries, the national premarital programs are mandatory and aimed at limiting marriage between carriers. In Iran, renouncing to marriage have been gradually replaced by PD and selective abortion and national incidence of TM has fallen from over 2000 cases a year to nearly zero affected newborn in some well managed regions of the country (9). Iran, with the majority being of Shia'a sub-religion, some of the Shia'a scholars allowed medical abortion before the 16th week of gestation under certain circumstances (10).

Although most Muslim countries are of Sunni sub-religion, some countries have accepted pregnancy termination under specific circumstances while others prohibited it absolutely. This is due to the four orthodox divisions in the Sunnis leading to diverse opinions towards medical abortion (11) as well as to the legal base system in a country which might not be exclusively religion-based but could be civil-based or a combination of both (11).

In Oman, medical abortion is thus far prohibited by religion and law. The majority of the people in Oman are of Abadhi sub-religion. The main Abadha scholar in Oman, who is also the country's Mufti, believe that human should not interfere with God's will and perceive that termination of a pregnancy should only take place if the pregnancy is putting the mother in a life threatening condition.

Despite well organised pre-marital clinics, the birth of HBP children is still very high in Oman. A large portion of the population may remain un-diagnosed due to social stigmatisation and unawareness. The concept of recessive disease might be difficult to understand for non-medically-educated couple and the reaction "why should I get an affected child if I am a totally healthy person?" might explain why people continue with their marriage plans regardless the 25% risk of getting a severely affected child.

Since PD is not offered to couples at risk in Oman thus far, the other primary prevention option besides not marrying, is selecting healthy gametes for fertilisation by pre-implantation genetic diagnosis (PGD) which is also not available in the country. PGD is permissible in Islam provided that the gametes are from the husband and wife (12). PGD may be socially a better option and more easily acceptable than PD in Muslim countries as parents do not

have to undergo the option of terminating the pregnancy (13). However, clinically it involves a considerable hormonal treatment for the woman. Moreover it is a technically complex procedure and has some limitation with concerns of multiple pregnancies or un-successful implantation and of course economically this procedure is quite expensive. Only couples who are financially capable to cover the costs can travel abroad for PGD where's the majority have to cope with the risk of getting another affected child or decide not to plan another pregnancy.

In a country such as Oman, where there are high rates of both consanguinity and hemoglobinopathies, it is the task of the health care authorities to debate the issue with the Country's Islamic leader or Mufti to consider medical abortion for severe genetic conditions. Moreover, public health authorities should be concerned with the awareness of the public and should educate couples at risk on the availability of alternative preventative services. For the time being, early population screenings and genetic counselling are the only available preventative measures for the control of sickle cell disease and β-thalassemia major. With good infrastructure and implementing successful strategies, incidence of hemoglobinopathies could be dramatically reduced in Oman.

ACKNOWLEDGEMENTS

The authors declare to have conducted this study according to local ethical regulations and to have no conflicts of interest on the presented matters.

REFERENCES

1. Al-Riyami AA, Suleiman AJ, Afifi M, et al. A community- based study of common hereditary blood disorders in Oman. East Mediterr Health J 2001;7:1004–11.
2. Alkindi S, Al Zadjali S, Al Madhani A, et al. Forecasting hemoglobinopathy burden through neonatal screening in Omani neonates. Hemoglobin 2010;34:135–44.
3. Hamamy HA and Al-Allawi NAS. Epidemiological profile of common haemoglobinopathies in Arab countries. J Community Genet. 2013;4:147–167.
4. Hoppe CC. Prenatal and newborn screening for hemoglobinopathies. Int J Lab Hematol. 2013;35(3):297-305.
5. Al Aqeel. Ethical guidelines in genetics and genomics. An Islamic perspective. Saudi Med J. 2005; 26(12): 1862-1870.
6. Chaabouni H, Chaabouni M, Maazoul F, M'rad R, Jemaa LB, Smaoui N, Terras K, Kammoun H, Belghith N, Ridene H, Oueslati B, Zouari F (2001) Prenatal diagnosis of chromosome disorders in Tunisian population. Ann Genet 44:99–104.
7. El-Beshlawy A, El-Shekha A, Momtaz M, Said F, Hamdy M, Osman O, Meshaal S, Gafaar T, Petrou M. Prenatal diagnosis for thalassaemia in Egypt: what changed parents' attitude? Prenat Diagn. 2012; 32:777–782.
8. Alkuraya FS and Kilani RA. Attitude of Saudi families affected with hemoglobinopathies towards prenatal screening and abortion and the influence of religious ruling (Fatwa). Prenat Diagn 2001; 21: 448–451.
9. Joulaei H, Shahbazi M, Nazemzadegan B, Rastgar M, Hadibarhaghtalab M, Heydari M, Ghaffarpasand F and Rahimi N. The Diminishing Trend of β-Thalassemia in Southern Iran From 1997 to 2011: The Impact of Preventive Strategies. 2014;38(1): 19-23.
10. Abolghasemi H,Amid A, Zeinali S, Mohammad H, et al. Thalassemia in Iran Epidemiology, Prevention, and Management. J Pediatr Hematol Oncol 2007;29:233–238.
11. Shapiro GK. Abortion law in Muslim-majority countries: an overview of the Islamic discourse with policy implications. Health Policy and Planning 2013;1–12.
12. Al-Sulaiman A, Al-Odaib A, Al-Rejjal R, Hewison J. Preimplantation genetic diagnosis in Saudi Arabia: parents' experience and attitudes. Prenat Diagn. 2010; 30:753–757.
13. Farra C, Nassar AH, Usta IM, Salameh P, Souaid M, Awwad J. Acceptance of preimplantation genetic diagnosis for beta-thalassemia in Lebanese women with previously affected children. Prenat Diagn. 2008; 28:828–832.

APPENDIX

Questionnaires used for the survey in this study

Name:
Tribe:
Sex:
Age:
Region:
Diagnosis:
Occupational status:
Relationship to partner (e.g 1st cousins)?
Screened prior/after marriage?
Counselled prior/after marriage?
Understood all the information provided by the counsellor? (yes/no).
Carried with the marriage regardless of the risk? (yes/no)
Accepted the risk of ¼ chance of getting an affected child? (yes/no)
Heard of PND before? (yes/no)
Would undergo PND if service is available in the country? (yes/no)
In case of carrying an affected fetus, would accept terminating the pregnancy? (yes/no) reasons? (e.g religion, legality, ethics, culture, personal believes?)

CHAPTER 14

SUMMARY, DISCUSSION AND CONCLUSION

Despite well-organized pre-marital clinics and the dramatic improvement in public health in the last few decades, the birth of children severely affected with sickle cell disease (SCD) or beta thalassemia major (BTM) is still high in Oman. Patients with severe hemoglobinopathoes (HBP) and their families are facing heavy suffering while treatment requires intensive, burdening and expensive efforts to be provided by public health which is offered free of charge to Omani citizens.

For further improvement of HBP management, effective prevention programs are needed. Better detection and counseling of couples at risk should be offered to make an informed reproductive choice possible for healthy carrier couples at risk of having children with severe hemoglobin disorders. Introducing these options is complex but essential and is currently limited by the restrictions imposed upon prenatal diagnosis (PD) and medical abortion in Oman.

Developing an efficient prevention strategy for HBP is a complex process both at the cultural and at the technical level. The essential technical and cultural elements involved in the process have been studied in this thesis providing the necessary knowledge to be used for the implementation of the most suitable strategy. Fundamental in the process towards establishing the necessary technical tools is an adequate knowledge of the molecular spectrum of HBP mutations in the country.

MOLECULAR SPECTRUM

We have studied the molecular spectrum of beta- and alpha- thalassemia in a large cohort of Omani patients demonstrating the widest spectrum of mutations reported thus far in the country, including the characterization of the most common mutations in the different regions of the country (Chapters 4-7). Our analysis has shown not only a broad range of common mutations, some of which are tribe specific and indicating a founder effect and genetic drift, but also many less common or rare mutations associated with historical migration patterns. From these studies, at least 32 different β-thal determinants were found among the Omanies (Table 5.1) with one novel beta-globin gene transversion in the promoter region (Table 6.2). In regard to the alpha globin gene, at least 21 defects were found with two novel mutations in the alpha 3.7 hybrid gene (Table 7.5). In Chapter 8, a total of 10 delta gene defect was characterised in the studied cases among which 2 novel delta determinants were found (Table 8.1). The necessity to characterize the molecular spectrum of delta globin gene defects is important in countries with high rate of beta thalassemia, especially when diagnosis of β-thalassemia carrier is based upon the measurement of HbA_2 fraction only as this can be overlooked when it is co-inherited with a delta mutation.

The broad range of HBP defects identified in these studies (Chapters 4-8), demonstrate the existence of a heterogenic pattern in the country accounting to gene-flow and the past trade with other countries. Molecular detection of hemoglobinopathies is essential for providing accurate genetic counseling following premarital screening as well as in tailoring treatment plan in affected patients.

Moreover thorough characterization of the spectrum of common and rare beta- and alpha- globin gene mutations in the Omani ethnic group allows the selection and application

of the most appropriate current molecular technologies. In addition the molecular spectrum is essential for genotype/ phenotype prediction and risk assessment, for treatment and prenatal diagnosis and also for designing advanced molecular methods for future application to detect all mutations simultaneously. For the latter we evaluated the use of the Ion Torrent PGM in beta thalassemia diagnostics (Chapter 12). Based on our findings, we have concluded that Ion Torrent could be a convenient sequencing platform for large scale diagnostic screening in Oman during premarital or in early pregnancy testing.

PHENOTYPE PREDICTION

Predicting the severity of the disease is not always straightforward. Also when the only available option is adapting partner choice, an accurate knowledge of the molecular spectrum examined in the totality of the genotype, is of great importance for a thorough premarital counseling of couples at risk. Therefore we have focused upon a number of technical elements essential for risk prediction and counseling and we have been able to show the following. Some defects, mild or silent, that cannot be easily detected at the hematological level may generate complex genotypes in association with the beta and/or alpha globin genes (Chapter 6).

Delta gene defects occur at a considerable frequency in Oman and may interfere with the diagnosis of beta thalassemia using hematological parameters (Chapter 8).

The co-existence of alpha thalassemia, very frequent in Oman, may modify the phenotype of the progeny of couples at risk and make risk prediction more complex. Therefore alpha thalassemia should not be overlooked during premarital screening when diagnosis is made on hematological parameters alone. If the couples are carriers of an alpha° or a relevant alpha+ defect, there is a 25% risk of getting a hydrops fetalis or severe HbH disease in the child and it is therefore necessary to perform molecular tests to define the alpha thalassemia defect and to differentiate between iron deficiency and/or alpha- or delta- thalassemia (Chapters 6 and 7).

PHENOTYPE PREDICTION AND CURE VERSUS TREATMENT

Treating a disease does not always lead to a cure. This is in general the case for most genetic disorders and for HBP's in particular.

Different HBP phenotypes may require different treatment and the only "cure option" for severe HBP is a successful stem cell transplantation using a HLA identical sibling as the donor. This expensive option can reduce morbidity and prolong survival but is not easily accessible to the great majority of HBP patients in Oman. Therefore we have focused in this thesis upon elements involved in modulating phenotype and influencing the efficacy of treatment.

We have conducted a countrywide analytical study on the correlation between the genetic makeup (beta-cluster haplotype, coexisting alpha-thalassemia and XmnI polymorphism) and phenotype (Chapter 9, 10 and 11) in patients with sickle cell disease (SCD).

Given the genetic heterogeneity of the Omani population, many different beta-cluster haplotypes were identified in SCD patients, and therefore we have explored the genotype-phenotype correlations which is important for healthcare providers so that better disease management of patients can be developed (Chapter 9).

We have further observed that the hyroxyurea drug (HU) can ameliorate the severe phenotype of the large majority of Omani SCD patients and that the XmnI C>T polymorphism is highly associated with a positive response to HU treatment. Few patients that did not show improvement after treatment with HU even after increasing the dose were all carries of an identical compound heterozygous beta gene cluster haplotype (Chapter 10). Thus we conclude that the presence of Xmn I polymorphism in Omani SCD population is a predictor of response to HU but other factors such as either haplotype and/or sub-haplotype are also involved. These findings allow an early planning for an alternative treatment for those who are less likely to respond to the drug.

Moreover we compared the clinical severity of SCD patients of identical homozygous HbS/S genotype that are categorized by identical beta globin cluster haplotype with different alpha globin genotypes and shown that alpha-thalassemia can modulate the hematological picture of Omani SCD patients but not clearly the overall severity manifestation in patients of all haplotypes (Chapter 11).

Overall, our results suggest that multiple determinants are involved in predicting disease severity in SCD patients and that these determinants should be considered for better assessment and treatment. Drawing correlation lines between the genetic make up and clinical severity is essential for prognostic purposes, accurate diagnosis and thus planning for the best tailored treatment.

PREVENTION OPTIONS

Primary prevention has been achieved in different countries through information and screening at different levels. School, premarital, early pregnancy and neonatal screenings have been applied with different results. The approach involving screening early in pregnancy followed by PD and eventually selective medical abortion has shown to be the most effective and the most accepted prevention method, lowering the incidence of severe HBP cases in many countries. Therefore in this thesis we have studied the attitude of a representative Omani cohort toward prevention by PD and medical abortion in view of the ethnic, cultural and religious backgrounds (chapter 13).

In our survey we have found that the majority of the couples inquired would have chosen for PD if this service would have been available in Oman but only if termination of pregnancy would be approved by law and by the country's main religious Mufti.

The issue of PD and selective termination of pregnancy in case of an affected fetus has been debated and legally approved in different Muslim countries but for the time being, early population screenings and the advice not to marry for carrier-couples at risk are the only available preventative measures in Oman. However, often the advice not to marry is ignored and high rates of both consanguinity and HBP frequency would benefit from the acceptability and availability of PD and medical abortion.

The results of our enquiry and the incidence of the diseases in the country show that these issues should be discussed and solved with the approval of the country's Islamic leader (Mufti) and public health authorities and that appropriate prevention measures should be offered to the many couples at risk in the country. Moreover, public health authorities should be

concerned with the awareness of the population and should educate couples at risk on the availability of alternative preventative options.

IN CONCLUSION

This thesis has investigated most of the technical aspects necessary for a state of the art prevention programme for HBP in Oman. Currently the most rational way to offer appropriate prevention in Oman, whilst simultaneously respecting cultural and religious aspects, is to provide the relevant information and screening at the preconception level because it leaves either the option not to marry or to seek PD and medical abortion abroad (the latter for those couples who can afford it). With appropriate infrastructure this strategy could dramatically reduce the incidence of severe hemoglobinopathies in Oman and herewith, the suffering of patients and parents and the need for long lasting supporting therapy with the associated financial burden on the public health budget.

SUMMARY/SAMENVATTING

SUMMARY

Hemoglobinopathies (HBP) are the most common autosomal recessive genetic disorder world-wide and in particular in the African continent, India, Far East, the Mediterranean and Middle Eastern countries including Oman. HBP can be the consequence of mutations causing structural abnormality in the hemoglobin molecule (abnormal hemoglobins) or a reduction/ abolishment in the overall synthesis of the hemoglobin components (thalassemia), leading to anemia. Carriers are usually asymptomatic but carrier couples are at 25% risk of getting a severely affected child.

One of the main challenges faced by most Arab countries and by Oman in particular are the high prevalence rates of HBP [mainly sickle cell disease (SCD) and beta thalassemia major (TM)]. The prevalence is high in these areas due to the positive selection in the presence of malaria tropica. Moreover, due to consanguinity, a socio-cultural habit, the incidence of severely affected children is high in Oman. Around 10 % of Omanis are SCD carriers, 3 % carry a defect causing β-thalassemia and at least half of the population are carriers of α-thalassemia. Although the disease could be treated, there is no definite cure until now except for a matched bone marrow transplant. Public health authorities have focused not only on state of the art management and patient care but also on prevention. National premarital clinics aiming at identifying partners at risk prior reproduction and offering genetic counselling and prevention have been working in the country for the last decade. The focus of this thesis is to study the molecular spectrum of HBP and the associated genetic determinants to work towards the development of prevention strategies for severe HBP's in Oman.

In order to develop and improve risk assessment during pre-matrimonial counselling we have defined the molecular spectrum of the disease all around the country. In Chapter 4, we present a first study on 87 un-related Omanies either heterozygous or homozygous for beta thalassemia mainly coming from four different regions in Oman. We found 11 beta determinants with at least 56% of the cases with heterozygosity or homozygositiy for the common alpha-thalassemia deletions; alpha 3.7kb and/or alpha 4.2kb. We have further extended our analysis to reveal a broader spectrum of beta globin gene mutations in Chapter 5, studying larger number of subjects (n=446) of different tribal origin, covering all the seven regions in the country. Thirty-two different beta mutations were identified with 11 being described for the first time among the Omani population. We then analysed the alpha globin gene defects in details in Chapter 7. A total of (n=634) subjects were divided into seven groups based on their hematological readings and were analysed at the molecular level. Twenty-one different alpha defects were categorised of which 15 were described for the first time among Omanies with two defects presumed to be new. We further reported in Chapter 6 two new cases among the Omani. The first is an alpha variant found in a consanguineous couple that probably resulted in a severe fetal hemolytic anemia while the second is a novel β-globin gene promoter mutation associated with borderline/slightly elevated HbA_2, indicating a very mild de novo β^+ thalassemia mutation. The high heterogeneity of common, rare and novel beta- and alpha- globin genes defects observed among Omanies (Chapters 4,5,6 and 7), outlines the historic migration pattern and the mixed ethnicity among the population and emphasize the necessity of implementing DNA testing during pre-marital screening for accurate risk prediction and genetic counselling

especially when both beta and alpha defects coexist making genotype prediction more complex and herewith providing the state of the art for prenatal diagnosis in the future.

In Chapter 8, we looked at the interfering effect of factors such as delta gene defect or iron deficiency that may influence HbA_2 measurement in the Omanies and herewith the diagnosis of beta thalassemia trait. This is particularly important during beta-thalassemia carrier screening because coexisting delta thalassemia defect with a beta thalassemia carrier status can normalise HbA_2 level and preclude a correct diagnosis. For that, we investigated 33 cases with low HbA_2 levels. Ten different defective delta alleles of which two are reported for the first time in literature were categorised in 20 subjects. The characterization of the delta-gene mutation spectrum is bound to make premarital screening and genetic counseling more reliable in the Omani population screened for beta thalassemia.

Patients with sickle cell disease (SCD) may show a strong variability in morbidity and response to therapy from case to case, depending from the often complex genotypes and haplotypes. The classical associated determinants were investigated in Chapters 9,10 and 11. In Chapter 9, the haplotype/sub-haplotype and phenotype in SCD patients (n=125) was investigated. A total of 11 different haplotype combinations were identified with the Asian haplotype being the most common and associated with a milder clinical form. In Chapter 10, a cohort of (n=52) SCD patients treated with hydroxyurea (HU) were tested for their response to HU based on their XmnI polymorphism. Patients homozygous or heterozygous for Xmn I (T/T or T/C) showed better response and improvement in clinical phenotype than patients bearing the (C/C) genotype. In Chapter 11, we assessed if presence or absence of alpha thalassemia in the same 125 SCD patients with identical beta genotype and haplotypes can ameliorate disease severity. We found that alpha thalassemia improves the overall hematological conditions but amelioration of the general disease severity is only noticed when compared in cohorts of the same haplotype. We conclude from these correlative studies that neither the haplotype or sub-haplotype nor the XmnI polymorphism nor alpha-thalassemia alone appears to be fully associated with the variable clinical phenotypes in SCD and that presumably other external factors can play a role in the different expression of the disease. Nevertheless, identifying genetic determinants is necessary for prognostic purposes, accurate diagnosis and planning for the best-tailored treatment to the affected children.

In Chapter 12, we tested the application of Ion Torrent PGM as a diagnostic ultra high-thoughput sequencing method for beta globin gene during beta thalassemia screening. We scanned a total of 297 Omani cases using a barcoded uni-directional sequence methodology and reliably identified beta-thal mutations in hundreds of patients simultaneously. Our results show that ion torrent can replace Sanger sequencing in the future and is a powerful diagnostic method to detect HBP carriers and carriers of other common genetic disorders in a national screening setting and that these molecular methods may become more practical if financially affordable.

Pre-matrimonial counselling, although thorough, is the only formal option available in Oman to couples at risk. This leaves only two options for couples whenever a presumed genetic risk has been suspected: to change the choice of partner or to continue with the marriage and hope for a healthy child from each pregnancy. Therefore the most effective prevention

method for HBP disorders thus far seems to be also in Oman, is prenatal diagnosis followed by the option of pregnancy termination. In Chapter 13 we investigated the attitude of 35 Omani couples at risk towards prenatal diagnosis and medical abortion. Although the majority would have accepted prenatal diagnosis if the service was available in the country, pregnancy termination was greatly influenced by the Islamic view as interpreted in the country by the main religious Muftee. However, prenatal diagnosis may be eventually considered in Oman with improved public awareness and once the public health authorities have reached a sensible agreement with religious authorities, as has been the case in other Muslim countries.

In conclusion, while providing tools for a better care and a better insight on the management of these severe diseases in Oman, our results will hopefully facilitate the prevention of HBP in the country.

SAMENVATTING

Hemoglobinopathieën (HBP) behoren tot 's werelds meest voorkomende autosomaal recessief overervende aandoeningen, en komen vooral voor in Afrika, India, het mediterrane gebied, het Verre- en Nabije Oosten, waaronder ook Oman. HBP zijn het gevolg van mutaties die structurele afwijkingen veroorzaken in het hemoglobine molecule (abnormale hemoglobines) of de expressie van de globine genen beïnvloeden waarbij de globine synthese verlaagd of zelfs geheel afwezig is, met anemie tot gevolg. Dragers van HBP zijn doorgaans asymptomatisch, echter als risicopaar hebben zij 25% kans op een ernstig aangedaan kind.

Een van de belangrijkste uitdagingen op het gebied van volksgezondheid voor de meeste Arabische landen en Oman in het bijzonder, is de hoge prevalentie van HBP, m.n. sikkelcelziekte (SCZ) en beta-thalassemie Major (TM). De hoge prevalentie voor HBP in deze gebieden is het gevolg van positieve selectie van dragers door malaria tropica. Bovendien draagt in Oman de sociaal-cultureel bepaalde traditie van consanguine huwelijken bij tot een verhoogde incidentie van kinderen met een ernstige vorm van HBP. Ongeveer 10% van de Omaanse populatie is drager van SCZ, 3% is drager van beta-thalassemie terwijl tenminste de helft drager is van alfa-thalassemie. Ook al is de ziekte behandelbaar, op een HLA identieke beenmergtransplantatie na zijn er tot op heden geen mogelijkheden tot genezing. De Omaanse autoriteiten op het gebied van publieke gezondheidszorg hebben zich niet alleen gericht op de beste behandeling en patiëntenzorg, maar ook op het terugdringen van het aantal aangedane geboorten. De laatste tien jaar heeft het premaritaal identificeren van risicoparen en het aanbieden van genetisch advies door speciaal hiervoor opgerichte nationale klinieken, een bijdrage geleverd aan de preventie van HBP. Dit proefschrift beschrijft het onderzoek naar het mutatiespectrum van HBP en de genetische determinanten die de ernst van de ziekte beïnvloeden om zo een bijdrage te leveren in het ontwikkelen van een preventie strategie voor de ernstige vormen van HBP in Oman.

Om via premaritale counseling het genetisch risico op HbP in het nageslacht beter te kunnen bepalen werd het moleculaire spectrum aan globine genmutaties die voorkomen in Oman onderzocht. Hoofdstuk 4 heeft betrekking op een eerste studie onder 87 niet-verwante Omani , homo- of heterozygoot voor beta-thalassemie, afkomstig uit vier verschillende gebieden in Oman. Daarbij werden 11 verschillende beta-thalassemie mutaties aangetoond, waarbij tenminste 56% van de individuen eveneens hetero- of homozygoot bleek te zijn voor de meest voorkomende alfa-thalassemie deleties, m.n. de alfa3.7kb en alfa4.2kb deletie. Een breder spectrum aan mutaties werd verkregen door de studie beschreven in hoofdstuk 5 waarbij nog eens een groot aantal individuen (n=446), behorende tot verschillende stammen en afkomstig uit zeven verschillende gebieden, werden onderzocht. Hierbij werden 32 verschillende beta-thalassemie mutaties gevonden, waarvan 11 voor het eerst beschreven in de Omaanse populatie. Hoofdstuk 7 beschrijft de analyse van de alfa-globine gendefecten. Op basis van de hematologische bepalingen werden de in totaal 634 individuen ingedeeld in 7 groepen en op moleculair niveau onderzocht. Er werden 21 verschillende alfa-gendefecten gevonden, waarvan 15 voor het eerst in Oman. Twee nieuwe alfa-gendefecten werden in deze studie voor het eerst beschreven. In hoofdstuk 6 worden twee casussen apart beschreven, de eerste is een alfa-globine variant gevonden in een consanguin echtpaar die vermoedelijk

de ernstige hemolytische anemie verklaart bij de foetus. De tweede betreft een nieuwe promotor mutatie van het beta-globine gen geassocieerd met een borderline/licht verhoogde HbA_2, indicatief voor een mild β^+-thalassemisch effect. De sterke heterogeniteit van veel voorkomende, zeldzame en nieuwe beta- en alfa-globine gendefecten (hoofdstuk 4, 5, 6 en 7) weerspiegelt het historisch migratiepatroon en etnische vermenging van de Omaanse bevolking en benadrukt de noodzaak van het implementeren van DNA tests om het genetisch risico in pre-maritale screening en counseling adequaat te kunnen bepalen. Vooral wanneer alfa- en beta-thalassemie defecten gecombineerd voorkomen, zijn DNA tests onmisbaar om een voorspelling van het risico voor het nageslacht op basis van het genotype te kunnen doen en cruciaal voor de 'state-of-the-art' prenatale diagnostiek in de toekomst.

In hoofdstuk 8 is gekeken naar factoren in de Omaanse HbP populatie die de HbA_2 meting en daarmee de diagnose beta-thalassemie trait beïnvloeden, zoals delta-gendefecten en ijzerdeficiëntie. Dit is met name belangrijk bij dragerschapscreening van beta-thalassemie aangezien een co-existerend delta-gendefect (delta-thalassemie of delta-globine variant) in een drager de HbA_2 normaliseert met een verkeerde diagnose tot gevolg. Hiertoe werden 33 gevallen met een verlaagd HbA_2 percentage onderzocht. Er werden 10 verschillende delta-gen defecten gevonden in 20 individuen, waarvan twee niet eerder in de literatuur gerapporteerd. Het bepalen van en kennis over het delta-globinegen mutatiespectrum draagt stellig bij tot de betrouwbaarheid van pre-maritale screening en genetische counseling voor beta-thalassemie in de Omaanse populatie.

Afhankelijk van de complexiteit van het genotype en het haplotype kunnen sikkelcelpatiënten onderling een enorme variabiliteit in morbiditeit en reactie op therapie vertonen. In hoofdstuk 9, 10 en 11 worden de klassieke hiermee geassocieerde determinanten onderzocht. In hoofdstuk 9 werd de associatie tussen fenotype en (sub)haplotype onderzocht in 125 patiënten met sikkelcelziekte. Van de in totaal 11 verschillende haplotype combinaties bleek het klinisch mildere Aziatische HbS haplotype het meest voor te komen. In hoofdstuk 10 werd in 52 met Hydroxy-Ureum (HU) behandelde sikkelcelpatiënten gekeken naar de responsie op behandeling in relatie tot de aanwezigheid van het XmnI polymorfisme (T/T of T/C) in de promotoren van de gamma globine genen. Hieruit bleek dat sikkelcelpatiënten, homo- of heterozygoot voor het XmnI polymorfisme (T/T of T/C), effectiever reageerden op HU behandeling en daarmee een milder klinisch fenotype vertoonden dan patiënten zonder dit XmnI polymorfisme (C/C). In hoofdstuk 11 werd in hetzelfde cohort van 125 sikkelcelpatiënten naar de invloed gekeken van de aan- of afwezigheid van alfa-thalassemie op de klinische ernst in patiënten met hetzelfde beta-genotype en haplotype. Daarbij werd gevonden dat de algehele hematologische condities door aanwezigheid van alfa-thalassemie gunstiger zijn, maar dat een verbetering in de algemene ernst van het ziektebeeld alleen merkbaar is wanneer groepen patiënten met eenzelfde haplotype met elkaar worden vergeleken. Van deze correlerende studies kan worden geconcludeerd dat noch haplotype of sub-haplotype, noch het XmnI polymorfisme, noch alfa-thalassemie alleen volledig de variatie in klinisch fenotype kan verklaren in patiënten met SCZ en dat andere, waarschijnlijk externe factoren een rol spelen in het verschil in expressie van de ziekte. Desalniettemin is het identificeren van genetische factoren noodzakelijk voor het stellen van de juiste diagnose en prognose en voor de beste behandeling op maat van aangedane kinderen.

In hoofdstuk 12 werd het nut van Next Generation Sequencing (NGS) tools zoals de Ion Torrent PGM als diagnostische screeningsmethode voor beta-thalassemie en HbS onderzocht. Hierbij werden DNA monsters van in totaal 297 Omaanse patiënten en dragers van beta-thalassemie en HbS getest op het voorkomen van mutaties in het beta-globine gen. Door gebruik te maken van barcode primers en uni-directionele sequentie-analyse werden van 100 individuen simultaan de mutaties in het beta-gen betrouwbaar gedetecteerd. Deze resultaten toonden aan dat de Ion Torrent in de toekomst de traditionele Sanger sequencing zou kunnen vervangen en dat het, mits financieel haalbaar, een krachtige diagnostische methode is voor toepassing in grootschalige nationale screeningsprogramma's voor HbP dragers of dragers van andere veelvoorkomende genetisch aandoeningen.

Een gedegen counseling voor het huwelijk is momenteel de enige formele optie in Oman om risicoparen te informeren over erfelijkheidsrisico. Hiermee zijn de opties voor preventie beperkt tot aanpassing van de partnerkeuze wanneer sprake blijkt te zijn van genetisch risico voor het nageslacht of continuering van het huwelijk in de hoopvolle verwachting van een niet-aangedaan kind. Het aanbieden van prenatale diagnostiek met als doel beëindiging van aangedane zwangerschappen is tot op heden het meest effectief in de preventie van ernstige vormen van hemoglobinopathie. In hoofdstuk 13 wordt beschreven wat de houding is van 35 Omaanse risicoparen ten aanzien van prenatale diagnostiek en abortus op medische indicatie. Hoewel de meerderheid van de risicoparen om prenatale diagnostiek zou hebben verzocht indien de mogelijkheid bestond, wordt de kijk op zwangerschapsbeëindiging sterk beïnvloed door de Islam en de opvattingen van de Muftee, de belangrijkste religieuze autoriteit van het land. Wanneer echter de publieke bewustwording over prenatale diagnostiek toeneemt en de autoriteiten op het gebied van volksgezondheid overeenstemming zouden bereiken met de religieuze autoriteiten, zou prenatale diagnostiek op den duur een mogelijkheid kunnen worden in Oman, zoals in andere moslim landen al het geval is.

Tot slot wil ik de hoop uitspreken dat de resultaten van dit proefschrift zullen bijdragen tot een betere zorg, een groeiend inzicht in de vraag hoe om te gaan met deze ernstige erfelijke ziekten en het voorkomen van ernstige vormen van HbP middels preventie in Oman.

CURRICULUM VITAE

CURRICULUM VITAE

Suha Mustafa Hassan was born on 8th of February, 1985 in Muscat, Oman. She finished her high school in 2002 with second highest score among Year 12 students at the national level and was granted a full scholarship to complete her bachelor degree in Melbourne – Australia. She got her Bachelor of Science certificate in 2006 and was qualified to do her research Honours year at Prince Henry's institute of medical research in affiliation with Monash Univeristy in Melbourne on XY females with Prof. Vincent Harley. In 2007 she was graduated with high class Honours degree and by 2008 she joined the Genetic Laboratory in Oman which she has been working in till now. In 2011, she was offered to do her PhD at Leiden University Medical Centre with Prof. Piero Giordano and Dr. Kees Harteveld. Her PhD project is based entirely on Omani cases either affected or carrier of hemoglobin disorders. Sample and data collection as well as hematological tests and DNA extraction was all conducted in Oman while advanced molecular analysis were held in Leiden during 3 months visit every year. During this PhD, Suha was exposed to new techniques such as melting curve analysis and ion torrent PGM sequencing. Her project was focused towards preventing hemoglobinopathies in Oman by identifying the spectrum of all the mutation alleles involved and drawing genotype-phenotype correlation by looking at various genetics determinants. She is now working at the hemoglobinopathy diagnostic laboratory in Oman, with the goal of implementing high quality work flow and advanced diagnostic tools which she gained during her PhD years in Leiden.

LIST OF PUBLICATIONS

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1. Hassan SM, Hamza N, Jaffer Al-Lawatiya F, Jaffer Mohammed A, Harteveld CL, Rajab A, Giordano PC. Extended molecular spectrum of bera- and alpha- thalassemia in Oman. Hemoglobin. 2010;34(2):127-34.

2. Hassan SM, Harteveld CL, Bakker E and Giordano PC. Broader spectrum of β-thalassemia mutations in Oman: Regional distribution and comparison with neighbouring countries. Hemoglobin. 2015;39(2):107-110.

3. Hassan SM, Harteveld CL, Bakker E and Giordano PC. Hb Lansing and a new β promoter transversion (- 52 G>T): An attempt to define the phenotype of two mutations found in the Omani population. Hemoglobin. 2015;39(2):111-4.

4. Hassan SM, Harteveld CL, Bakker E, Giordano PC. Molecular spectrum of α-globin gene defects in the Omani population. Hemoglobin. 2014;38(6):422-6.

5. Hassan SM, Harteveld CL, Bakker E, Giordano PC. Known and new δ-globin gene mutations and other factors influencing HbA_2 measurement in the Omani populations. Hemoglobin. 2014;38(4):299-302.

6. Hassan SM, Al Muslahi M, Al Riyami M, Al Balushi A, Bakker E, Harteveld CL and Giordano PC. Haplotypes, sub-haplotypes and geographical distribution in Omani patients with Sickle Cell Disease. Thalassemia reports. 2015; 5(4739): 6-11.

7. Hassan SM, Al Muslahi M, Al Riyami M, Bakker E, Harteveld CL and Giordano PC. Association of XmnI (-158 γ^G) polymorphism and response to hydroxyurea in Omani S/S and S/β patients. Genetic Genome Research. 2014, 1:1 ISSN:2378-3648

8. Hassan SM, Al Muslahi M, Al Riyami M, Bakker E, Harteveld CL, Giordano PC. Sickle cell anemia and α-thalassemia: A modulating factor in homozygous HbS/S patients in Oman. Eur J Med Genet. 2014;57(11-12):603-606.

9. Hassan SM, Vossen RH, Chessa R, den Dunnen JT, Bakker E, Giordano PC, Harteveld CL. Molecular diagnostics of the HBB gene in an Omani cohort using bench-top DNA Ion Torrent PGM technology. Blood Cells Mol Dis. 2014;53(3):133-7.

10. Hassan SM, Bakker E, Harteveld CL, Giordano PC. Primary prevention of hemoglobinopathies by prenatal diagnosis and selective pregnancy termination in a Muslim country: Oman. Thalassemia Reports. 2014; 4(4171):19-21.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

My greatest gratitude goes to Dr. Piero Giordano, first for allowing me to take this PhD at Leiden University and second for his endless support and continuous effort throughout the PhD years.

I would also like to thank Dr. Kees Harteveld and Prof. Egbert Bakker for their help and contribution. Also would like to thank Rolf Vossen and Roberta Chessa for helping me in with the Ion Torrent protocol and Marion Phylipsen for melting curve analysis. And not to forget, thanks to all the other LDGA team for their constant assistance.

Many thanks to all the clinicians and hematologist who contributed with their help in Oman and special thanks goes to my boss Dr. Anna Rajab for allowing me to take this opportunity to complete my PhD while still working at the Genetic Center in Oman.

And last I would like to thank my parents, especially my mother, and my husband Hani for their continuous encouragement and support throughout the difficult years. I would not have made it without them. Hani, you have sacrificed your annual leave for the past 4 years just to escort me during my practical months in Leiden and that means a lot to me. And of course my daughter Joori who have came into life with the start of this PhD project and my baby son Ali who was born towards the end of my thesis preparation. Joori and Ali, I am sorry for not being available to you at all times but you were the source of my inspiration and the reason that pushed me to keep going.