Chapter

Newborn Screening and Thalassaemia Syndrome

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Abstract

Haemoglobin variants or haemoglobin disorders are a group of clinical disorders characterised by impairment of synthesis of normal adult haemoglobin, due to genetically determined abnormality in the formation of the globin moiety of the molecule. These disorders fall into two broad groups, that is qualitative (haemoglobinopathies) and quantitative (thalassaemias). In the anthropoids, the most common congenital single-gene disorder is the alteration of the globin genes which account for about 270 million carriers globally. These globin gene alterations cause low/no globin expression (thalassaemia) or abnormal globin protein production (haemoglobinopathy). The clinical manifestation of haemoglobin disorder is the culminated measure of one's genetic and molecular makeup. Summarily, the study, diagnosis and management of thalassaemia are models of biological principles of human disease. Newborn screening, however, is a system that aims at improving management and/or eradication of genetic disorders from the neonatal stage of life. This chapter will be dealing with the definition and steps involved in newborn screening for thalassaemia.

Keywords: thalassaemia, newborn screening, confirmatory diagnosis

1. Introduction

The haemoglobin (Hb) is a tetramallic, metalloprotein consisting of two alpha α and/or α-like alleles (α or ζ) and two β and/or β-like alleles (ε, γ, δ or β) globin chains with a chemical formula ($C_{2952}H_{4664}O_{832}N_{812}S_8Fe_4$) [1, 2]. Each polypeptide globin chain is folded around a haem molecule (**Figure 1**). The major function of Hb is a gaseous transfer between the tissue and the lungs. The globin chains are encrypted by their various genes sited on chromosome 11 and chromosome 16 and they all have more than one allele [4]. These alleles codes for the various globin chains at various stages of human life from the embryonic to adult life in varying concentrations (**Tables 1** and **2**). Many of these alleles undergo point mutations during DNA sequencing resulting in single amino acid substitution in the globin portion, leading to the production of haemoglobin derivatives (variants) [6]. Haemoglobin variants or haemoglobin disorders are a group of clinical disorders characterised by impairment of synthesis of normal adult haemoglobin, due to genetically determined abnormality in the formation of the globin moiety of the molecule. These disorders fall into two broad groups, that is, qualitative (haemoglobinopathies) and quantitative (thalassaemias) [7]. In the anthropoids, the most common congenital

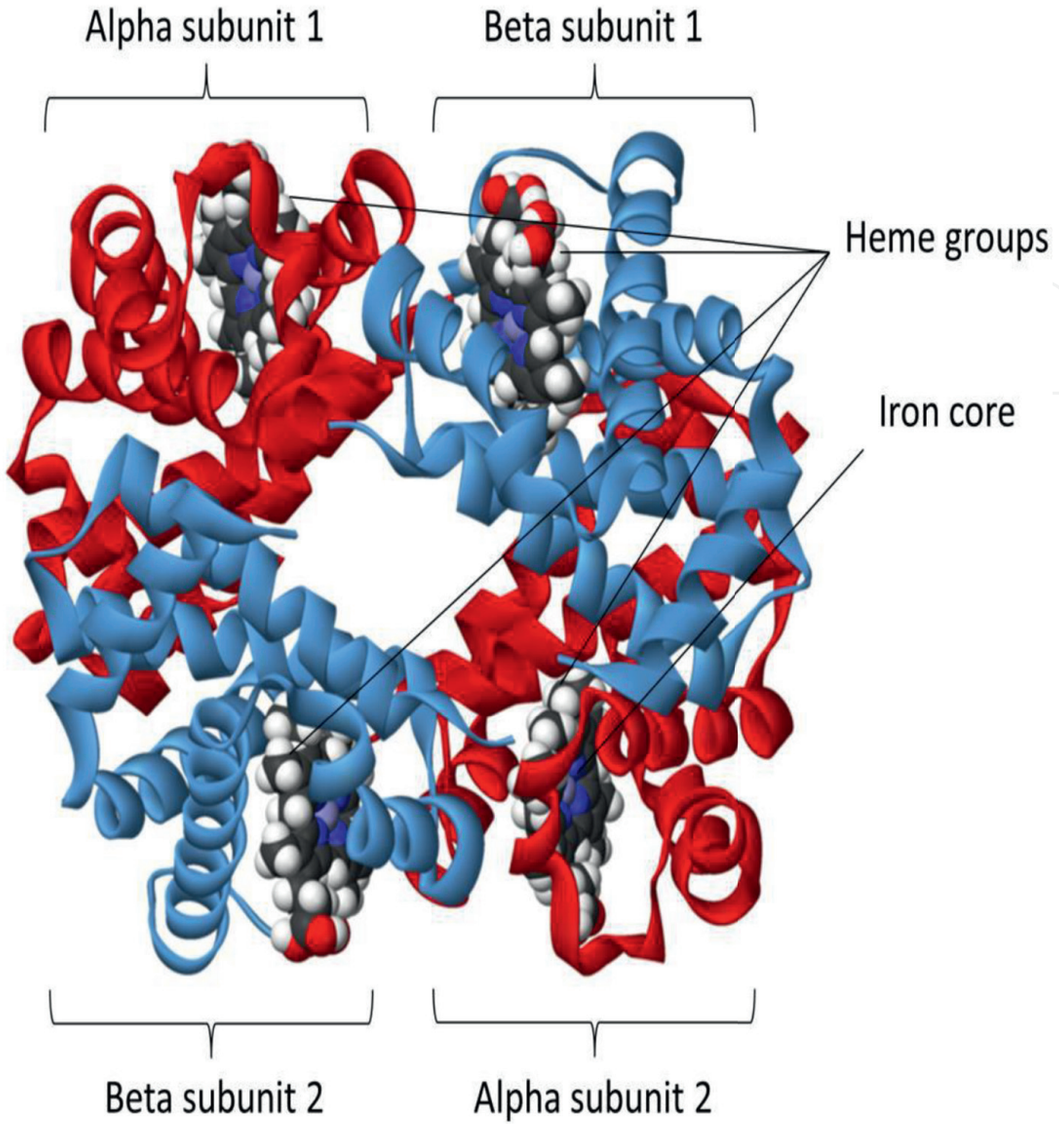

Figure 1.
Haemoglobin structure. A molecule of haemoglobin is made up of four subunits; two alpha-like subunits and two beta-like subunits. Each subunit contains a haem group with a ferrous core to which an oxygen molecule can reversibly bind. Image adapted from [3].

single-gene disorder is the alteration of the globin genes which account for about 270 million carriers globally and 0.4 million of such births annually [8]. These globin gene alterations cause low/no globin expression (thalassaemia) or abnormal globin protein production (haemoglobinopathy). The clinical manifestation of haemoglobin disorder is the culminated measure of one's genetic and molecular makeup. Summarily, the study, diagnosis and management of thalassaemia is a model of biological principles of human disease. Newborn screening however is a system that aims at improving management and/or eradication of genetic disorders from the neonatal stage of life.

1.1 The haemoglobin nature and structure

The anatomy and genetic structure of the human haemoglobin are demonstrated in the tables and figures below.

Haemoglobin specie	Globin chains present	Period when normally present
A	$\alpha_2\beta_2$ (alpha$_2$ & beta$_2$)	Major haemoglobin in adult life
A_2	$\alpha_2\delta_2$ (alpha$_2$ & delta$_2$)	Minor haemoglobin in adult life; even more minor in foetal and neonatal life
F	$\alpha_2\gamma_2$ (alpha$_2$ & gamma$_2$)	Minor haemoglobin in adult life; major haemoglobin in foetal life with a declining percentage through the neonatal period
Gower 1	$\zeta_2\varepsilon_2$ (zeta$_2$ & epsilon$_2$)	Significant haemoglobin during early intrauterine life
Gower 2	$\alpha_2\varepsilon_2$ (alpha$_2$ & epsilon$_2$)	Significant haemoglobin during early intrauterine life
Portland I or Portland II	$\zeta_2\gamma_2$ or $\zeta_2\beta_2$ (zeta$_2$ & gamma$_2$ or beta$_2$)	Significant haemoglobin during early intrauterine life

Modified from: [5].

Table 1.
Haemoglobins normally present during adult, foetal and embryonic periods of life.

Haemoglobin	Structure	Levels at birth	Levels in adult	Comments
A	$\alpha_2\beta_2$	20–25%	97%	Reaches adult levels by one year of age.
A_2	$\alpha_2\delta_2$	0.5%	2.5%	Elevated in β thalassaemia trait
F	$\alpha_2\gamma_2$	75–80%	<1%	Reaches adult levels by one year of age
HbH	β_4	15–20% in HbH disease	NA	HbH produces Heinz bodies in the erythrocytes and haemolysis
Hb Bart	γ_4	100% in hydrops feotalis, 15–25% in HbH disease	NA	Increased in carriers of α thalassaemia trait at birth

Courtesy: [3].

Table 2.
Normal and variant haemoglobin at birth and in older children.

1.2 What is newborn screening?

Newborn screening is an entire system of identification, treatment, management and possibly eradication of genetic disorders from the neonatal stage of life. Newborn screening starts from the recruiting stage through the diagnosis of the infant and management. It is generally applied for the early recognition of infants affected by disorders that benefit from early treatment to avoid irreversible health problems [9]. It is supreme for early diagnosis and enrolment of affected children into a comprehensive health care programme. This has created diagnostic and treatment opportunities for several children with genetic or metabolic diseases across the globe with a resultant healthier life. However; in many countries, the screening

programmes have not started or have been limited to a few disease conditions [10]. These delays are tied to financial incapabilities of the citizens and lack of government-established organisation for screening. However, the physician's suspicion and or awareness is heightened by accurate identification of risk factors of haemoglobin disorders and family history [11]. The ultimate benefit of newborn screening programmes is the improved health status in patients diagnosed early and treated optimally. However, issues such as false positives and false negatives results might occur but the use of novel and molecular technologies for confirmation has over that [12]. Each part of the newborn screening system is important and needs evaluation for any weaknesses. Newborn screening for genetic disorders can be undertaken from two dimensions:

1. Random selection from all neonates aged 0–9 months of life to ensure full development of every haemoglobin gene and mutational characteristics.

2. Collation of family history during antenatal visits or recruiting of all willing pregnant women whose family histories are suggestive of a haemoglobin disorder or the "don't know" subjects.

The family history method might be cost-effective but will likely miss out on the few misinformed questionnaires, so for a start, a random newborn screening of a population site should be of best interest for subsequent studies, government policies and data storage.

1.2.1 Algorithm for newborn screening

1. Create awareness through seminars and meetings for parents/guardians and plead or persuade them to cooperate for the success of the programme.

2. Formulate centres/medical outlets that will be equipped with personnel for recruiting, counselling, sampling and testing.

3. Alternatively, incorporate NBS into an already existing programme such as child immunization and include personnel for haemoglobin disorders screening as in no. 2 above.

4. All prospective thalassaemia syndromes from a well-structured questionnaire are issued a consent form to the parents/guardians.

5. Every consented subject should be sampled for screening via dry blood sampling (DBS) in six to eight spots.

6. Communicating positive screening results to a clinician and/or parents and tracking outcomes of confirmatory testing are of primary concern for the final outcome.

7. Follow-up or regular data review processes for newborn screening ensure total inclusion of all infants with thalassaemia syndrome for management and treatment

8. For the collection of whole blood samples for confirmation, ethylenediaminetetraacetic acid (EDTA) is the typical anti-coagulant used. Heparin may interfere with DNA amplification by polymerase chain reaction (PCR). DBS collected from a finger prick; preferably the last finger or the heel is commonly used. To maintain the integrity of haemoglobin molecules, the medium of transportation and storage of DBS must be dry and cool, possibly by means of dry ice.

1.3 The thalassaemias

Thalassaemia is a group of heterogenous genetic disorders of haemoglobin synthesis. These disorders arise from a decrease in production rate of one or more globin chain [13]. The thalassaemias are named α, β, δβ- and γδβ-thalassaemias depending on the globin chain that is produced in a reduced amount. In the occasion where one of the globin chain is not synthesised at all, the condition is known as thalassaemia Null (°), that is, α^0 or β^0 thalassaemias. This condition usually occurs amongst the populace with structural Hb abnormalities, therefore the inheritance of one thalassaemia gene from one parent and the second gene with a structural Hb variant from the next parent is a common finding in such places [14]. Other minor haemoglobins in adults include HbF (foetal haemoglobin, $\alpha_2\gamma_2$) and HbA2 ($\alpha_2\delta_2$) [15].

The α-Thalassaemias: These groups of thalassaemias result from the deletion of one or more alpha-globin genes and are subgrouped in order of the number of the α-globin gene deletions. Hence, one gene deletion is α^+-thalassaemia, α^0-thalassaemia is two gene deletion from the same chromosome, Hb-H syndrome is a three-gene deletion while hydrops feotalis with the Hb-Barts is a four-gene deletion. The haemoglobin being a tetrametric protein of 4 globin gene $\alpha_2\beta_2$, it has been observed that deletion of only one α gene will not result in a significant haematological abnormality and is therefore referred to as a "silent carrier" state. On the other hand, deletions of two α-genes can occur in two ways i.e. from the same chromosome (in cis) or of the opposite chromosome (in trans). The two α gene deletion is the homozygous state or homozygous α^+-thalassaemia and has similar clinical presentations as mild hyperchromasia and microcytic anaemia but the cis-genotype is common amongst Asians while the trans-form is common in Black African origins [16].

The Hb-H disease is usually associated with haemolysis due to the excessive accumulation of β-globin subunits that self-bond to form soluble β-chain tetramers which are the Hb-H. Because of the relatively unstable nature of the Hb-H, it does not precipitate as the erythrocytes age leading to the formation of inclusion bodies which distorts the red cells' life span [17].

Hydrop's foetalis with Hb-Barts is usually detected at the third trimester or within the early post-natal period. Haemoglobin Barts is not an effective oxygen transporter because it has a very high affinity for oxygen. It is a tetramer of 4γ globin subunits so the foetus or infant will lack Hb F&A, resulting in hypoxia and extreme organ swelling and subsequent deaths [17].

The β-Thalassaemias: The Hb variant resulting from the point-mutation of the β-globin gene is known as the β-thalassaemias. This variant has two main sub-types, that is, the β^0-thalassaemia in which there is complete absence of normal β-globin subunits. And the second is β^+-thalassaemia which has remarkably reduced synthesis of normal β-globin. It was noted that some forms of β-thalassaemia might be due to an unequal crossing over of bridges of the δ & β-globin genes leading to a fusion of δβ-globin gene (thalassaemia), εγδβ-thalassaemia and hereditary persistence of foetal

haemoglobin (HPFH) syndromes [18]. It has been reported that β-thalassaemia has over 200 molecular different subtypes but in spite of their heterogeneity; they still possess similar clinical manifestations since they all lack HbA with excess accumulation of α-subunits [19].

1.4 Newborn screening methods for thalassaemias

In the recent past, most newborn screening programmes uses high-performance liquid chromatography (HPLC) as the primary screening method to make a presumptive screening of possible haemoglobinopathy [20]. However, for low-income nations, a simple alkaline or acid globin chain electrophoresis with $_{DL}$-dithiothreitol ($_{DL}$-DTT) and urea in Tris EDTA-borate buffer can suffice for the detection of abnormal haemoglobins [21]. Also, manual HbF quantification and inclusion body detection can serve as a good NBS source for low-income states. All suspected abnormal Hbs or neonates can then be subjected to fully automated, high throughput HPLC for identification, and quantitation of Hb F, HbA_2 and Hb Bart's, enabling thalassaemic screening and classification in the newborn period [20]. According to literatures, an understanding of the specific HPLC retention times will aid the probable identification of thalassaemic disorders such as a Hb S/Hb A ratio >2.0 is highly suggestive of Hb S/β+ thalassaemia rather than Hb AS trait. Secondary or primary screening with HPLC can thus help to streamline the subsequent tests needed for the identification/confirmation of a thalassaemia syndrome in most cases [22].

Globin chain electrophoresis: This is used in the separation of α- and β-globin chains by adding 6 M urea and 2-mercaptoethanol to the buffer. When electrophoresis is applied at alkaline or acid pH, these chains migrate differently revealing the characteristic patterns of migration of abnormal α- and β-chains. This method provides a means of identifying abnormal haemoglobin variants that cannot be identified by routine electrophoretic methods. It is especially helpful when variants other than S and C are present and which have identical migration on both cellulose acetate and citrate agar systems [23].

Determination of distribution of HbF in red cells: This is employed to distinguish hereditary persistence of foetal haemoglobin (HPFH) from β thalassaemias. The acid elution test of Betke-Kleihauer is used to evaluate the distribution of HbF, where fresh thin-blood film fixed with ethanol is examined microscopically. The principle is that HbA on fixing readily wash off from red cells by acid solution while HbF resists acid-elution and remains within the cells. Cells containing more HbF appear dark after staining, while those with no HbF appear unstained and empty or ghost-like [24].

Tests for inclusion bodies: Inclusion bodies that can be detected in thalassaemias include HbH and α chain inclusion and they can be detected as follows:

a. HbH inclusions: These are detected when peripheral blood is incubated with methyl violet in splenectomised patients. They are also precipitated in mature or nucleated red cells as multiple, small, ragged inclusions due to the redox action of dyes such as brilliant cresyl blue. HbH inclusions are usually seen in Hb Bart's hydrops foetalis syndrome, HbH disease, and α-thalassaemia carrier states [23].

b. α chain inclusions: In homozygous β-thalassaemia, α chain inclusions are seen only in nucleated red cells in bone marrow, or in peripheral blood after splenectomy. α chain inclusions appear as single, ragged structures closely attached to

the nucleus when peripheral blood or bone marrow sample is incubated with methyl violet, and the prepared films observed under microscope [23].

High-performance liquid chromatography: This is used as a screening test for thalassemias and for the detection, identification and quantification of haemoglobin variants. It is also used for the quantitation of HbA2 and HbF. HPLC is well suited for neonatal screening since it can detect small amounts of haemoglobin and needs small amount of blood. Haemoglobins A, F, S, C, E/A2, D_{Punjab}, $O\text{-}_{Arab}$ and $D_{Philadelphia}$ can be separated and identified with HPLC. In this technique, blood sample is introduced into a column packed with silica gel. Different haemoglobins get adsorbed onto the resin. Elution of different haemoglobins is achieved by changing the pH and ionic strength of the buffer. Haemoglobin fractions are detected as they pass through a detector and are recorded by a computer [25].

1.5 Confirmatory diagnostic testing for the thalassaemias

Demographic information and an EDTA/DBS blood sample from one or both parents are required with that of the newborn to help guide the sequence of confirmatory diagnostic tests for specific thalassaemias. Methods of gene-typing for thalassemia based on PCR techniques are as follows: dot-blot analysis, reverse dot-blot analysis, the amplification refractory mutation system, denaturing gradient gel electrophoresis, mutagenically separated polymerase chain reaction, gap-PCR, restriction endonuclease analysis, real-time polymerase chain reaction, Sanger sequencing, pyrosequencing, multiplex ligation-dependent probe amplification and gene array [26–29] Gap-PCR is used to test for common α-thalassaemia deletions or duplications, as well as all forms of HPFH and Hb Lepore deletions. will identify point mutations in the γ-, α- and β-globin genes are usually captured in direct DNA sequencing but mutations within the alleles as well as large deletions are often missed out. Large β-globin locus deletions account for only a very small number of β-thalassaemia mutations but are the most difficult to detect because gap-PCR relies on knowledge of the deletion breakpoints. Multiplex ligation-dependent amplification (MLPA) becomes a handy method to determine the presence of an unidentified α- or β-globin gene deletion, by assessing DNA ploidy quantity changes [30]. Long-range sequencing using comparative genomic hybridisation (CGH) or microarray-based comparative genomic hybridisation (matrix CGH) method to identify deletion breakpoints and DNA copy numbers with high resolution is employed for beta thalassaemia confirmation [31]. This is a molecular cytogenetic method for analysing "copy number variation" which is related to the number of complete sets of chromosomes in a cell and hence the number of possible alleles for autosomal and pseudo-autosomal genes [32]. These novel methods are summarised in **Table 3**.

The method employed for the detection of unknown mutations is the restriction fragment length polymorphism (RFLP) analysis. This method is based on the fact that each restriction enzyme targets different nucleotide sequences in a DNA strand hence different enzyme cuts at different sites. The distance between the cleavage sites of a certain restriction endonucleotide differs between individuals. Hence, the length of the DNA fragments produced by a restriction endonuclease will certainly differ from organisms and species [33]. The variations that affect restriction sites and produce different fragmentation sizes after digestion are known as restriction fragment length polymorphisms (RFLPs). This polymorphism serves as 'markers' for genetic disorders, especially thalassemias. If the linkage is not close then the crossing

Disorder and mutation type	Diagnostic method
α^0-Thalassaemia	Gap-PCR, MLPA
α^+-Thalassaemia: deletion nondeletion	Gap-PCR, MLPA ASO, RE, DGGE, Sanger sequencing
β-Thalassaemia: deletion non-deletion	Gap-PCR, MLPA ASO, RDB, ARMS, RE-PCR, Sanger sequencing
δβ-Thalassaemia	Gap-PCR, MLPA
HPFH deletion	Gap-PCR, MLPA ASO, ARMS, RE-PCR, Sanger sequencing

The main diagnostic approaches commonly used for the diagnosis or confirmation of thalassaemias [28].

Table 3.
The novel methods of DNA diagnosis for thalassaemia.

over of chromosomal material between homologous chromosomes during meiosis may 'separate' the polymorphic site from the abnormal gene; this will lead to a false negative result in the foetus [23].

2. Next-generation sequencing (NGS)

Recently, however, the introduction of technologies such as dosage mutation tests to detect large deletion or duplication mutations and multiple gene panel tests by massively parallel sequencing (next-generation sequencing; NGS) facilitates a more precise molecular diagnosis of thalassaemias and a better understanding of the genomic mechanisms of the disease [34].

In next generation sequencing (NGS), the diagnosis of thalassaemias is based on massively parallel sequencing of clonally amplified DNA molecules, alongside sufficient computational power and appropriate software for efficient data analysis [35]. The procedure can be applied to a whole genome or exome, and to specific targeted regions of the genome. The most critical step in NGS manipulation is the design of the probe-set to be applied for DNA capture which requires a high level of homology between the genes in the alpha and beta clusters. Another critical point for NGS is that it is useful for the detection of single nucleotide substitutions and insertions or small deletions, but it is less accurate for other types of genomic variation.

3. Conclusion

Newborn screening is a system of identification, treatment, management, and possibly eradication of genetic disorders from the neonatal stage of life. The procedure begins at the recruiting site or stage through the diagnosis of the infant and management. It is generally applied for the early recognition of infants affected by disorders that benefit from early treatment to avoid irreversible health problems. It is supreme for early diagnosis and enrolment of affected children into a comprehensive health care programme thus; thousands of children with genetic and/or metabolic diseases have had an opportunity for a healthy life with early diagnosis and

treatment. Thalassaemia syndrome has a high financial and national health burden, national policies and intervention are needed for its success. Each nation should adopt newborn screening and diagnostic/confirmatory methods for thalassaemia syndromes within their financial or economical capacity, maintaining standards. Communication, documentation and follow-up is the key to the success of newborn screening for thalassaemia syndromes.

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