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POLICY

EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies

This article has been amended since online publication. A corrigendum also appears in this issue.

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Haemoglobinopathies constitute the commonest recessive monogenic disorders worldwide, and the treatment of affected individuals presents a substantial global disease burden. Carrier identification and prenatal diagnosis represent valuable procedures that identify couples at risk for having affected children, so that they can be offered options to have healthy offspring. Molecular diagnosis facilitates prenatal diagnosis and definitive diagnosis of carriers and patients (especially 'atypical' cases who often have complex genotype interactions). However, the haemoglobin disorders are unique among all genetic diseases in that identification of carriers is preferable by haematological (biochemical) tests rather than DNA analysis. These Best Practice guidelines offer an overview of recommended strategies and methods for carrier identification and prenatal diagnosis of haemoglobinopathies, and emphasize the importance of appropriately applying and interpreting haematological tests in supporting the optimum application and evaluation of globin gene DNA analysis.
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INTRODUCTION

These Best Practice guidelines offer an overview of recommended strategies and methods for carrier identification and prenatal diagnosis of haemoglobinopathies. They are complementary to, and can be used in conjunction with, other guidelines, for example from the British Society for Haematology,[1] the ENERCA recommendations for preconception or antenatal screening, prenatal diagnosis and genetic counselling of haemoglobinopathies[2] and the UK NHS Sickle Cell and Thalassaemia screening programme.[3] They cover not only DNA analysis but include also details of haematological (biochemical) tests. This is to emphasize the importance of appropriately applying and interpreting haematological tests in supporting the optimum application and evaluation of globin gene DNA analysis. Most of the methods recommended in these guidelines, especially for haematology, have been in use for several years, and few technical advances have been translated into clinical practice.[4] Due to worldwide population migrations, carrier identification and prenatal diagnosis of the haemoglobinopathies is currently appropriate in most countries, even the traditionally non-endemic countries of Northern and Western Europe. Thus these guidelines are expected to be useful for laboratories in all regions of the world, and not only those where the haemoglobinopathies are traditionally endemic.

External Quality Assessment (EQA) is an intrinsic part of Best Practice. EQA provides a long-term, retrospective assessment of laboratory performance. Participation in EQA (when available) is encouraged and essential, for any laboratory already accredited or seeking accreditation to international standards, for example ISO 17025, ISO 15189 or equivalent.[5,6] Unless dictated by legislation, choice of EQA provider lies with the laboratory, but use of an accredited EQA programme (to ISO 17043) is recommended, wherever possible.

Throughout the guidelines, for reasons of space, some globin gene variants have been referred to using 'traditional' names rather than nomenclature recommended by the Human Genome Variation Society (HGVS). Relevant HGVS nomenclature can be found in the supporting information (Supplementary Table 1) and the HbVar database: http://globin.bx.psu.edu/hbvar/menu.html.

Description of the disease group

Haemoglobinopathies constitute the commonest recessive monogenic disorders worldwide.[7,8] They are caused by variants that affect the genes that direct synthesis of the globin chains of haemoglobin, and may result in altered synthesis (thalassaemia syndromes and hereditary persistence of fetal haemoglobin (HPFH)) or structural

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changes (sickling of the red blood cells, haemolytic anaemia, polycythaemia or more rarely cyanosis).

Thalassaemia variants and various abnormal haemoglobins interact to produce a wide range of disorders of varying degrees of severity. There are four main categories of severe disease states, for which genetic counselling, and possibly prenatal diagnosis, is indicated, as follows (Tables 1, 2 and 3).[9]

- Thalassaemia major (co-inheritance of two β-thalassaemia variants including inheritance of $\delta\beta$-thalassaemia variants and Hb Lepore).
- Sickle cell syndromes (for example Hb S/Hb S, Hb S/Hb C, Hb S/bthalassaemia, Hb S/Hb D-Punjab, Hb S/Hb O-Arab, Hb S/Hb Lepore, Hb S/Hb E).
- Hb E thalassaemia (co-inheritance of β-thalassaemia variants with Hb E).
- Hb Bart's Hydrops Fetalis syndrome (homozygous α^0-thalassaemia, genotype $-/-$), and (rarely) Hb H Hydrops Fetalis syndrome (genotype $-/\alpha^T\alpha$ or $\alpha^T\alpha/\alpha^T\alpha$).

In many populations β-thalassaemia syndromes (and related conditions caused by haemoglobin variants such as Hb S and Hb E) are clinically more relevant than the α-thalassaemias, since the severe forms are more common and require life-long treatment and clinical management. However, in populations that have a high prevalence of α°-thalassaemia defects, such as the Chinese and many South East Asian populations, or in countries with significant immigrant populations from these areas, α-thalassaemias are also relevant. The severest form of α-thalassaemia, Hb Bart's Hydrops Fetalis, is usually fatal as infants either die *in utero* (23–38 weeks) or shortly after birth (unless subjected to intrauterine blood transfusion therapy). Even with perinatal treatment it is a very severe condition with these patients requiring lifetime transfusion therapy and iron chelation and some children also have long term neurological complications.[10] Furthermore, hydropic pregnancies are frequently associated with serious complications in the mother, and most pregnancies in which the fetus is diagnosed as affected are terminated due to the increased risk of both fetal and maternal morbidity.

The genes and disease-causing variants

The major haemoglobin in adult life is Hb A, a tetramer composed of two alpha and two beta globin chain subunits ($\alpha_2\beta_2$). Each subunit consists of a globin chain wrapped around a haem group containing iron to which O_2 can bind. The single gene encoding β-globin chains is located on the short arm of chromosome 11 (11p15.5), within the so-called β-globin gene cluster, and the two genes encoding the α-globin chains are located on the short arm of chromosome 16 (16p13.3), within the α- globin gene cluster.

In the *HBB* gene locus, more than 280 genetic variations (variants) causing β-thalassaemia have been described, the majority of which are point variants. In the *HBA1* and *HBA2* genes and wider locus, more than 100 α-thalassaemia variants have been reported most of which involve deletions within the α-globin gene cluster.[11] In addition, more than 1150 DNA variants causing structural protein variants have been

Table 1 β**-Thalassaemias and** β**-globin gene disorders—genotype interactions, disease states and recommendations for prenatal diagnosis and preimplantation genetic diagnosis (PGD)**

Genotype interaction	*Disorder expected*	*Appropriate to offer PND*
Homozygous		
β° or severe β^+-thalassaemia	Thalassaemia major	Yes
Mild β^+-thalassaemia	Thalassaemia intermedia	Occasionally[a]
Mild β^{++}-thalassaemia (silent)	Very mild thalassaemia intermedia	No
$\delta\beta^\circ$-thalassaemia	Thalassaemia intermedia	Occasionally[a]
Hb Lepore	Thalassaemia intermedia to major (variable)	Occasionally[a]
HPFH	Not clinically relevant	No
Hb C	Not clinically relevant	No
Hb D-Punjab	Not clinically relevant	No
Hb E	Not clinically relevant	No
Hb O-Arab	Not clinically relevant	No
Compound heterozygous		
β°/severe β^+-thalassaemia	Thalassaemia major	Yes
Mild β^+/β° or severe β^+-thalassaemia	Thalassaemia intermedia to major (variable)	Occasionally[a]
Mild β^{++}/β° or severe β^+-thalassaemia	Mild thalassaemia intermedia (variable)	Occasionally[a]
$\delta\beta^\circ/\beta^\circ$ or severe β^+-thalassaemia	Thalassaemia intermedia to major (variable)	Occasionally[a]
$\delta\beta^\circ$/mild β^+-thalassaemia	Mild thalassaemia intermedia	Occasionally[a]
$\delta\beta^\circ$/Hb Lepore	Thalassaemia intermedia	Occasionally[a]
Hb Lepore/β° or severe β^+-thalassaemia	Thalassaemia major	Yes
Hb C/β° or severe β^+-thalassaemia	β-thalassaemia trait to intermedia (variable)	Occasionally[a]
Hb C/mild β^+-thalassaemia	Not clinically relevant	No
Hb D-Punjab/β° or severe β^+-thalassaemia	Not clinically relevant	No
Hb E/β° or severe β^+-thalassaemia	Thalassaemia intermedia to major (variable)	Yes
Hb O-Arab/β°-thalassaemia	Severe thalassaemia intermedia	Yes
$\alpha\alpha\alpha/\beta^\circ$ or severe β^+-thalassaemia	Mild thalassaemia intermedia	No
$\alpha\alpha\alpha\alpha/\beta^\circ$ and $\alpha\alpha\alpha\alpha\alpha\alpha/\beta^\circ$-thalassaemia	Mild to severe thalassaemia intermedia (variable)	Occasionally[a]

Note: The decision to have prenatal diagnosis belongs to the couple, once they have had comprehensive counselling.
[a]Couples with genotypes that may lead to offspring with unpredictable phenotypes occasionally select to have prenatal diagnosis or PGD.

Table 2 Sickle cell disorders—interactions and indications for prenatal diagnosis and preimplantation genetic diagnosis (PGD)

Genotype interaction	*Disorder expected*	*Appropriate to offer PND*
Homozygous		
Hb S	Sickle cell disease	Yes
Compound heterozygous		
Hb S/β° or severe β^{+}-thalassaemia	Sickle cell disease	Yes
Hb S/mild β^{+}-thalassaemia	Mild sickle cell disease	Occasionally[a]
Hb S/$\delta\beta^{\circ}$-thalassaemia	Mild sickle cell disease	Occasionally[a]
Hb S/Hb Lepore	Mild sickle cell disease	Occasionally[a]
Hb S/HbC	Sickle cell disease (variable severity)	Yes
Hb S/Hb D-Punjab	Sickle cell disease	Yes
Hb S/Hb O-Arab	Sickle cell disease	Yes
Hb S/Hbs C-Harlem, S-Southend, S-Antilles	Sickle cell disease	Yes
Hb C/Hb S-Antilles	Sickle cell disease	Yes
Hb S/Hbs Quebec-Chori, C-Ndjamena, O-Tibesi	Sickle cell disease	Yes
Hb S/Hbs I-Toulouse, Shelby, Hope, North Shore	Haemolytic anaemia	No
Hb S/Hb E	Mild to severe sickle cell disease	Occasionally[a]
Hb S/HPFH	Very mild sickle cell disease	No

Note: The decision to have prenatal diagnosis belongs to the couple, once they have had comprehensive counselling.
[a]Couples with genotypes that may lead to offspring with unpredictable phenotypes occasionally select to have prenatal diagnosis or PGD.

characterized.[11] Some structural variants are additionally associated with an altered rate of synthesis. (Summarized in the HbVar database: http://globin.bx.psu.edu/hbvar/menu.html).

Table 3 α-Thalassaemias—interactions and indications for prenatal diagnosis and preimplantation genetic diagnosis

Genotype interaction	*Disorder expected*	*Appropriate to offer PND*
Homozygous		
α°-thalassaemia ($-/-$)	Hb Bart's hydrops fetalis	Yes
α^{+}-thalassaemia ($-\alpha/-\alpha$)	Not clinically relevant	No
α^{+}-thalassaemia ($\alpha^{T}\alpha/\alpha^{T}\alpha$)	Severe α-thalassaemia carrier to severe Hb H disease	Occasionally[a]
Compound heterozygous		
α°-thal/α^{+}-thal ($-/-\alpha$)	Hb H disease	No

Note: The decision to have prenatal diagnosis belongs to the couple, once they have had comprehensive counselling.
[a]Couples with genotypes that may lead to offspring with unpredictable but potentially severe phenotypes occasionally select to have prenatal diagnosis or PGD. Reported examples of potentially severe phenotypes include genotype combinations involving variants in the polyadenylation signal in the *HBA2* gene, Hb Adana, Hb Agrino, Hb Constant Spring and Hb Taybee (see Supplementary Table S1 for HGVS nomenclature).

Approaches for carrier detection

Within the context of managing the health burden of the haemoglobinopathies, laboratory diagnosis plays a key role, identifying carrier couples and offering them reproductive choices. Haemoglobinopathies are unique among all genetic diseases in that detection of carriers is possible by haematological and biochemical tests rather than DNA analysis. Thus, it is recommended that, with currently available technologies, carrier detection should be mandatory using haematological and biochemical tests. However, DNA analysis should be employed for the determination of carrier status in complex cases or when the haematological/biochemical results are unclear. Depending on acceptable practices in each society, at-risk couples can then be offered the reproductive choice to avoid the birth-affected children, or helped to prepare for the possible birth of a severely affected child. Among the available options to avoid having affected children are, before marriage, a change in partner choice; or, after marriage, by remaining childless, opting for gamete donation or adoption. However couples rarely take these options. Instead it is more common for at-risk couples to avoid the birth of an affected child by opting for a conventional prenatal diagnosis in early pregnancy, or before pregnancy by choosing preimplantation genetic diagnosis (PGD). Both conventional prenatal diagnosis and PGD involve prior variant characterization in the parents and subsequent fetal (or embryo) DNA analysis. Thus genetic services for haemoglobinopathies require close collaboration between several specialities, most notably haematology, genetic counselling, molecular genetics and fetal medicine.

These guidelines will focus on best practice in laboratory methods and interpretation of results for carrier identification and prenatal diagnosis. For the best strategy to detect at-risk couples there are certain factors that should also be taken into consideration, including the frequency of the disease in endemic and non-endemic immigration countries,[12,13] the heterogeneity of the genetic defects in the target society, the knowledge of genotype–phenotype correlation (supported by access to original papers as well as to genomic databases), the resources available and finally the social, legal, cultural and religious factors.

In addition screening may target newborns or adolescents, or premarital, preconceptional or antenatal stages. For families wishing to avoid the birth of affected children, preconception or antenatal screening is the most effective approach and is widely applied in many high-risk populations. Newborn screening is less effective for primary screening and prospectively informing carriers about their

reproductive risks, but it is applied in certain countries to support better infant healthcare through prompt identification of affected babies. Pre-operative screening of individuals from populations with increased incidence of sickle cell is also indicated.

HAEMATOLOGICAL METHODS FOR CARRIER DETECTION

'Screening' is distinct from 'definitive' diagnosis in that the purpose of screening is to test for a defined set of conditions using simple haematological/biochemical tests. Screening programme strategies use first- and second-line methods in order to obtain a reliable diagnosis, albeit essentially a presumptive diagnosis. If an unequivocal, definitive diagnosis is required, characterization methods based on either protein or DNA analysis must be used.

With the thalassaemias, screening will detect most cases of raised Hb A_2 β-thalassaemia trait. However, there are often samples that present with haematology that is not consistent with typical β-thalassaemia trait (Tables 4 and 5) and, furthermore, there is no specific screening test of high-enough sensitivity for the clear identification of heterozygous α-thalassaemia, which is usually indicated once other states have been excluded. Definitive diagnosis of atypical cases and α-thalassaemia can only be made by DNA analysis.

When an abnormal haemoglobin is identified by screening methodology, the results obtained constitute a presumptive identification of the variant haemoglobin. It is important to remember that with phenotypic screening it is possible that some rarer conditions will not be detected and this has to be taken into account in the interpretation and reporting of results. For all samples, screening using haematological methods[14] precedes genetic diagnosis.[9]

Good laboratory practice also includes the minimization of clerical errors, particularly a danger in haematology laboratories undertaking carrier screening on large numbers of samples, sometimes numbering more than 1000 blood counts each day. Careful sample identification is essential (including: Full Name, Date of Birth, Sample date, if transfused in last 4 months). Bar coding is recommended for sample tracking. If acceptable and permitted, it is useful to note the ethnic origin, since many haemoglobin disorders are population-specific.[7,11–13] Laboratory error rates for the methods utilized (if known), or a link to their availability, should be stated on reports.

Table 4 Interpretations to consider when the haematology is not consistent with typical β-thalassaemia trait

Haematological parameters	*Possible interpretation*
Reduced red cell indices (MCV < 79 fl, MCH < 27 pg), normal Hb electrophoresis/HPLC/CE, normal %Hb A_2 & %Hb F)	(i) Iron deficiency
	(ii) heterozygous α-thalassaemia
	(iii) heterozygosity for mild β-thalassaemia variants (sometimes Hb A_2 is borderline raised)
	(iv) co-inheritance of heterozygous δ- with β-thalassaemia
	(v) heterozygous e$\gamma\delta\beta$-thalassaemia
Normal/borderline reduced red cell indices with raised Hb A_2	Interaction of α- with β-thalassaemia
	Carriers of β-thalassaemia with folic acid or vitamin B12 deficiency or hepatitis
Normal or reduced red cell indices with raised Hb F (>5%) and normal or low Hb A_2	Heterozygous $\delta\beta$-thalassaemia, $^{A}\gamma\delta\beta$-thalassaemia or HPFH
Normal red cell indices with normal/borderline Hb A_2	Triplication of α-genes (when implicated in family studies), KLF1 variants or mild β-thalassaemia variant
Severely reduced red cell indices and raised Hb A_2	Multiple α-globin genes (>4) and heterozygous β-thalassaemia

Note 1: Some Hb variants are not detected by electrophoretic or chromatographic procedures, but may be suspected due to the presence of abnormal haematological parameters and/or clinical symptoms. In such cases it is recommended that samples are analysed using mass spectrometry or DNA methods. Occasionally hyperunstable variants are present and these may only be found by DNA methodology as the protein produced is so unstable.
Note 2: When evaluating cases be aware of potential complex genotype interactions.

Table 5 Genetic variations associated with normal/borderline Hb A2 levels—a guideline of related haematological and biosynthetic characteristics

Variation HGVS nomenclature NM_000518.4 (HBB)	*Variation traditional nomenclature*	*MCV fl*	*MCH pg*	*Hb A_2*	*α/β ratio*
c.−151C>T	β −101 (C→T)	88.5±7.8	30.1±1.0	3.1±1.0	1.3±0.4
c.−142C>T	β −92 (C(T)	83.0±6.0	28.3±2.0	3.5±0.4	1.3±0.8
c.−18C>G	β +33 (C(G)	82.0±9.2	27.1±3.4	2.5±1.4	1.3±0.6
c.316-7C>G	βIVS2-844 (C→G)	96.0±4.0	30.3±1.8	3.2±0.2	1.0±0.6
c.*6C>G	β +1480 (C→G)	88.3±9.5	27.9±6.0	2.7±0.8	1.6±0.4
	$\alpha\alpha\alpha/\alpha\alpha$	85.5±7.8	30.4±5.0	2.8±0.6	1.2±0.4
	KLF1 variants (29)	82.7±5.7	27.8±2.2	3.6±0.2	
c.−50A>C	Cap+1 (A(C)	23–26*	75–80*	3.4–3.8*	—
c.92+6T>C	β IVS1-6 (T→C)	71.0±4.0	23.1±2.2	3.4±0.2	1.9±1.0
	$\delta+\beta$ thalassaemia	64.3±4.0	20.9±1.4	3.6±0.2	1.7±0.6

Values (mean±2SD or range (*)) are a guideline and represent those reported in various studies on carriers of these variants (prepared by R Galanello).
Note: It is recommended that subjects with borderline Hb A2 levels, particularly if their partner is a typical β-thalassaemia carrier, should be extensively investigated (α and β gene analysis, globin biosynthesis), although the majority usually have normal HBB and HBA genes. Borderline-raised Hb A2 levels in normal individuals are usually explained as the extreme distribution of the normal range of the Hb A2.
Furthermore, in couples where one partner is heterozygous for a severe α-thalassaemia defect and the other is a β-thalassaemia carrier, it is recommended that the *HBA* gene cluster be fully characterized in the β-thalassaemia carrier in order to preclude any risk of offspring with severe Hb H disease or Hb Bart's hydrops.

It is strongly recommended that all haematological parameters be co-evaluated to avoid spurious conclusions. In addition, the extent to which the genetic test results explain the haematology results should be evaluated and stated.

FIRST-LINE HAEMATOLOGY METHODS

All haematological testing[14] should be carried out on blood samples that are as fresh as possible. The complete blood count (CBC) or full blood count (FBC), haemoglobin pattern analysis and haemoglobin component quantification requires whole blood drawn into anticoagulant (EDTA). If necessary, bloods can be transported and stored at 4ºC.

Complete blood count

Electronic measurement is recommended, especially for MCV (mean cell volume), for which the measurements should be direct. All red cell indices (and other parameters) are important in evaluation, including Hb (haemoglobin), RBC (red blood cell count), MCH (mean corpuscular haemoglobin content), MCV and some labs use RDW (red cell distribution width), which is the standard deviation of the red cell size measurements expressed either as a percentage of the mean or a coefficient of variation.

RDW can potentially discriminate between thalassaemia carriers and iron deficiency and sometimes between thalassaemia carriers and a thalassaemia disorder or other rare causes of microcytosis, as indicated by decreased MCV (see notes below). It is a measure of the degree of anisocytosis and is simpler and faster than performing red cell morphology analysis (see Supplementary Information), although not as comprehensive. RDW alone is not a diagnostic parameter for β-thalassaemia trait.

The RBC count is also a parameter that can potentially distinguish between iron deficiency and thalassaemia. High RBC (erythrocytosis) results from a mechanism that compensates for the chronic low MCH present in thalassaemia carriers. This compensating mechanism needs folic acid levels to be maintained and may restore the Hb level of a markedly microcytic thalassaemia carrier to near normal values, raising RBC to 6–7 (10^{12}/l) or higher, without exceeding the normal packed cell volume (PCV) level. On the other hand, RBC compensation might be less evident in case of folic acid deficiency, a vitamin with limited body reserves, which is essential for cell division and thus for erythropoiesis. In case of folic acid deficiency carriers may become more anaemic, and microcytosis may become less evident, even disappearing in cases with coexisting vitamin B12 deficiency. In addition, RBC compensation is less evident in cases when MCH is only moderately reduced.

Interpretation of CBC, should consider the following:

- Key cut-off values in adults are MCV below 79 fl and an MCH below 27 pg, below which heterozygosity for thalassaemia is indicated. However, each laboratory should establish their own cut-off ranges for these parameters, based on the ethnicity of their patient population(s) and patient age group(s).
- Evaluation of CBC in samples more than 24 h after sampling should be made with caution, as the red cells increase in size, leading to falsely raised MCV. On the other hand, the MCH may be stable for up to 5 days, depending on storage conditions (4–20 °C);
- In advanced pregnancy the RBC is not a useful parameter, due to possible haemodilution. Furthermore, iron-deficient women who are responding to iron supplementation may have increased RBC.
- The RDW may be altered in several cardiac and hepatic conditions[15] and may have a limited discriminating potential especially in cases with combined iron deficiency.

Haemoglobin (Hb) pattern analysis

For a presumptive identification of abnormal haemoglobins at least two methods should be used. These methods include haemoglobin electrophoresis at pH 8.6 using cellulose acetate membrane, haemoglobin electrophoresis at pH 6.0 using acid agarose or citrate agar gel, isoelectric focusing (IEF), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE).[16–18] More details of these methods are described in the Supplementary information.

There are many *recommendations* related to the optimum application and interpretation of haemoglobin pattern analysis. The use of both HPLC and CE will maximize the detection of any Hb variant present in a sample and minimize false evaluation of artefacts. With automated systems, standards and controls are essential to verify that the equipment is working satisfactorily.[16,17] Other important recommendations are summarized in the Supplementary Information.

Interpretation of Hb pattern analysis when common β-variants interact with α-thalassaemia

In carriers of the most common β-chain variants (Hb S, Hb D-Punjab, Hb E and Hb C) the percentage of the variant haemoglobin is directly dependent on the number of α-globin genes. In the presence of α-thalassaemia it is usually decreased, e.g., to less than $\sim$35% for Hb S, D-Punjab and Hb C and less than $\sim$25% for Hb E,[19] although precise measurements may vary depending on the technology used. In the absence of iron deficiency, the percentage of Hb variant may therefore indicate the presence of co-inherited α-thalassaemia and help exclude β-thalassaemia as a cause of red cell microcytosis; it is a particularly useful observation to make in people of ancestral origins where both Hb E and α^0-thalassaemia are prevalent. However, this information is not reliable enough to definitely *exclude* the presence of α-thalassaemia because of the possibility of additional co-inheritance of multiple α-globin gene duplications (e.g. triplicated or quadruplicated rearrangements).

It is of note that for some stable β-chain variants that are more negatively charged than Hb A, such as Hb J, the relationship is inversed, as the number of α-globin genes decreases, so the percentage of Hb variant *increases* in the presence of α-thalassaemia.

Quantification of Hb A_2

Measurements should be carried out on freshly drawn anti-coagulated blood. Methods include[16,20,21] electrophoresis and elution, micro-chromatography, HPLC, capillary electrophoresis, Hb electrophoresis (not recommended).

It is important to note that the quantification of Hb A_2 using both HPLC and CE may still be compromised in the presence of common or rare α-chain variants, or δ chain variants, which split the Hb A_2 peak, or coexisting δ thalassaemia, which decreases the Hb A_2 peak.[22] The use of internal controls are *recommended*[16] as well as adherence to instructions provided by the manufacturers.

Haemoglobin variants which elute with or close to Hb A_2 on HPLC or capillary electrophoresis may affect Hb A_2 quantification.[16]

Important points for the interpretation of results for Hb A_2 quantification include:

- Hb A_2 levels above 3.5% is the standard cut-off value, above which heterozygosity for β-thalassaemia is indicated.
- Borderline levels of 3.1–3.5% Hb A_2 (depending upon the method, the laboratory reference range and coefficient of variation) indicate *further investigation required* (see Tables 5, 6 and Supplementary Table 3).
- Reference intervals in normal subjects are usually between 2.0 and 3.3%, but have been observed to differ slightly depending on the method and population group.
- Rare genetic[23] and acquired factors that may increase or reduce Hb A_2 levels are reported in Supplementary Table 3.

Quantification of Hb F

Methods reliable for the measurement of Hb F levels include alkali denaturation,[24] HPLC and CE, with the latter two methods being more recent and much more accurate.[16] More details on these methods are outlined in the Supplementary Information.

Important points for the interpretation of results for Hb F quantification include:

- Normal levels of Hb F are usually below 1% after the age of 2 years. Adults with Hb F > 2% should undergo further investigation.
- In carriers of typical elevated Hb A_2 β-thalassaemia or in carriers of Hb S, Hb F levels are usually within the normal range. When elevated (up to $\sim$7–8%) this is usually due to co-inheritance of other globin gene variations (e.g. co-inheritance of a triplicated α-globin allele with heterozygous β-thalassaemia) or specific β-globin gene cluster haplotypes (e.g. γ-globin gene promoter variations such as the $^{G}\gamma$ gene promoter Xmn-I polymorphism or the Arab-Indian or Senegal haplotypes in Hb S carriers) or variants of non-globin genes that affect γ-globin gene transcription.
- Elevated Hb F values, usually >5%, are associated with $\delta\beta$ or $\gamma\delta\beta$-thalassaemia heterozygotes (along with normal or low Hb A_2 levels and reduced RBC indices) or hereditary persistence of fetal haemoglobin (HPFH).
- Raised Hb F levels can also be caused by bone marrow malignancies, aplastic anaemia, Fanconi anaemia, erythropoietic stress, treatment with certain cytotoxic agents (e.g. hydroxyurea) or pregnancy.[17]
- In pregnancy Hb F may increase up to around 3%, making values in the range of 3–5% difficult to interpret. Values of Hb F above 5% during pregnancy may indicate the presence of heterocellular HPFH, and follow-up 6 months after delivery is recommended to clarify the Hb F levels.
- Hb F can be equally distributed in all cells (pancellular) or limited to a sub-population of red cells called Hb F cells (heterocellular) depending upon the underlying cause. Since Hb F cells in heterozygous β- or $\delta\beta$-thalassaemia are larger than those with low amounts of Hb F, microcytosis may be masked. Alternatively an increased RDW may be observed.

Iron (Fe) status

There are several parameters that can be measured to evaluate the the iron status of an individual, including Zinc protoporphyrin (ZnPP) serum ferritin and transferrin saturation measurements.

The interpretation of all three parameters should be done with some caution.[25] Measurement of iron status in samples with hypochromic, microcytic indices but with normal Hb A_2 and F is useful to distinguish between cases of iron deficiency and those with possible α-thalassaemia trait or certain form of 'silent' β-thalassaemia trait, the latter of which should have normal iron status. This distinction is useful to prevent unnecessary further investigation as well as inappropriate iron therapy. However, it is important to note that iron deficiency can co-exist with the thalassaemias, which could lead to misinterpretation. If an individual is found to be iron deficient, it is recommended to repeat the haematology screen once the individual is iron replete, although this may not always be practical or feasible in a couple with an on-going pregnancy.

SECOND-LINE HAEMATOLOGICAL METHODS

There are several methods which are not necessarily first-line methods for identifying thalassaemia carriers but may be useful in supporting the diagnosis in cases which do not have a clear diagnosis with first-line methods described previously. These (Supplementary Methods) include measurement/evaluation of red cell morphology (RCM), reticulocytes and F-cells,[14,26] HbH inclusions,[14] the single tube osmotic fragility test (OF),[27] globin chain synthesis,[28] globin chain separation, solubility or sickling tests for Hb S, DCIP (2.6 dichlorophenolindophenol) test,[29] Heinz body formation and the measurement of an oxygen dissociation curve. Mass spectrometry is also described as a second-line method for the characterization of Hb variants identified by first-line methods such as HPLC.[30] However, it potentially has a role as a first-line test, where it may be used more widely for carrier screening. All these second-line tests are discussed in the Supplementary Information.

REPORTING HAEMATOLOGY RESULTS

Haematology results should be given following complete evaluation of all relevant haematological parameters. Reporting should be done in a format complying with local legislation, regulations and acceptable practice. The report should be written clearly to avoid misinterpretation. If possible it should be accompanied by relevant information materials, currently most important for health practitioners in non-endemic countries who may not have experience with the haemoglobinopathies. The report should recommend referral of carrier couples or affected children to a relevant healthcare professional and encourage that testing be offered to additional family members.

Recommended formats for reporting haematology results can be found in the UK NHS Sickle Cell and Thalassaemia: Handbook for Laboratories.[3]

METHODS FOR DNA ANALYSIS

Almost all methods for DNA analysis of haemoglobinopathies currently in use are based on the polymerase chain reaction (PCR). There are many different PCR-based techniques that can be used to detect the globin gene variants. Those in current use for detecting and/or characterizing nucleotide variations include the amplification refractory mutation system (ARMS), restriction-endonuclease PCR (RE-PCR), denaturing gradient gel electrophoresis (DGGE), high resolution melting analysis (HRMA), Sanger sequencing, pyrosequencing, real-time PCR, reverse dot blot analysis and microarrays (the latter two usually as commercially available systems rather than as in-house protocols) (Tables 6a and 6b). For detecting and/or characterizing deletions, Southern blot analysis has been largely replaced by PCR-based methods including gap-PCR, multiplex ligation-dependent probe amplification (MLPA) and array comparative genome

Table 6a Advantages and limitations of methods for detecting and/or characterizing globin gene nucleotide variations (point variants) in carriers and conventional PND

Method	*Advantages*	*Limitations*
Reverse dot blot hybridization (RDB)	Multiplexed variant screening Relatively inexpensive Simple, rapid and reliable	Difficult to standardize and validate in-house Commercial kits/systems available* (see Note 2 below)
ARMS-PCR	Simple, rapid and inexpensive Can be modified for multiplexed variant screening	Stringent PCR conditions paramount for accuracy If primers degrade at the allele-specific 3′end, then PCR will be non-specific
Restriction enzyme (RE)-PCR	Simple and rapid Reliable	Not all variants are amenable Needs care to avoid partial digestion problems 'Frequent cutter' enzymes not very useful Some enzymes costly
Real-time PCR	For quantitative or qualitative evaluation of PCR products Rapid and high throughput No post-PCR processing Wide dynamic range of detection and high sensitivity	Instruments are relatively costly Sample diagnosis can be costly when screening for many variants (although it can be cost effective for prenatal diagnosis with prior knowledge of parental samples) Protocols require in-house validation
Denaturing gradient gel electrophoresis (DGGE)	Allows medium-scale screening Predictive software can support optimization Robust heteroduplex detection Relatively cheap	Variant samples need definitive characterization with another method Some regions (especially when CG-rich) may be difficult to optimize and analyse Overall technically demanding
High-resolution melting analysis (HRMA)	Simple (once standardized) Rapid and suited to automation Predictive software can support optimization	Technically demanding, with stringent assay design Variant samples need definitive characterization with another method Specialized and relatively costly instrumentation
Sanger sequencing (automated)	Generic method for detecting point variants Economical running costs	Instrument costly Can be technically demanding (laboratory processing and data interpretation)
Pyrosequencing	Results are quantitative Faster and more sensitive than Sanger sequencing	Targeted DNA sequence is only 20–50 nucleotides Any variant in target sequence must be known

Table 6b Advantages and limitations of methods for detecting and/or characterizing globin gene cluster rearrangements (deletions/duplications) in carriers and conventional PND

Method	*Advantages*	*Limitations*
GAP-PCR	Simple, rapid and inexpensive Can be multiplexed	Only for deletions with known breakpoint sequences Amplification of GC rich region technically difficult Susceptible to allele drop-out (not recommended as a stand-alone method for prenatal diagnosis)
Multiplex ligation-dependent probe amplification (MLPA)	Once primer-probe sets are validated, it is simple, rapid and suited to automation Can detect any copy number variation within the locus Commercial kits available (see Note 2 below)	Automated sequencer required for fragment analysis (costly) Unknown/sporadic SNPs may interfere with primer-probe hybridization DNA quality and concentration may be critical
Micro-arrays (deletions and insertions)	High throughput Useful for detecting any deletion or insertion (copy number variation)	Does not provide precise characterization of deletion/insertion Instruments and assays quite costly
Southern blotting	Generic method for detecting large deletions/insertions	Time consuming and cumbersome, technically demanding

Note 1: All PCR methods should be run simultaneously with positive and negative controls to avoid spurious results and conclusions.
Note 2: There are a limited number of commercial kits available, but as with any other method they should not be used in the absence of alternative methods in the diagnostic lab.
Note 3: Methods such as allele-specific oligonucleotide hybridization (ASO) are no longer widely used.
Note 4: Samples identified to vary from normal with DGGE and HRMA need definitive characterization with another method. In some cases this may also apply to samples identified to have deletions or duplications using MLPA.

hybridization (aCGH). All are recommended for in the context of best practice, each method having its own advantages and limitations (Tables 6a and 6b). The particular methods chosen by a laboratory for the diagnosis of the globin gene nucleotide variants or deletions depends not only on the technical expertise available but also on the type and variety of the variants likely to be encountered in the individuals (population groups) being tested. All molecular genetic methods should be validated before clinical use.

It is paramount that all DNA diagnostic laboratories take appropriate measures to preclude false-positive and especially false-negative results. The correct characterization of genotypes in carriers is extremely important to support appropriate genetic counselling and

essential prior to performing prenatal diagnosis if this is the choice of the woman (or carrier couple).

Recommendations

For genotype characterization in carriers, optimal DNA analysis results can be achieved by adhering to the following:

- Before DNA analysis, to evaluate haematology so as to direct the most appropriate molecular analysis for each sample.
- Post DNA analysis, to co-evaluate haematology to preclude a serious misinterpretation of results (misdiagnosis).
- Evaluation of family history and haematology in some cases if relevant.
- Preferably to have available for use more than a single DNA analysis protocol for characterizing the genotype in carriers (prospective parents), in order to support cross-checking of results (see Tables 6a and 6b).
- If Sanger sequencing is the only method available for characterizing nucleotide variations then it is recommended that the target region in the gene of interest be analysed in duplicate, and in both the forward and reverse directions. Ideally the Sanger sequencing reaction should be performed on templates amplified by two alternative sets of primers, or, if this is not feasible, at least on duplicate templates independently amplified from the original DNA sample. In the latter case, false-negative results should be prevented by careful co-evaluation of haematology results (see above).
- If a GAP-PCR analysis produces a negative result in individuals with haematology otherwise indicating a carrier status involving a likely deletion-variant (for example α-thalassaemia), then it is recommended that either MLPA or aCGH be used to further investigate the DNA sample.

DNA extraction methods for the diagnosis of haemoglobinopathies are the same as for the molecular analysis of any other genetic disorder. General guidelines for DNA extraction methods can be found within the Best Practice Guidelines for Laboratory Internal Quality Control (http://www.cmgs.org/).

Diagnostic strategy for DNA analysis

The most common haemoglobinopathies (which have autosomal recessive inheritance) are traditionally population-specific, with each population having a unique combination of abnormal haemoglobins and/or thalassaemia disorders. The spectrum of variants and their frequencies have been published for most populations, and usually consist of a limited number of common variants and a slightly larger number of rare variants.[12,13] Therefore knowledge of the ethnic origin and family history (including consanguinity) of a patient may support the diagnostic strategy, to expediate identification of the underlying defects in most cases. With the advent of global migration, however, this is becoming less practical and useful.[7,12]

α-Thalassaemia: Gap-PCR (amplification across the breakpoints of a deletion) provides a quick diagnostic test for most of the known α^+-thalassaemia and α°-thalassaemia deletion variants. For prenatal diagnosis Gap-PCR requires careful application and interpretation, since it may be susceptible to false negative results caused by allele drop out (ADO). The first gap-PCR assays were subject to technical failure through ADO, but the more recently published primers and conditions support more robust assays.[31,32]

Many of the common α^0-thalassaemia deletions can be diagnosed by gap-PCR: the $-^{SEA}$ allele, found in Southeast Asian individuals; the $-^{MEDI}$ and $-(\alpha)^{20.5}$ alleles found in Mediterranean individuals; the $-^{FIL}$ allele, found in Filipino individuals and finally the $-^{THAI}$ allele, found in Thai individuals. The two most common α^+-thalassaemia deletions (the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ alleles) can be detected by gap-PCR. The $-\alpha^{3.7}$ deletion is found most commonly in African, Mediterranean, Asian and Southeast Asian populations, while the $-\alpha^{4.2}$ deletion is found most commonly in Southeast Asia and the Pacific populations. However it is good practice to screen for both deletions in all individuals suspected of being an α^+-thalassaemia carrier. (For HGVS nomenclature of variants, see the HbVar database: http://globin.bx.psu.edu/hbvar/menu.html and Table 1 in the Supplementary Information.)

Southern blotting using ζ-gene and α-gene probes was traditionally used to diagnose all other α^0- and α^+-thalassaemia deletion variants as well as α-gene triplications and quadruplications. However, it can only detect larger segmental duplications or deletions for which probes are available. For these rare copy number variations (deletions and duplications) involving larger chromosomal regions multiplex ligation-dependent probe amplification (MLPA) represents a robust method, which has largely replaced Southern blotting in many laboratories.

MLPA is a technology based on ligation of multiple probe-pairs hybridized across the entire locus of interest, followed by amplification, facilitated through the use of universal-tag PCR primers for all ligated probe pairs, and subsequently fragment analysis.The use of universal PCR means that amplicons are generated with comparable efficiency and thus the method is semi-quantitative. In this way MLPA can detect deletions or duplications across the locus analysed and represents a valuable alternative or supplementary method to gap-PCR when investigating known and unknown deletions and duplications underlying α-, *β*- or δ*β*-thalassaemia.[33–36] Commercially available MLPA kits for the α- and the *β*-globin gene clusters are available[35] (www.lgtc.nl).

α^+-Thalassaemia may also be caused by nucleotide variants in either of the duplicated α-globin genes. All non-deletion alleles can be detected by PCR using a technique of selective amplification of each α-globin gene, followed by DNA sequence analysis.[37,38] Alternatively if the common non-deletion variants in the local population are known, the use of variant-specific tests is practical and can be recommended (Table 6a).

β-Thalassaemia: Traditionally, a limited number of *β*-thalassaemia variants were prevalent in most of the populations at risk for severe thalassaemia syndromes and this permitted the most appropriate targeted methods to be selected according to the ethnic origin. The most commonly used procedures for known variants included the reverse dot blot analysis with allele specific oligonucleotide probes,[39] primer specific amplification (ARMS),[40] and RE-PCR for a limited number of variants.[41] Currently, with the trend of global migration, variant spectrums within geographical regions have become much broader. Thus the more generic method of direct Sanger sequencing (automated) has become more relevant for detecting and characterizing point variants. Real-time PCR[42] and pyrosequencing[43] are robust alternative methods in laboratories with the necessary instrumentation.

Gene scanning methods such as DGGE[44] or HRMA[45] are also useful for locating (or excluding) possible variants within the *β*-globin gene, *HBB*. They are advantageous as they provide a means to reduce the use (effort and cost) of targeted assays or sequencing, and have proved reliable and relatively inexpensive to run. However, since they do not definitively characterize nucleotide changes, when they are used within a diagnostic setting, it is imperative to subsequently

characterize any nucleotide variation indicated, using either targeted direct variant assays or automatic sequencing.

Small deletions can be detected by polyacrylamide gel electrophoresis of an amplified β gene product. Some of the known larger deletions that remove the β-globin gene may be identified by gap-PCR (including Hb Lepore, some $\delta\beta$-thalassaemia and HPFH deletions)[46] or more recently by MLPA using one of the commercially available kits.[33]

Common Hb variants: The clinically important variants, Hb S, Hb C, Hb E, Hb D-Punjab and Hb O-Arab, can be diagnosed by dot blot hybridization, the ARMS technique or direct sequencing. All except Hb C can also be diagnosed by RE-PCR. For the many other haemoglobin variants, positive identification at the DNA level is achieved by selective globin gene amplification and DNA sequence analysis.

Reporting molecular genetic (DNA) analysis results

It is strongly recommended that molecular genetic analysis for all haemoglobinopathy cases be reported in relationship to the haematology screening results, and confirm that the genotype and phenotype are consistent. If the haematology screening results are unavailable to the DNA lab then the molecular haemoglobinopathy report should recommend that the referring physician/clinician co-evaluate the molecular result with the patients' haematology and (if relevant) clinical phenotype.

Reporting should be done in a format which conforms to local legislation, regulations and acceptable practice. The HGVS nomenclature should be used to avoid ambiguity between laboratories but whenever possible the traditional variant description should also be given. Most of these are available on the HbVar Globin Gene Server: http://globin.bx.psu.edu/hbvar/menu.html.

There are several guidelines available for reporting results including those from the Swiss society of Medical Genetics (http://www.sgmg.ch/user_files/images/SGMG_Reporting_Guidelines), the Clinical Molecular Genetics Society (CMGS) (http://www.cmgs.org/BPGs/Reporting%20guidelines%20Sept%202011%20APPROVED.pdf), the OECD (http://www.oecd.org/science/biotechnologypolicies/38839788) and the UK NHS Sickle Cell and Thalassaemia screening programme.

FETAL DNA ANALYSIS

It is best practice for all couples undergoing prenatal diagnosis to be counselled by a qualified health professional well versed in the molecular diversity of the haemoglobinopathies. No woman should undergo prenatal diagnosis unless she has been counselled by a qualified health professional, and preferably been provided with appropriate information materials. A good selection of these are available on the web-pages www.chime.ucl.ac.uk/APoG1, http://www.enerca.org, and the NHS web-page http://sct.screening.nhs.uk/.[2,3] Irrespective of the couples' previous experience, counselling should be offered for each pregnancy found to be at risk.

Prenatal diagnosis laboratories may or may not be associated with a cytogenetics laboratory. Either way it is highly recommended that women are also evaluated for the risk of transmitting other conditions, and should be offered any relevant tests. One common example is karyotype analysis for women with a high-risk of carrying a trisomy 21 pregnancy. The cytogenetics laboratory may also support the option of fetal sample backup cultures.

Due to the number of individuals involved in the care of a patient, many of whom may be at different geographical locations, good communication between all health professionals involved is essential. The laboratory also needs to have a good relationship with the genetic counsellors' team and the fetal medicine centres which carry out the sampling. When a woman is booked for fetal sampling, the molecular laboratory should be informed and the sample should be tracked from one location to another, with each site confirming the exchange of the sample (sending and receipt). The risk of misdiagnosis by the laboratory, or the possible incidental detection of non-paternity during testing, should be communicated during the counselling session.

PCR-based prenatal diagnosis is highly sensitive to maternal DNA contamination and is technically demanding. Laboratories should have access to a broad battery of probes and primers to support the detection of a wide range of thalassaemia and variant haemoglobin variants. The following procedures are intended to minimize the diagnostic error rate.

Parental blood samples

Blood samples should be obtained from both parents to confirm the phenotype of parents (by a full blood count and a haemoglobinopathy screen such as electrophoresis), and as a source of control DNA for the molecular analysis. Copies of haematology results should be sent to the molecular diagnostic laboratory. This should be repeated with every prenatal diagnosis that a couple undergoes, unless the identity of both parents is absolutely certain.

There are cases where a carrier woman requests prenatal diagnosis although her partner is unavailable for testing. In such situations it is important to evaluate the risk of a major haemoglobinopathy in the fetus. If the fetal DNA diagnosis identifies the sickle cell or thalassaemia variant inherited from the mother then it is recommended that the entire β-globin gene of the fetal DNA is sequenced to exclude a second *HBB* variant. In the report it should be stated that the paternal genotype is unknown and that DNA analysis cannot exclude all haemoglobin disorders.

Fetal sampling

There are three possible procedures for fetal samples, including chorionic villus sampling (CVS), amniocentesis and fetal blood sampling. The risk of miscarriage is low if the fetal sampling is performed in an experienced centre.

Chorionic villus sampling. Prenatal diagnosis of haemoglobinopathies is preferably carried out by analysing a chorionic villus sample in the first trimester of pregnancy from 11 weeks of pregnancy.

The CVS provides a source of high-quality DNA in more than sufficient quantity to complete the prenatal DNA analysis. Any risk of maternal contamination is low, especially if careful microscopic dissection to remove contaminating maternal deciduas is performed prior to DNA extraction and analysis. There is a risk of maternal contamination if the sample is cultured, although this should not be necessary if the sample is of adequate size.

Amniocentesis. Amniocytes can be used for molecular analysis directly spun down from the amniocentesis sample (amniotic fluid, AF). The DNA yield is generally lower than from CVS samples but usually sufficient for analysis with PCR-based methods. Of note is that direct analysis from uncultured amniotic fluid cells should be carried out with caution as the fetal cells can be contaminated with maternal cells. Greater amounts of fetal DNA may be achieved if the sample is cultured for 10-14 days and this additionally decreases the risk of maternal contamination, although the reverse has been observed, on very rare occasions. For these reasons it is recommended that the laboratory evaluates all samples undergoing prenatal

diagnosis for maternal contamination. The prenatal diagnosis result based on an amniocentesis is available later in pregnancy compared to CVS, as amniocentesis is not usually performed earlier than the 15th week.

Fetal blood sampling. With fetal blood sampling, 1–2 ml of fetal blood is obtained, which can be used for molecular analysis, globin chain synthesis studies or HPLC. Fetal blood sampling may be more useful in women at-risk of α-thalassaemia hydrops fetalis when ultrasound examination shows hydropic features in the fetus. In these cases a quick diagnosis, available in a few minutes, may be obtained on the fetal blood using haematological techniques such as HPLC, as Hb F will be absent if the fetus is affected. For β-haemoglobinopathies globin chain synthesis in fetal blood is no longer used by most centres as it is technically more demanding than current DNA analysis methods. In addition, fetal blood sampling is associated with a higher rate of miscarriage and results are available much later in pregnancy (after 18–20 weeks).

Molecular analysis

The laboratory carrying out the molecular analysis should choose the technique(s) best suited to their laboratory infrastructure, expertise and target population. The techniques, along with relative summary of advantages and limitations, are listed in Tables 6a and 6b.

Diagnostic errors may be introduced by either technical pitfalls, for example partial digestion by restriction enzymes, or inherent properties of the DNA sample such as rare nucleotide variations that may prevent annealing of the PCR primers or probes used in the protocol/method, leading to ADO. All logical steps should be taken to monitor, and thus preclude, such events. If identical methods are used to identify the variants in the parents and subsequently to analyse the fetal DNA, then any pitfalls caused by rare nucleotide variations will be previously identified and can be addressed by adapting the diagnostic strategy accordingly.

Recommendations:

- Before performing a prenatal diagnosis, the genotypes in the prospective parents should be accurately characterized and confirmed (if the women's partner not available for testing, see above).
- Simultaneously with the fetal DNA, always analyse parental DNA sample(s) and appropriate control DNA's within the test batch, preferably all as duplicates. Always include PCR blanks and, optionally, sample blanks (e.g. some labs include a DNA extraction control sample using reagents from the extraction procedure).
- Perform repeat variant test(s) on the fetal DNA, along with relevant controls, possibly with different DNA concentrations.
- Use a limited number of amplification cycles to minimize co-amplification of any maternal DNA. This is especially important when using ARMS PCR for prenatal diagnosis, as there may be a risk of preferential amplification of maternal alleles.
- For optimal accuracy of a prenatal result, one approach is to base all prenatal diagnosis results on two independent diagnostic methods to identify/investigate each parental risk allele (variant). In laboratories where Sanger sequencing is the only method available for nucleotide variations, then all sequencing reactions (on fetal and parental DNA) should involve both the forward and reverse directions. If rare nucleotide variations are indicated within a sample/family when analysing the parental DNA samples, this pitfall can be addressed by performing a sequencing analysis using alternative primer sets to generate the target PCR template.

Maternal contamination

It is recommended that a maternal cell contamination test be performed on all prenatal specimens in order to rule out significant contamination of fetal DNA with maternal DNA. It is important to consider that all CVS and AF samples (with or without culture) may have maternal contamination. Although chorionic villus samples should be carefully dissected to remove maternal tissue,[47] it is still important to check that maternal contamination is not present. Monitoring for maternal contamination is achieved by the analysis of polymorphic DNA sites in the fetal versus parental DNA samples.[48,49]

Recommendations:

- The possibility of maternal DNA contamination should be investigated (and preferably excluded) in every case. It is recommended to use a panel of short tandem repeat polymorphisms (STRs). There are several commercially available STR kits, such as the Amp FISTR Identifier kit (ABI), which analyses 16 STR markers. When the fetal globin genotype is the same as that of the mother, and there are no informative markers to indicate the presence or absence of maternal contamination, the fetal diagnosis report should state these findings and indicate a greater risk of error in the fetal result.
- In dichorionic diamniotic twins (DCDA) where it is important to obtain an accurate diagnosis for each twin, STR analysis should be used to confirm there has been no twin-to-twin sample mixing, during fetal sampling. This is particularly important if the sample was obtained by CVS. In such cases, reporting the fetal sex may also be beneficial to obstetricians in the event that they have to carry out a selective termination.
- If the paternal DNA sample is analysed in addition to the fetal and maternal DNA sample, then these tests may also identify non-paternity. Such incidental findings should be handled according to local practice. In laboratories which do not routinely analyse paternal samples when performing prenatal diagnosis, paternity is assumed to be true and the prenatal report should state that the accuracy of the diagnosis is based on declared relationships.[49]

Patient consent and reports

According to local practices and legislation, there should be a consent form signed by the patient and counsellor accompanying the diagnostic samples, consenting to DNA testing, DNA storage and, if appropriate, the use of the remaining DNA for standardizing and developing new tests.

The fetal DNA report should detail the types of DNA analysis performed and clearly state the risk of misdiagnosis based on the reported technical errors of the protocols utilized.

Prenatal diagnosis follow-up

If there are no restrictions by local conditions and practices, it is desirable to confirm the fetal DNA diagnosis at birth through a request for a cord blood sample (the request may be sent out with the fetal diagnosis report). Haematological, haemoglobin and DNA analysis are also requested by some centres. It is important that centres are trained in the collection of pure cord blood samples, otherwise there is a risk the cord blood is contaminated with maternal blood.

Where neonatal screening programmes for sickle cell and haemoglobinopathies are available then networking to obtain neonatal

screening results for babies that have undergone prenatal diagnosis, will negate the need for requesting a cord blood sample.

When affected pregnancies are terminated, ideally fetal material should be requested to confirm the prenatal diagnosis result. However, these samples are rarely received by the requesting laboratory.

Audit

National registers should exist to audit services for prenatal diagnosis. In the UK the three diagnostic laboratories enter data for each diagnosis onto a shared register and aggregated data can be used for national audit of antenatal carrier screening and utilization of prenatal diagnosis by risk, ethnic group and region. It can also be used to report on the accuracy of prenatal diagnosis.[50–52] Audit should be an on-going activity that aims to identify any weaknesses in the prenatal diagnosis services, directing ways for improvement.

DEDICATION

Following the very sad news about the premature death of Professor Renzo Galanello in May 2013, the authors of these Best Practice Guidelines have decided unanimously to dedicate them to him, as he made substantial contributions to all sections. Renzo was a pioneer in the field of thalassaemia research, diagnosis and prevention, and also in the treatment and management of patients with haemoglobinopathies. The global thalassaemia community has lost a great scientist, researcher, compassionate clinician and colleague.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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