EXPERT REVIEWS

Prenatal, noninvasive and preimplantation genetic diagnosis of inherited disorders: hemoglobinopathies

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Disorders of hemoglobin synthesis have been used as a prototype for the development of most approaches for prenatal diagnosis (PND). PND for hemoglobinopathies based on molecular analysis of trophoblast or amniocyte DNA has accumulated approximately 30 years of experience. Disadvantages with conventional PND include 'invasive' fetal sampling and the need to terminate affected ongoing pregnancies. New developments are directed towards improving both the timing and/or safety of procedures. Preimplantation genetic diagnosis, an established procedure with 20 years of clinical application, avoids the need to terminate affected pregnancies through the identification and selective transfer of unaffected *in vitro* fertilization embryos. Approaches towards 'noninvasive' PND, through analyzing fetal cells or free fetal DNA present in the circulation of pregnant women, are a focus of ongoing research. Overall, PND, preimplantation genetic diagnosis (and potentially 'noninvasive' PND) represent valuable reproductive options for couples at risk of having a child affected with a severe inherited disease.

Keywords: noninvasive prenatal diagnosis • PCR • PGD • preimplantation genetic diagnosis • prenatal diagnosis

Prenatal diagnosis (PND) represents a highly important reproductive option for couples wishing to initiate a healthy family, and the hemoglobinopathies represent one of the best examples of the importance of PND for combating severe inherited disease.

Mendelian or monogenic diseases account for approximately 5% of the overall disease burden in developed countries, and over two-thirds of patients admitted to pediatric clinics have an underlying disorder that is, at least partly, genetically determined. The hemoglobinopathies (including the thalassemia syndromes and hemoglobin disorders, such as sickle-cell syndromes and HbE thalassemia) represent the most common monogenic disorders worldwide, which, with minimal exceptions, follow an autosomal recessive pattern of inheritance. It is estimated that approximately 7% of the global population carry a defective globin gene and that approximately 350,000 new patients are born each year [1]. Although traditionally common in all the subtropical regions of the world where falciparum malaria is (or was) endemic, the hemoglobinopathies are also becoming globally distributed as a result of worldwide population movements [2]. For example, it has recently been estimated that up to 10% of the US population is at risk of sickle-cell anaemia, and between 2 and 9% of the population in Europe belong to ethnic minorities at risk of hemoglobin disorders [3].

The ultimate goal of molecular medicine is to offer fundamental therapeutic and curative interventions for severe genetic diseases. However, despite some progress, such as the recent success with gene therapy reported in a case with HbE/β-thalassemia major [4], the main clinical approach for confronting severe inherited disorders remains the identification of couples or pregnancies at risk of producing an affected child, with the option of PND.

In the mid-1970s, the first approaches to PND of hemoglobinopathies were based on analysis of hemoglobin synthesis (protein biosynthesis studies) in fetal blood samples. Subsequent development of recombinant DNA technologies supported the gradual elucidation of the molecular

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genetics of inherited diseases, supporting the use of DNA analysis to characterize globin gene mutations in amniotic fluid cells and trophoblast samples [5].

New developments in PND are directed towards improving both the timing and/or safety of prenatal diagnostic procedures, and involve the analysis of alternative sources of fetal genetic material along with the application of new analytical technologies. The main disadvantages with current PND protocols include the need for 'invasive' fetal sampling procedures, along with the need to terminate affected ongoing pregnancies, the latter associated with widely debated ethical and social implications. With 'invasive' fetal sampling there is an, albeit very small, risk of fetal loss of up to 2% [6,7], and approaches to achieve 'noninvasive' prenatal analysis, based on the analysis of either fetal cells or cell-free fetal DNA (cffDNA) both confirmed to exist in the circulation of the pregnant mother, are a focus of ongoing research [8]. An approach that avoids the need to terminate affected pregnancies is represented by preimplantation genetic diagnosis (PGD). PGD involves the identification and selective transfer of unaffected embryos established from *in vitro* fertilization (IVF) and, following 20 years of clinical application, is now an established procedure within the reproductive options available to couples at risk for transmitting a severe genetic disorder, especially appropriate for couples who also have fertility problems or an unsuccessful reproductive history [9,10].

Conventional PND

Prerequisites

Prenatal diagnosis for inherited disorders aims to provide an accurate, rapid result as early in pregnancy as possible. To support this, couples at risk of an affected pregnancy have to be identified and their disease-causing mutations characterized. For less common monogenic diseases, at-risk couples are usually identified following the birth of an affected child. With respect to the hemoglobin disorders, their high frequency and severity means that many countries now apply either population-wide or targeted carrier screening, mainly through preconception or antenatal screening, and for sickle-cell syndromes, some countries have introduced newborn screening [11,12].

It is of paramount importance to note that the hemoglobin disorders differ from other genetic diseases in that identification of carriers is possible and, most importantly, should be mandatory, by hematological (biochemical) tests rather than DNA analysis since there are many categories including α-thalassemia, β-thalassemia, δβ-thalassemia, hereditary persistence of fetal hemoglobin and hemoglobin variants, which can all interact with one another to cause complex genotypes (Table 1) [13]. The clinically severe interactions for which PND is indicated are summarized in Table 1. At the molecular level, more than 1000 different mutations have been described [201]. Thus for carrier identification, the first step involves screening by hematological methods, followed by evaluation of the hematological parameters, which is fundamental to direct the use of the most appropriate molecular tests and also to evaluate the DNA result. A standalone negative molecular analysis is at serious risk of misinterpretation. Once a carrier couple has been identified based on their hematology, it is obligatory to characterize their underlying mutations in order to counsel them on the risk of a serious hemoglobin disorder and finally proceeding to the PND if indicated.

Another fundamental prerequisite for PND is to obtain fetal material promptly and safely. Established methods require close collaboration with gynecologists experienced in either trophoblast sampling (also known as chorionic villi sampling) at 10–12 weeks of pregnancy or amniocentesis after the 15th week.

Molecular methods based on examples from the hemoglobinopathies

The hemoglobinopathies have constituted a model for the development of many molecular analytical methods for mutation characterization in patients, carriers and PND samples.

The mutations underlying the hemoglobinopathies give examples of almost all mutation categories associated with inherited diseases (notable exceptions include triplet repeat mutations and imprinting defects). Point mutations within, or close to, functional genes are the most frequent mutations found to cause β-thalassemia and abnormal hemoglobin variants, and deletions removing functional genes are the most common defects underlying the α- and δβ-thalassemias (see [201]).

For genetic-based testing, and most especially PND, methods must be rapid, efficient and reliable, with sensitivity and specificity approaching 100%. The requirements of methods for identifying mutations in candidate parents differ slightly from those needed for the analysis of the prenatal sample. Since many couples request PND following the initiation of a pregnancy, methodologies for characterizing parental mutations should preferably be rapid, capable of detecting a wide range of mutations and, if there is a high sample workload, preferably high throughput. For the PND itself, once the parental mutations have been definitively characterized, the methods and strategies used should be focused to ensure 100% accuracy, additionally precluding pitfalls such as fetal sample contamination and tube switching, all according to best practice recommendations [14,202].

The choice of method(s) used for genotyping samples also depends on factors including the types of mutations underlying the disease (point mutations or deletions), their heterogeneity, whether there are population-specific and/or recurrent mutations, the infrastructure of the laboratory, the available expertise and budget. Each method has relative advantages and limitations, as summarized in Table 2. For example, with the hemoglobinopathies, in the populations where they are endemic, the majority of variant alleles are accounted for by a few common mutations, along with a number of rarer mutations. Knowledge of the population-specific mutation spectrum(s) guides the selection of the most appropriate methods for molecular analysis, although with the advent of global migration and multiethnic societies, population-specific targeted diagnostic approaches are becoming less relevant.

The first method applied for molecular genetic analysis was Southern blotting, which was suitable for detecting large deletions or insertions [15]. However, it was generally inadequate when detecting mutations affecting a single or a few nucleotides,

Table 1. Genotype–phenotype interactions in the hemoglobinopathies and indications for prenatal diagnosis.

Genotype interaction	Clinical phenotype	Prenatal diagnosis indicated
α-thalassemia		
Hom. deletion α^{o}-thal	Hb Bart's hydrops fetalis	Yes
Hom. deletion α^{+}-thal	Not clinically relevant	No
Hom. nondeletion α^{+}-thal	HbH disease, to severe α-thal carrier phenotype	Occasionally
Deletion α^{o}-thal/α^{+}-thal	HbH disease	No
Deletion α^{o}-thal/nondeletion α^{+}-thal	Severe Hb disease	Occasionally
β-thalassemia and β-hemoglobinopathies		
Hom. β^{o} or severe β^{+} mutation	Thal major	Yes
Hom. mild β^{++} mutation	Thal intermedia	Rarely
Hom. δβ-thalassemia	Thal intermedia	Occasionally
Hom. HPFH	Not clinically relevant	No
Hom. Hb Lepore	Thal intermedia to major (variable)	Occasionally
Hom. Hb S	Sickle-cell syndrome	Yes
Hom. HbC	Not clinically relevant	No
Hom. HbD	Not clinically relevant	No
Hom. HbE	Not clinically relevant	No
Hom. HbO Arab	Not clinically relevant	No
β^{o}/severe β^{+} thal	Thal major	Yes
Mild β^{++}/β^{o} or severe β^{+} thal	Thal intermedia to major (variable)	Occasionally
δβ/β^{o} or severe β^{+} thal	Thal intermedia to major (variable)	Occasionally
δβ/mild β^{++} thal	Mild thal intermedia	Rarely
δβ/Hb Lepore	Thal intermedia	Rarely
Hb Lepore/β^{o} or severe β^{+} thal	Thal major	Yes
HbC/β^{o} or severe β^{+} thal	β-thal trait to intermedia (variable)	Rarely
HbC/mild β^{++} thal	Not clinically relevant	No
HbD/β^{o} or severe β^{+} thal	Not clinically relevant	No
HbE/β^{o} or severe β^{+} thal	Thal intermedia to major (variable)	Usually
HbO Arab/β^{o} thal	Severe thal intermedia	Yes
HbS/β^{o} or severe β^{+} thal	Sickle-cell syndrome	Yes
HbS/mild β^{++} thal	Mild sickle-cell disease	Occasionally
HbS/δβ thal	Mild sickle-cell disease	Occasionally
HbS/HbC	Sickle-cell disease (variable severity)	Yes
HbS/HbD Punjab	Sickle-cell syndrome	Yes
HbS/HbO Arab	Sickle-cell syndrome	Yes
HbS/HPFH	Sickle-cell trait	No
Double-heterozygous states		
ααα/β^{o} or severe β^{+} thal	Mild thal intermedia	No
αααα/β^{o} and αααααα/β^{o}	Mild to severe thal intermedia (variable)	Rarely

Indications for prenatal diagnosis 'rarely' and 'occasionally' require case-by-case evaluation.
Hom.: Homozygous; HPFH: Hereditary persistence of fetal hemoglobin; Thal: Thalassemia.

Table 2. Advantages and limitations of methods for characterizing globin gene mutations in carriers and conventional prenatal diagnosis.

Method	Advantages	Limitations
Southern blotting	Generic method for detecting large deletions/insertions	Time consuming and cumbersome
Sanger sequencing (automated)	Generic method for detecting point mutations Relatively cheap running costs	Instrument costly Can be technically demanding (laboratory processing and data interpretation)
Allele-specific oligonucleotide probe dot-blot hybridization	Widely applicable and reliable	Traditionally used radioactively labeled probes Screens one mutation at a time (time consuming)
Reverse dot-blot hybridization	Multiplexed mutation screening Relatively inexpensive Simple, rapid and reliable	Difficult to standardize and validate in-house Commercial kits/systems may be costly
ARMS-PCR	Simple, rapid and inexpensive Can be modified for multiplexed mutation screening	Stringent PCR conditions paramount for accuracy If primers degrade at the allele-specific 3′ end, then PCR will be nonspecific
Denaturing gradient gel electrophoresis	Allows medium-scale screening Predictive software can support optimization Robust heteroduplex detection Relatively cheap	Variant samples need definitive characterization with subsequent method Some regions (especially when CG-rich) may be difficult to optimize and analyze Overall technically demanding
Denaturing high-pressure liquid chromatography	Allows large-scale screening (automation) Highly sensitive with distinct elution profiles Relatively cheap running costs	Variant samples need definitive characterization with subsequent method Instrument costly
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 dropout (not recommended as a standalone method for prenatal diagnosis)
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 mutations, (although it can be cost effective for prenatal diagnosis with prior knowledge of parental samples)
Multiplex ligation dependent probe amplification	Once primer-probe sets validated, it is simple, rapid and suited to automation Can detect any copy number variation within the locus Commercial kits available	Automated sequencer required for fragment analysis (costly) Unknown/sporadic SNPs may interfere with primer–probe hybridization DNA quality and concentration may be critical
High-resolution melting analysis	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 subsequent method Specialized and relatively costly instrument
Microarrays	Potentially high throughput	Inadequate for samples with polymorphisms or new mutations Most microarray platforms technically demanding (sample processing and data interpretation) Instruments and assays quite costly Few systems are fully validated

SNP: Single-nucleotide polymorphism.

with the exception of mutations, which (coincidentally) altered a restriction enzyme cleavage site. In addition, Southern blotting is slow and very cumbersome for routine diagnostic use, especially for PND. The discovery of the PCR in 1985 [16] led to a revolution in molecular genetics, providing the basis for numerous new methods, concurrently facilitating the elucidation of the molecular basis of many inherited diseases. Almost all the methods currently used in a diagnostic setting for both point mutations

and even many large deletions/insertions are based on PCR, and are suitable for characterizing the mutations in carrier couples, as well as prenatal samples [14].

Among all the methods available for detecting nucleotide variations (point mutations), direct sequencing using an automated DNA sequencing instrument is the most comprehensive, capable of detecting any nucleotide variation within a selected region [17]. However, as is the case for any laboratory procedure, it is prone to laboratory errors (technical or human) and/or errors in data interpretation [18]. Furthermore, its use is restricted to laboratories with budgets that can cover the initial purchase of equipment and the relatively high running costs. Other mutation detection methods have been traditionally separated into two categories: direct (targeted) mutation detection methods and scanning methods. Direct mutation methods are designed to interrogate samples for the presence/absence of specific mutations likely to be present underlying the disease and/or within the population group to which the carrier parents belong. Many such protocols have been customized to identify numerous point mutations [14], and are commonly based on methods such as allele-specific oligonucleotide probe hybridization, amplification-refractory mutation system (ARMS) PCR or restriction endonuclease analysis of PCR (RE-PCR) products, some of which are available as commercial kits. For detecting common deletions with known breakpoints within the α- or δβ-thalassemias, many 'Gap-PCR' protocols have been described [14]. Generally, for laboratories that only need to identify a relatively limited number of mutations, all of the aforementioned methods should be adequate for the reliable and accurate (if applied correctly) identification of parental mutations, and the subsequent PND. With the advantage of simplicity, relatively low cost and requiring only basic laboratory infrastructure, they are ideal for laboratories with limited resources, as is commonly the situation in less developed regions of the world where the hemoglobinopathies are traditionally endemic.

However, with the trend of globalization, the mutation spectrums within geographical regions are broadening, as already observed in several countries in Northern Europe [2]. In this context, more generic mutation detection methods are appropriate. For point mutations, one approach is to apply gene-region scanning methods that are capable of identifying (or excluding) sequence variations in the selected region. Methods in this category that have been validated and successfully applied to PND include denaturing gradient gel electrophoresis (DGGE) [19] and denaturing high-pressure liquid chromatography (dHPLC) [20]. When scanning methods are used within a diagnostic setting, it is imperative to subsequently definitively characterize any nucleotide variation indicated, using either targeted direct mutation assays or automatic sequencing. In this way, once the parental mutations have been identified and linked to a DGGE mobility pattern or a dHPLC chromatogram, these protocols can be reliably applied to PND. Scanning methods are advantageous as they provide a means to reduce the use (effort and cost) of targeted assays or sequencing, and have proved to be reliable and relatively inexpensive to run. Their disadvantages include the requirement of specialized equipment, which may be costly to purchase (especially for dHPLC).

Several newer techniques are emerging for detecting disease-causing mutations (including either nucleotide variations or deletions/duplications), although to date, experience of their use within clinical diagnostic settings are limited for most. In addition, all rely on the use of expensive and in most cases highly specialized equipment. These methods include real-time (RT)-PCR [21–23], high-resolution melting curve analysis (HRMA) [24–32], multiplex ligation-dependent probe amplification (MLPA) [33–35], pyrosequencing [36] and genotyping microarrays [37–39].

Protocols based on RT-PCR have been described for detecting both point mutations and evaluating gene copy number, the latter appropriate in samples with suspected deletions or duplications in the α- or β-globin gene clusters (*HBA* and *HBB* respectively) [21]. Point mutations are analyzed using qualitative RT-PCR with the use of allele-specific probes or primers [22], whereas deletions or insertions (duplications) are analyzed by performing gene-region dosage measurements using quantitative RT-PCR [23]. Some protocols have been reliably applied in the context of analyzing carrier parents but only a few have been applied for PND. Generally, a wider application of RT-PCR protocols in PND is limited by both the costs of the assays and the initial outlay for the specialized instruments.

High-resolution melting curve analysis is a relatively new method used usually to detect nucleotide variations in gene regions, analogous to the principle of DGGE. The melting profile of a PCR product depends on its GC content, the length and the sequence composition; whereby changes in melting profile, as well as heteroduplex formation, will occur when mutations are present. The melting profiles are monitored through the use of saturating dyes, which show fluorescence when intercalated within double-stranded PCR products. HRMA is advantageous as a closed-tube method, involving no post-PCR manipulations, although it does require the use of specialized equipment for the melting analysis. With relatively low running costs and potential simplicity, HRMA is a promising alternative for DGGE, dHPLC and, with the inclusion of appropriate mutation detection probes, is transformed into a mutation-targeted method [24,25]. High-resolution melting analysis is reported to have a sensitivity and specificity of 100% for PCR products smaller than 400 bp in length, and should thus be well suited to the relatively small globin genes, although the high number of mutations in the *HBB* gene within a relatively small region complicates the design of convenient primers that give amplicons with robust melting curves [Traeger-Synodinos J, Vrettou C, Kanavakis E, Unpublished Data]. Previous studies have successfully analyzed heterozygotes and homozygotes for several common β-gene variants [25–31] and even α-thalassemia deletions in combination with Gap-PCR [32]. Although it has been suggested that it may also be appropriate for PND, further clinical validation of HRMA for globin gene mutation detection is pending, and the need to purchase relatively expensive, specialized equipment may limit its wider use.

Multiplex ligation-dependent probe amplification is a recently described method that can detect copy number genomic variants within a targeted chromosome region of up to several hundred kilobases. It is based on ligation of multiple probe pairs hybridized across the region of interest, followed by PCR using universal-tag PCR primers to amplify the ligated probe pairs (each designed

to have very slight size differences), and finally fragment analysis of the multiplex PCR products [33,34]. The PCR step in MLPA is essentially quantitative due to the use of universal PCR tag-primers at the 5′ and 3′ ends of the similarly sized ligated probe pairs (one of which is fluorescently labeled), such that when the PCR fragments are analyzed with capillary electrophoresis, relative quantification allows detection of deletions or duplications across the locus being analyzed. With availability of commercial kits for many disease-associated loci, including the *HBA* and *HBB* gene clusters [35], MLPA is being applied in an increasing number of diagnostic laboratories for a wide spectrum of inherited diseases (see MRC-Holland and Service[XS]). As a new technology, it has not yet been widely applied for PND, although since it is a robust method, this will potentially change. Overall, MLPA represents a valuable alternative for Southern blot analysis or a supplementary method to Gap-PCR.

Pyrosequencing is a real-time 'sequencing by synthesis' technique that differs from the Sanger sequencing and is highly suited to sequencing of small target gene regions to detect nucleotide variations. Recently, its application has been reported for characterizing clinically significant nondeletion α-thalassemia mutations [36], where it proved to be rapid, flexible, accurate, reliable and less costly than sequencing based on the widely established Sanger-sequencing technology devices. The authors suggest that pyrosequencing is also potentially suited for PND, but wider clinical validation is required [36].

Microarray-based genotyping methods held the promise of simplified multiplex diagnostic protocols. However, although several microarray methods have been described for application to various monogenic diseases, including the hemoglobinopathies, their application has been very limited so far in clinical settings, especially for PND [37–39]. To date, the few examples illustrate that microarray technologies probably do not offer many advantages over current methods, especially DNA sequencing. They are inadequate when samples have polymorphisms and new mutations within the gene region (otherwise easily detected by DNA sequencing), highlighting a substantial disadvantage for match/mismatch-based microarray technologies. For both sample processing and data interpretation, microarray platforms tend to be quite technically demanding, and thus require highly trained operators. For optimal high-throughput efficiency, microarray systems require support by integrated automated DNA extraction and PCR preparation systems. Overall, large-scale validation of microarrays platforms remains incomplete and the costs of all platforms are quite high [38]. Moreover, although microarrays potentially facilitate rapid diagnosis of parental mutations, high-throughput methods are inappropriate for processing PND samples, which should preferably be approached with a 'personalized' strategy to preclude pitfalls such as sample misidentification.

Other emerging methods, such as MALDI TOF [40] or next-generation sequencing, will not be discussed in detail here since they have not yet been validated in stringent diagnostic settings such as PND [41], although this may change in the near future (see the 'Expert commentary' and 'Five-year view' sections).

Finally, supplementary to the main PND genotyping is the need for protocols to monitor for the presence of maternal contamination, which, if present, may lead to spurious genotyping results, and is a major pitfall in all PND based on PCR methods. This is usually achieved by the use of analyzing highly polymorphic regions (variable nucleotide tandem repeats, or short tandem repeats) in the genome in the parental and PND samples, whereby any inconsistent results will indicate maternal contamination (and also nonpaternity) [14].

Preimplantation genetic diagnosis

General considerations

Preimplantation genetic diagnosis avoids the need to terminate affected pregnancies, through the identification and selective transfer of unaffected IVF embryos. Although the regulatory frameworks vary between countries, even within Europe [42], PGD is generally considered an established reproductive alternative for couples with a high risk of transmitting an inherited disorder, and is especially suited to those with a difficult or unsuccessful reproductive history [9]. The first clinical cycles were applied over 20 years ago [43] and it is currently offered in a substantial number of specialized centers throughout the world [203]. With respect to monogenic diseases, PGD can theoretically be applied for any genetic disease with a definitive molecular diagnosis and/or defined linkage within a family.

Preimplantation genetic diagnosis requires close collaboration between experts in assisted reproductive techniques (ART) and genetics, and involves many stages: evaluation of the couple with respect to genetics and assisted reproduction, counseling of the couples, all stages of ART, the biopsy of genetic material representing each embryo and the genetic analysis, and, if implantation occurs subsequent to embryo transfer, follow-up of pregnancy and baby (or babies) delivered.

With the exception of the step for oocyte/zygote or embryo biopsy, ART within PGD is the same as for infertility treatment, irrespective of whether the couple is fertile or not. Genetic analysis may be performed at various stages postfertilization, including the oocyte/zygote biopsied on the first day postinsemination (polar body analysis), on one to two blastomeres from cleavage-stage embryos biopsied on the third day postinsemination (blastomere biopsy) or on five to ten trophectoderm cells biopsied from blastocysts on the 5th day postinsemination (blastocyst biopsy). There are relative advantages and limitations for each choice (outside the scope of this article), but to date most PGD cycles involve blastomere biopsy [9,44].

Whatever the stage of biopsy, the quantity of sample available for genetic analysis is very limited, and is usually only a single cell. The limited sample is considered the most technically challenging aspect of PGD, compounded by the often suboptimal quality of the embryo and/or embryo cell biopsied. For monogenic diseases PGD involves methods that are almost exclusively based on PCR. The protocol has to be rapid to produce a result within approximately 24–72 h, depending on stage of biopsy and transfer. Prior to clinical application, the protocol must be stringently optimized to address the innate limitations of

single-cell PCR, including total PCR failure, failure to detect both alleles (allelic dropout [ADO]) and sample contamination, the latter potentially occurring during any stage of the PGD procedure (ART, embryology and genetic analysis). PCR failure, although undesirable, will not lead to an unacceptable misdiagnosis. On the other hand, ADO and contamination may lead to serious misdiagnosis. It is recommended that optimized PGD-PCR protocols are based on multiplex and fluorescent-PCR (F-PCR), to facilitate analysis of several linked markers across the disease-associated locus, addressing the aspects of ADO and monitoring contamination. This, along with stringent laboratory procedures applied during PGD analysis, should ensure optimal efficiency and accuracy of the PGD result, supporting the transfer of unaffected embryos [45,46].

Examples from the hemoglobinopathies

Preimplantation genetic diagnosis for hemoglobinopathies is now one of the most common applications for monogenic diseases, as based on the annual data collections published by the European Society of Human Reproduction and Embryology (ESHRE) [44].

The first PGD cycles for β-thalassemia were reported in 1998, based on analysis of polar bodies and using genotyping protocols involving nested PCR with restriction enzyme analysis of the disease-causing mutations [47]. With the development of more sophisticated molecular analytical tools, methods have evolved towards highly multiplexed, F-PCR protocols, allowing accurate, sensitive assays [45,46]. As with classic PND, PGD applications are restricted to the hemoglobinopathies with severe clinical expression (Table 1), most frequently the β-thalassemia and sickle-cell syndromes.

Despite the large number of mutations underlying the β-hemoglobinopathies, the small size of the *HBB* gene, along with the clustering of the majority of common mutations within the 5′ region of the *HBB* gene has facilitated the development of more generic PGD protocols. This precludes the need to develop patient-specific protocols each time, and is particularly appropriate for PGD centers expecting a relatively high number of cycles, as is potentially the case where the hemoglobinopathies are traditionally endemic. Among the more recent methods described for PGD of hemoglobinopathies are mini-sequencing or RT-PCR [48,49]. Mini-sequencing, coupled with microcapillary analysis, permits the quick and accurate detection of the disease-causing mutation and additionally any linked single-nucleotide polymorphisms (SNPs), the latter to enhance the accuracy of the PGD, analogous to the use of linked microsatellite analysis. Mini-sequencing is ideally suited for single-cell PCR, since the amplification of small stretches of DNA is much more efficient than for larger fragments in a single cell [48]. RT-PCR with hybridization probes also provides a rapid and accurate approach for genotyping single cells for PGD and is ideally suited to PGD of β-thalassemic hemoglobinopathies since the relatively small size of the β-gene allows multiplex genotyping for the majority of mutation interactions [49,50].

Protocols for PGD applied to preclude the severe forms of α-thalassemia commonly found in Southeast Asia and China, which are usually caused by homozygosity for deletions of both functional *HBA* genes from the α-globin gene cluster, (Hb Bart's hydrops fetalis; see Table 1), have been described and are usually based on either multiplex fluorescent Gap-PCR [51] or linkage analysis of polymorphic sites within the disease-associated locus [52].

To overcome the limitations of testing single cells with PCR-based protocols, whole-genome amplification (WGA) on the single (minimal) cell samples prior to the genotyping step represents an emerging approach. Initial PCR-based protocols such as degenerated oligonoucleotide-primed PCR or primer extension PCR had limited success [53], the emergence of multiple displacement amplification (MDA) utilizing bacteriophage ϕ.29 polymerase provides a more efficient and faster means for DNA amplification [53,54]. However, levels of ADO post-MDA are observed to be quite high (up to 30%) [54,55; Traeger-Synodinos J, Vrettou C, Kanavakis E, Unpublished Data], making MDA worthwhile only when PGD protocols are targeted for diseases associated with large genes and many potential mutations (e.g., cystic fibrosis or Duchenne muscular dystrophy). In such cases, a more generic approach for clinical PGD cases is possible involving initial WGA followed by disease-specific PCR multiplexes applied under standard laboratory conditions [56]. WGA is not so useful for PGD protocols applied to hemoglobinopathies. An exception may be when the PGD is applied for HLA typing in addition to the selection of embryos unaffected for a hemoglobinopathy; although, even for such applications it has been observed that direct single cell analysis without prior WGA gives robust results for up to approximately 20 multiplexed loci [57; Traeger-Synodinos J, Vrettou C, Kanavakis E, Unpublished Data]. The selection of a histocompatible sibling to facilitate a bone marrow transplant in a thalassemia major patient is considered appropriate in a number of societies, where many of the ethical controversies associated with donor-sibling selection have been resolved [58,59]; although the ultimate success, that is, the birth of an unaffected histocompatible baby, is very limited in practice: if the chance of two siblings being HLA-matched (25%) is combined with the chance that only 75% of embryos will be unaffected for the hemoglobinopathy, then only 18.8% of all embryos fertilized in any cycle will be suitable for transfer. When combined with the overall success of implantation and pregnancy delivery rates, which are approximately 30% on average, the overall success rate for PGD-HLA matching rarely surpasses approximately 10–15% for any cycle initiated [60]. Before embarking on this reproductive option all couples should be clearly counseled and informed about this, although to date, over 200 clinical cycles have been reported [44,61].

Noninvasive PND

The well-established existence of fetal cells and free fetal nucleic acids (including DNA and RNA) in the maternal circulation has promoted much research effort towards developing approaches for 'noninvasive' PND (NIPD), applicable to the exclusion of both single-gene and chromosome disorders [8]. Fetal cells represent the ideal source of fetal genetic material for NIPD, since they offer the potential of achieving a 'full' genetic analysis. This is in contrast to cffDNA where only paternally inherited alleles that differ from those carried by the mother can be distinguished with most current methods.

Among fetal cell categories found in the circulation are trophoblasts, fetal leukocytes and fetal nucleated erythroblasts (nucleated red blood cells [NRBCs]). Each cell type has some disadvantages as targets for NIPD analysis: trophoblast cells may be multi- or even anucleate, and there is also a slight risk (1%) of placental mosaicism; fetal leukocytes may persist in the maternal circulation after the pregnancy has been delivered, a disadvantage for NIPD in women who have had previous pregnancies; and NRBCs may be of both fetal and maternal origin. However, most importantly, fetal cells are extremely rare, with an estimated single fetal cell in every 1 ml of whole maternal blood. Despite these drawbacks, many fetal versus maternal cell-separation methods have been investigated. They have tried to exploit differential cell size (fetal cells tend to be larger than maternal cells), differential expression of cell-surface antigens and magnetic properties of cells, using optical, magnetic or size-based methods [62]. To date, none have achieved yield and stringency of fetal cell isolation acceptable for clinical applications, including efficiency, effectiveness and cost [8,63]. An additional drawback is the often poor quality of the genetic material in fetal cells, which, at least in fetal erythroblasts, is related to the process of enucleation that erythrocytes undergo as they mature [64,65]. With respect to the diagnosis of monogenic disorders based on PCR analysis, this presents a major limitation, and probably explains the high rates of ADO that have been observed when analyzing isolated fetal NRBCs [66,67]. However, recently, efforts have been revived to isolate fetal cells using approaches that are based on more automated cell-sorting techniques. To date, technologies that have been investigated include automated microscopy [68], microfluidics [69], light-scattering spectroscopy [70] and a combination of automatic screening for enriched target cells (based on immunofluorescence labeling) with isolation of single candidate microchimeric cells (by laser microdissection and subsequent laser catapulting) and finally low-volume on-chip multiplex PCR for DNA fingerprint analysis [71]. Preliminary results with these methods have been encouraging and indicate potential for NIPD applications, assuming all limitations and technical problems encountered so far can be addressed [8,63].

With respect to cffDNA, its presence in the maternal circulation was first reported in 1997 [72]. Studies have demonstrated that cffDNA is mainly derived from trophoblastic cells [73], that it is stable but cleared rapidly following birth, and that it comprises a minority fraction of total (maternal and fetal) cell-free DNA, ranging from 3 to 20% (up to 100 genome-equivalents/ml), depending on the stage and state of the pregnancy and method of quantification [74–76]. Another characteristic of cffDNA molecules is that they are generally shorter (<200–300 bp) than the cell-free DNA fragments of maternal origin [77]. Based on this observation, protocols for the relative enrichment of fetal DNA fragments have been investigated, ranging from simple electrophoresis in agarose gels [78–80] to more sophisticated microfluidic microsystems [81].

Protocols to detect paternally derived genetic loci in cffDNA that are not shared by the maternal genome are relatively uncomplicated. These include the rhesus D locus, Y-chromosome sequences and dominant diseases, when transmitted by the father. Many protocols have been described and some applications for testing the Rhesus status or the sex of an ongoing pregnancy have already been transferred into clinical practice [82–84]. However, progress has been slow for the more demanding NIPD applications, which aim to distinguish the status of the pregnancy relative to autosomal recessive hereditary disorders. The fact that 50% of the fetal genome is shared with the mother precludes achieving a 'full' genotype in NIPD with most current methods. The detection of paternally transmitted alleles is limited to those with sequences that differ from those of the mother, either the pathological mutation directly, or SNPs with known linkage and phase relative to the paternal alleles. In fact, analysis of paternally transmitted linked SNPs is the only option when a couple shares identical disease-causing mutations. The highly homologous maternal alleles compete with the cffDNA during PCR amplification, making detection of paternally transmitted sequences, which do not usually differ by more than a single nucleotide, extremely difficult. To address the very low and minority levels of cffDNA, methods for NIPD must combine high sensitivity with high specificity in order to fulfill the criteria of a clinically robust assay.

Examples from the hemoglobinopathies

The hemoglobinopathies, particularly the β-thalassemias, constitute one of the main model monogenic diseases for which methodologies are being investigated for NIPD.

With respect to fetal cells, there are minimal reports describing approaches for NIPD of hemoglobinopathies. One protocol described the application of NIPD for hemoglobin Bart's hydrops fetalis (Table 1). The protocol involved the isolation of NRBCs from at-risk pregnancies (10–26 weeks), enriched using magnetic cell sorting with an anti-CD71 antibody, followed by staining of fetal NRBCs with rabbit antihuman α-globin antibody and immunofluorescent microscopic evaluation of samples [85]. No further applications of this approach have been reported and, generally, analogous applications to other inherited disorders will be limited by the absence of appropriate target cells and antibodies to distinguish disease versus the unaffected status of ongoing pregnancies. Two other protocols for NIPD of β-hemoglobinopathies have also involved the analysis of CD71 magnetic cell sorting-enriched NRBCs, which were then individually isolated from slides by microdissection [86] or PALM microbeam laser microdissection and pressure catapulting [67], and then subject to PCR-based genotyping of the *HBB* gene. One of these studies also included the analysis of polymorphic microsatellite loci to differentiate maternal versus fetal NRBCs [67]. Finally, another study applied PCR-based analysis of the mutation-containing region of the *HBB* gene in individual NRBCs enriched by high-speed gradient centrifugation and immunofluorescent staining with anti-ξ-globin [87]. As mentioned previously, to date, the results of all studies do not meet criteria acceptable for clinical application, highlighting the limitations of isolating sufficient quantity and satisfactory quality of fetal NRBCs for accurate analysis.

With respect to cffDNA, many different approaches have been investigated for the noninvasive exclusion of the paternal β-thalassemia allele. More simple protocols have used RT-PCR for the

paternally inherited codon 41/42 (-CTTT) β-thalassaemia mutation [88], although of note is that this mutation is a 4-bp deletion rather than a single-nucleotide substitution, somewhat facilitating its more robust detection in the context of NIPD. Another simple approach describes restriction enzyme analysis of PCR products to detect the paternally transmitted HbE mutation [89].

A more sophisticated approach described the use of MALDI-TOF mass spectrometry (MS) to detect paternally transmitted alleles, either based on conventional homogeneous MassEXTEND assay, or for the analysis following the nucleotide-specific single-allele base extension reaction or the allele-specific base extension reaction [90,91]. These methods were applied to exclude the fetal inheritance of common Southeast Asian β-thalassaemia mutations or, when couples shared identical β-thalassaemia mutations, the exclusion of informative SNPs linked to the *HBB* locus. These MS-based methods demonstrated high sensitivity and specificity, with potential for high-throughput automated analysis, but the use of sophisticated and expensive equipment limits the wider potential of this approach in the diagnostic laboratories.

Another approach for NIPD involved enriching the cell-free DNA samples by exploiting the smaller size of cffDNA (<200–330 bp) compared with circulatory maternal DNA sequences (>500 bp) [77,78]. Even using separation based simply on agarose gel electrophoresis, cffDNA could be enriched over five- to six-times [79]. In one study, downstream analysis of enriched paternally derived alleles involved PCR reactions in the presence of a peptide–nucleic acid (PNA) clamp, designed to suppress amplification of the normal maternal allele. PNAs are DNA analogues that have a peptide backbone instead of a ribose–phosphate backbone. Totally complementary PNA–DNA hybrids have much higher thermal stability than corresponding DNA–DNA hybrids, but are more destabilized by single-pair mismatches, whereby PNAs can be designed to specifically impede amplification of a wild-type allele target, while allowing amplification of the analogous mutant allele. Following size fractionation of the isolated free DNA, the paternal mutant allele could be detected by an allele-specific PCR/PNA protocol with an overall sensitivity of 100% and specificity of 93.8% [79]. This approach did not involve sophisticated equipment, although the gel electrophoresis/elution-based size enrichment of fetal sequences is time consuming and prone to contamination. As a principle, enrichment of cffDNA has also been demonstrated with downstream analysis based on RT-PCR and MALDI-TOF MS, with apparently robust results [80].

Another protocol applied the use of PNAs to impede amplification of wild-type sequences of six β-thalassemia mutations, and subsequent mutation detection based on microelectronic microchip analysis, with confirmation of results in some samples using direct sequencing or pyrosequencing. Although the results were accurate, showing complete concordance with the classical PND results, the chemistry of each PCR/PNA protocol was not very robust, requiring careful assay-specific optimization [92].

Finally, another microchip assay, the arrayed primer extension microchip assay, which is based on enzymatic extension of allele-specific probes immobilized via their 5′ end on a glass surface in an array format when hybridized to PCR amplified samples [38,39], was tested to detect 11 different paternally inherited SNPs within the *HBB* locus in pregnancies from couples sharing identical β-thalassaemia mutations [93]. The paternally transmitted fetal allele (mutant or normal) was determined in six out of seven maternal plasma samples through linkage, demonstrating both a high degree of sensitivity and specificity. However, further optimization of the microarray assay is required, and a major disadvantage is the use of expensive, specialized equipment for the microarray analysis.

Expert commentary & five-year view

Since the first applications of PND for inherited disorders over three decades ago, there have been impressive advances in molecular genetic technologies, obstetrics and ART, which have supported the development of numerous robust protocols for genetic testing and PND.

The early post-PCR era in the 1990s saw the development of numerous methods for mutation detection. These were translated by diagnostic laboratories into 'home-brew' tests, considered to be simple, robust, relatively inexpensive diagnostic protocols, capable of producing a reliable result within a time frame acceptable for conventional PND (including the characterization of carrier-couples). However, these 'home-brew' tests, are not easily amenable to automation and high throughput. This can be an issue in diagnostic laboratories with a heavy workload when confronting the characterization of parental mutations before a PND, especially when targeting a potentially wide range of candidate mutations and mutation categories (point mutations or gene deletions and duplications). Furthermore, 'home-brew' tests may present issues with quality control and standardization of laboratory protocols. Ideally, a diagnostic test should be applicable for detecting a wide range of mutations, and be rapid, reliable, automated and affordable. The latter is especially relevant for PND centers in developing countries, where hemoglobinopathies tend to be extremely common and where resources are often limited. To date, no single method yet fulfills the ideal criteria of a universal, rapid, reliable, automated and inexpensive method. However, new technologies, most noteably new-generation sequencing, are expected to facilitate the development of ideal diagnostic tests, possibly within the next 5 years. Several studies have already attempted to validate new-generation sequencing for analyzing samples for disorders such cardiomyopathies or breast cancer susceptibility [94,95]. They have highlighted the current limitations and pitfalls (accuracy and costs), which remain to be addressed before it can be more widely applied in a clinical diagnostic setting, especially critical for carrier identification and all forms of PND. Finally, the existence of commercially bench-marked (*in vitro* diagnostic-certified) kits for a more targeted approach for mutation detection would also be desirable.

During the 20 years during which PGD has been applied in a clinical context, continuous technical improvements have supported progression from an experimental procedure to a widely acceptable alternative to conventional PND. Most of the technical, practical and ethical issues have been addressed, and extremely reliable and accurate single-cell genetic diagnostic protocols are now available [42,59,96–98]. However, despite many advances, PGD remains, for the most part, a technically challenging, multistep and

labor-intensive procedure, requiring close collaboration between many specialists. Although the genetic analysis can be stringently optimized, limitations to wider application of clinical PGD cycles include the requirement to involve ART, even if the couple is fertile, the high cost of a complete PGD cycle and the fact that pregnancy and birth rates rarely surpass 30–35% in most cases. The latter can be partly attributed to chromosomal mosaicism and chaotic cell division frequently observed in preimplantation embryos, even from normal fertile couples [99,100]. Aneuploidy screening of preimplantation embryos (known as preimplantation genetic screening [PGS]) has been applied for many years using fluorescent *in situ* hybridization on biopsied polar bodies or blastomeres. However, there is much controversy regarding whether fluorescent *in situ* hybridization-based PGS actually improves pregnancy and live-birth rates [101]. Recently, a method coined 'karyomapping' has been developed, representing a universal approach for genome-wide analysis of genetic disease in preimplantation zygotes or embryos. It is based on mapping the crossovers between parental haplotypes, involving the use of whole-genome amplification followed by genome-wide SNP microarrays [102]. 'Karyomapping' potentially combines PGD applied to single gene defects with aneuploidy screening, allowing the selection of embryos unaffected for the targeted monogenic disease, which also have a normal complement of chromosomes (ploidy). Although wider clinical validation is pending, it can be foreseen that in the next 5 years, further optimization of array technologies should facilitate simpler and more automated protocols, to support even wider application of PGD, as well as possibly addressing the improvement of ART outcome.

Despite considerable progress in the field of NIPD, there are many technical challenges to be resolved before it can be considered a reliable alternative for conventional PND applicable to autosomal recessive inherited disorders (including hemoglobinopathies). Issues that have been raised by technologies tried so far include the need to improve assay specificity for robust detection of the paternally transmitted fetal alleles, and that no information is derived about the alleles inherited from the mother. However, recently, new technologies that facilitate single molecular counting have been described, including digital PCR and next-generation sequencing [103–106]. They demonstrate enormous analytical potential, supporting the possibility to determine the 'full' fetal genotype in cffDNA associated with a monogenic disorder, including the detection and characterization of the maternally derived alleles. These methods require expensive equipment and reagents, and substantial optimization and improvements are needed before they can be considered for clinical application. However, they certainly represent a breakthrough that may potentially allow NIPD for all pregnancies and monogenic diseases, irrespective of the mutations involved. It can be foreseen that within the next 5 years, digital PCR and next-generation sequencing may be among the choices available for diagnostic strategies applied for NIPD.

The current strategies and methods for conventional PND, PGD and NIPD have their relative advantages and drawbacks (Table 3). Overall, the next 5 years can expect to witness technological developments that will contribute towards further improvements in prenatal diagnostic strategies for inherited disorders, even extending to simultaneously include an overall evaluation of

Table 3. Relative advantages and drawbacks between conventional prenatal diagnosis, preimplantation genetic diagnosis and 'noninvasive' prenatal diagnosis.

Parameter	Conventional PND	PGD	NIPD
Timing of genetic analysis	Second or third trimester (based on CVS or amniocentesis sampling, respectively)	Before embryo transfer during ART	From ~7 weeks gestation
Risk to fetus, pregnancy or baby	Miscarriage ~2% (Other rare complications include infection and fetal injury)	No greater risk than conventional ART	Not expected
Accuracy of genetic analysis	>99%	>99% (using multiplex single-cell assays)	Not yet validated
Chance of healthy baby delivered	75% (based on risk of transmitting recessive monogenic disease)	30% per embryo transfer (mainly limited by embryo implantation rates and pregnancy outcomes)	Not yet validated
Major drawback(s)	Need to terminate affected pregnancy	Technically challenging, multi-step and labor-intensive procedure Requires IVF (even if couple fertile) Relatively low pregnancy and birth rate Relatively costly	'Low technology' methods lack specificity and robustness, and provide no information on contributing maternal allele Emerging 'high technology' methods require expensive equipment and reagents Still await optimization of accuracy and reduction of costs prior to clinical application
Major benefit(s)	Well-validated procedures	Precludes need to terminate affected pregnancies	Precludes invasive fetal sampling

ART: Assisted reproductive techniques; CVS: Chorionic villi sampling; IVF: *In vitro* fertilization; NIPD: 'Noninvasive' prenatal diagnosis; PGD: Preimplantation genetic diagnosis; PND: Prenatal diagnosis.

fetal wellbeing. This should provide couples with wider and even safer reproductive options when planning a family. Of course, it cannot be excluded that some advances may also be through approaches other than genetic testing, such as proteomics or metabolomics. Finally, it should also be considered that advances in genetic and/or molecular therapies may eventually reduce the wider need for PND of inherited diseases.

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Key issues

For all prenatal diagnosis (PND) applications (conventional PND, preimplantation genetic diagnosis and 'noninvasive' PND):

- Tests should always be applied within strict clinical practice, including all aspects of counseling and ethical considerations appropriate for the couples and wider society.
- The highest standards in laboratory practice are mandatory to preclude any diagnostic errors (best-practice and quality control).
- Laboratories should understand the potential and pitfalls of any methods they apply.
- Methods should ideally be accurate, robust, simple, rapid and preferably cost effective.
- It is expected that within the next 5 years, new technologies will facilitate the development of diagnostic PND tests, fulfilling the ideal criteria of being: universal, rapid, robust, automated and cost effective.

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