Non-invasive prenatal testing for aneuploidy: current status and future prospects

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ABSTRACT

Non-invasive prenatal testing (NIPT) for aneuploidy using cell-free DNA in maternal plasma is revolutionizing prenatal screening and diagnosis. We review NIPT in the context of established screening and invasive technologies, the range of cytogenetic abnormalities detectable, cost, counseling and ethical issues. Current NIPT approaches involve whole-genome sequencing, targeted sequencing and assessment of single nucleotide polymorphism (SNP) differences between mother and fetus. Clinical trials have demonstrated the efficacy of NIPT for Down and Edwards syndromes, and possibly Patau syndrome, in high-risk women. Universal NIPT is not cost-effective, but using NIPT contingently in women found at moderate or high risk by conventional screening is cost-effective. Positive NIPT results must be confirmed using invasive techniques. Established screening, fetal ultrasound and invasive procedures with microarray testing allow the detection of a broad range of additional abnormalities not yet detectable by NIPT. NIPT approaches that take advantage of SNP information potentially allow the identification of parent of origin for imbalances, triploidy, uniparental disomy and consanguinity, and separate evaluation of dizygotic twins. Fetal fraction enrichment, improved sequencing and selected analysis of the most informative sequences should result in tests for additional chromosomal abnormalities. Providing adequate prenatal counseling poses a substantial challenge given the broad range of prenatal testing options now available. Copyright © 2013 ISUOG. Published by John Wiley & Sons Ltd.

INTRODUCTION

The past quarter century has been witness to a series of remarkable advances in the screening of pregnancies for aneuploidy, particularly in the identification of Down syndrome (trisomy 21). During the 1970s and early 1980s, advanced maternal age, defined in most localities as over 35 years, was the only means by which the general population was assessed as to risk of a fetal chromosomal abnormality. Fewer than one third of Down syndrome pregnancies were diagnosed prenatally and of those undergoing invasive prenatal diagnosis only about 2% had fetal karyotype abnormalities, a figure comparable to the 0.5–1% chance of procedure-related fetal loss associated with amniocentesis or chorionic villus sampling (CVS). In the late 1980s and early 1990s, the introduction of second-trimester maternal serum markers, in the form of ‘double’, ‘triple’ and ‘quad’ marker testing, improved significantly the screening performance for aneuploidy. The proportion of Down syndrome pregnancies diagnosed more than doubled and a chromosomal abnormality was found in as many as 4% of those designated as ‘screen-positive’. In the late 1990s and early 2000s, aneuploidy screening shifted to the first trimester with the ‘combined’ test, which uses ultrasound measurement of nuchal translucency thickness (NT) together with maternal serum concentration of placental proteins human chorionic gonadotropin (hCG) (free β, intact or total) and pregnancy-associated plasma protein-A (PAPP-A). Currently available screening protocols also incorporate additional ultrasound markers and sequential screening using two blood samples, one in the first and one in the second trimester, with or without NT. Consequently, screening performance has improved such that more than nine-tenths of Down syndrome cases can be diagnosed prenatally and the yield from invasive testing has risen to about 6%.

Recently, analysis of cell-free (cf)DNA in maternal blood for non-invasive prenatal testing (NIPT) has been shown to be highly accurate in the detection of common fetal autosomal trisomies. In 1997, Lo et al. first reported their seminal discovery that plasma from pregnant women contained cfDNA, including a fraction designated ‘fetal’, although this is thought to be placental in origin, resulting...
from apoptotic trophoblasts. This and other early studies suggested that the ‘fetal fraction’ was only 3–6%, but more recent studies have found that it may be closer to 10–20%.8 Fetal cfDNA can be detected as early as 4 weeks’ gestation9 and exceeds 4% of all the cfDNA in nearly all women from 10 weeks onward. The typical size of the cfDNA fragments is approximately 150 basepairs10,11 and, importantly, the entire fetal genome is represented. It has been shown that the half-life of fetal cfDNA is very short12; fetal fragments are no longer detectable very soon after birth13,14. There is therefore no serious concern that a prenatal cfDNA test could be confounded by a prior pregnancy in multigravid women. Maternal plasma cfRNA, unlike RNA extracted directly from cells, appears to be relatively stable and can also potentially be used in screening15–17.

The commercial introduction of NIPT raises several concerns. One major issue is the optimal integration of NIPT into current screening practice18. Should NIPT and conventional screening tests be treated as related but independent measures when assessing aneuploidy risk or should they be complementary to one another? How can healthcare providers be educated and prospective patients counseled about all the prenatal testing options now available?

In this Review we provide the information needed by clinicians and public health providers when considering to whom NIPT will be offered and regarding the consequences of doing so, practical suggestions on how to implement this new and powerful technology into routine clinical practice, and some indications of how we expect this testing to expand. We focus primarily on the detection of fetal chromosomal abnormalities, recognizing that the technology will eventually be used for testing for a broad range of other genetic disorders.

**ESTABLISHED SCREENING MODALITIES**

In the current context it is important to distinguish prenatal diagnosis of aneuploidy from antenatal screening. A diagnostic test performed on chorionic villi, amniotic fluid or fetal blood needs to have very few false negatives (aneuploid pregnancies misdiagnosed as euploidy) and false positives (euploid pregnancies misdiagnosed as aneuploid), since the result will inform the decision as to whether to terminate the pregnancy. In contrast, antenatal screening does not aim to be definitive; rather, it is designed to identify women who are at sufficiently high risk of common aneuploidies as to warrant invasive prenatal diagnosis. Since the invasive diagnostic procedures, mainly CVS and amniocentesis, are hazardous and expensive, only a relatively small group of women are identified as being at high risk. Established screening programs determine the risk of Down syndrome, many also including Edwards syndrome (trisomy 18) and some including Patau syndrome (trisomy 13) and Turner syndrome (45,X). The model-predicted aneuploidy detection rates (DR) for fixed false-positive rates (FPR) of established prenatal screening protocols has been reviewed elsewhere4.

Among those undergoing invasive prenatal diagnosis because of a high risk of Down and Edwards syndromes, a large proportion have a different chromosomal abnormality. Alamillo et al.19 reported on 10 years’ experience of a combined test, giving risks of Down syndrome and Edwards or Patau syndromes. The 97 cases with chromosomal abnormalities included Down syndrome (48%), Edwards syndrome (16%), Patau syndrome (6%), Turner syndrome (9%), other sex chromosomal aneuploidies (4%), other aneuploidies including mosaics (9%) and chromosomal rearrangements (6%). The California state-wide screening program20 used a second-trimester quad test for Down and Edwards syndromes and detected a total of 1316 chromosomal abnormalities. Excluding balanced translocations, these were Down syndrome (48%), Edwards syndrome (14%), Patau syndrome (2%), Turner syndrome (8%), triploidy (2%), Klinefelter syndrome (47,XXY) (1%) and other abnormalities (25%). Some of these were chance findings but many were related to maternal age or abnormal marker profiles.

Conventional aneuploidy screening modalities also need to be considered in the context of the substantial number of non-chromosomal fetal abnormalities and pregnancy complications that may be identified as a result of this testing. Particularly important is the role of first-trimester ultrasound, which has the potential to identify a non-chromosomal abnormality in approximately 1% of cases4.

**ESTABLISHED INVASIVE PRENATAL DIAGNOSIS**

**Conventional cytogenetics**

Chromosomal analysis of cultured chorionic villi and amniotic fluid cells can identify fetal aneuploidy, structural rearrangements, such as translocations and inversions, and relatively large duplications and deletions (generally exceeding 5 Mb in size)21. Chromosomal analysis of amniotic fluid cells is considered to be the ‘gold standard’ in prenatal testing because error rates are exceedingly low, probably less than 0.01–0.02%, and mostly appear to be due to maternal cell contamination, laboratory error or typographic mistakes22. Error rates with CVS are higher than those seen with amniocentesis, and these can be due to confined placental mosaicism (CPM), maternal cell contamination or lower resolution banding of chromosomes23. In a small proportion of cases in which CVS karyotyping is carried out there are ambiguous results which may require resampling or amniocentesis for clarification.

**Molecular cytogenetics**

Because of the time taken to obtain a complete chromosomal analysis, the occasional finding of a karyotype of minimal or uncertain significance and cost considerations, some centers perform a rapid aneuploidy test (RAT) using molecular genetic techniques as an adjunct to or
replacement for karyotyping. Methods include the use of fluorescence in-situ hybridization (FISH), quantitative fluorescence polymerase chain reaction (QF-PCR) testing and multiplex ligation-dependent amplification (MLPA). These tests are generally designed to test only a restricted range of cytogenetic abnormalities. Although proven to be very accurate, confirmatory testing using conventional cytogenetics has been recommended.

In contrast, relative to conventional karyotyping, other molecular approaches can now improve substantially on the detection of clinically significant genetic imbalances. In high-risk pregnancies with a normal chromosomal constitution, the increased resolution of array comparative genome hybridization (aCGH) coupled with single nucleotide polymorphism (SNP) array has the potential to identify submicroscopic copy number variations (CNVs), specifically microdeletions and microduplications, as well as regions of homozygosity.

When compared to conventional chromosomal analysis, aCGH and SNP array are purported to have significant advantages in terms of: (1) faster turnaround time; (2) higher throughput while being less labor-intensive; (3) higher resolution independent of the ability of the cells to grow and/or generate good metaphase spreads; (4) amenability to automation and quality control; and, (5) direct mapping of aberrations to the genome sequence. Faster turnaround time is possible because arrays can be performed on DNA obtained from uncultured villi and amniotic fluid cells, thus avoiding the need for tissue culture and the associated time delay required for conventional chromosomal analysis.

A National Institute of Health (NICHD) multicenter, prospective, blinded study evaluated the application of microarrays in over 4000 high-risk pregnancies undergoing prenatal invasive testing largely because of advanced maternal age, a positive first-trimester screen or the presence of an ultrasound anomaly. The use of microarrays resulted in the identification of clinically relevant CNVs that were unrecognizable by conventional karyotyping in 2.5% of all cases. In those cases referred for testing because of an ultrasound abnormality and showing a normal karyotype, 6% were found to have a clinically relevant CNV. These studies were performed on low-resolution aCGH platforms; newer arrays have much higher resolution. The use of SNP arrays has expanded the ability to detect triploidy and significant regions of homozygosity, thereby detecting uniparental disomy and potentially identifying disorders associated with consanguinity. The NIH multicenter study also revealed the limitations of all aCGH and SNP array platforms, the principal limitation being the identification of variants of uncertain significance (VOUS).

Hazard of invasive testing

The principal hazard of amniocentesis is miscarriage, but the excess risk associated with the procedure is difficult to quantify precisely. Some 3–4% of mid-trimester pregnancies will miscarry without amniocentesis and in a particular case of fetal loss following the procedure it is possible only rarely to attribute directly the adverse outcome to the procedure; cases of amnionitis or chronic amniotic fluid leakage would be attributable but these are relatively rare consequences. In a randomized trial of amniocentesis at a single center, the fetal loss rate was 0.8% higher in the amniocentesis group compared with controls. There have been no similar randomized trials for CVS, but a number of studies have compared late first-trimester CVS with second-trimester amniocentesis. A Cochrane Review showed that, when performed by a skilled operator, the fetal loss rates of the two procedures were comparable. An updated review concluded that the excess miscarriage rate for either procedure is between 0.5% and 1%. An initial concern that CVS may cause fetal limb reduction defects has not been sustained, as the reported excesses occurred when CVS was performed early in gestation, before 10 weeks.

NIPT METHODS

The fact that fetal cfDNA and cfRNA are present only as minority components of the total cell-free nucleic acids in maternal plasma specimens has posed a significant technical obstacle in the development of NIPT. A number of approaches have been suggested that allow enrichment of the fetal component. These include taking advantage of size differences between fetal and maternal DNA fragments, using formaldehyde in the samples, taking advantage of the nucleosome binding property of the DNA and immunoprecipitation of hypo- or hypermethylated DNA, but none is currently being used in clinical practice. Even without enrichment a number of technical approaches have now been proven to be effective. Each approach relies on the extraordinary advances in molecular biology and sequencing achieved in recent years. We briefly review the major methods below, without discussing in detail all of the possible refinements and other developments that are currently under consideration.

Shotgun massively parallel sequencing (s-MPS)

This approach relies on the identification and counting of large numbers of the DNA fragments in the plasma specimen. Using massively parallel sequencing (MPS), millions of both fetal and maternal DNA fragments can be sequenced simultaneously and, since the entire human genome sequence is known, each piece that maps to a discrete locus can be assigned to the chromosome from which it came. If fetal aneuploidy is present, there should be a relative excess or deficit for the chromosome in question. The difference in counts will be small; for example, if fetal trisomy 21 is present and the fetal fraction is 20%, the relative excess in chromosome 21 DNA fragments will be \((0.8 \times 2) + (0.2 \times 3) = 2.2\) compared with the situation for a euploid fetus \((0.8 \times 2) + (0.2 \times 2) = 2\), i.e. a relative increase in number of chromosome 21 counts of only 10%. To be able to

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detect reliably these differences, large numbers of counts are necessary, the fetal fraction needs to be appreciable and the counts need to be compared with the expected counts for euploid cases. The latter can be achieved either by normalizing counts for the chromosome of interest against other chromosomes that are expected to be disomic within the same test run or by comparing the fraction of counts assigned to a particular chromosome against the fractions seen for a set of known euploid cases. Results can be expressed as a z-score which can be converted into a probability that a specific chromosome result departs from the normal diploid situation. Although it is possible to convert the z-score to a patient-specific risk, major commercial providers of this testing currently choose to present their results only in terms of ‘positive’ or ‘negative’ based on z-scores exceeding predefined thresholds. Another variation of the statistical analysis calculates two Student t-test statistics, one based on the hypothesis that the result is from a euploid fetus and the other that it is from an aneuploid one based on the hypothesis that the result is from a statistical analysis also include adjustment for guanine-cytosine (GC) base content of the sequences.

The approach is referred to as ‘shotgun’ because it relies on sequencing and counting all informative chromosome regions. Sequences which do not map to a unique locus are uninformative. In some studies, only those fragments that have sequences identical to the reference genome sequence are accepted in the analysis, while in others one or two mismatches in the sequence will be accepted. Because of costs, it is common to run multiple samples simultaneously (‘multiplexing’) by adding a ‘bar-code’ sequence to each fragment that will allow identification of the sample origin. The number of cases run together can be variable and is an important consideration because high-level multiplexing places a limitation on the total number of sequence reads that are carried out on each case (i.e. the ‘depth of sequencing’). The number of fetal sequence reads will also vary according to the fetal fraction and therefore a minimum fetal fraction, typically 4%, is currently required. Most studies have used Illumina sequence analyzers but there are also studies using Solid and Helicos platforms.

Targeted massively parallel sequencing (t-MPS)

It is also possible to amplify selectively only those chromosomal regions that are of interest (e.g. chromosomes 21, 18 and 13) and then to assess whether there is a departure from euploidy based on the relative number of DNA fragment counts for this subset of chromosomes. This ‘targeted’ approach has the advantage of requiring considerably less sequencing and thereby reduces costs. The major commercial provider of this approach includes an adjustment to allow for the variability in the proportion of DNA that is fetal and then combines the results of the laboratory test with maternal age to provide a patient-specific risk for Down, Edwards and Patau syndromes.

In theory, results could also be combined with other factors, for example results of other screening tests or prior history of an aneuploid pregnancy.

Single nucleotide polymorphism (SNP)-based approaches

The feasibility of a non-invasive aneuploidy test that takes advantage of DNA polymorphisms was first demonstrated by Dhallan et al. In their approach, the paternal, maternal and fetal SNPs present in blood (paternal), buffy coat (maternal) and maternal plasma (maternal and fetal) were evaluated as bands on sequencing gels. Quantification of band intensities of the uniquely inherited paternal SNPs in the plasma allowed an estimation of the fetal fraction. Comparison of the maternal plus fetal bands with the unique fetal band intensity also allowed estimation of the fetal chromosome 21 dosage.

A similar approach was used by Ghanta et al., who identified combinations of paired SNPs to focus on highly informative regions and then used cycling temperature capillary electrophoresis for evaluating fetal fraction and dosage. The method was thought to be useful in situations in which the fetal fraction was as low as 2%.

A more complex approach that relies on polymorphisms has been proposed by Zimmermann et al. The approach involves a multiplex amplification of 11 000 SNP sequences in a single PCR reaction carried out on the plasma DNA followed by sequencing. Each product is evaluated based on the hypothesis that the fetus is monosomic, disomic or trisomic. After considering the positions of the SNPs on the chromosomes and the possibility that there may have been recombination, a maximum likelihood is calculated that the fetus is either normal, aneuploid (chromosome 21, 18, 13 or sex chromosome) or triploid, or that uniparental disomy is present.

In theory, with SNPs there should be advantages over methods based on total sequence counting. SNPs can provide information about parent of origin of aneuploidy, recombination and inheritance of mutations. On the other hand, SNPs account for only 1.6% of the human genome and therefore enrichment for fetal DNA, deeper sequencing or higher levels of high-fidelity amplification may be required to identify unambiguously affected pregnancies with small imbalances. When few loci are used in the analysis, greater attention needs to be paid to the exclusion of regions in which there are rare benign CNVs. The SNP approach could result in the inadvertent detection of non-paternity or perhaps consanguinity. In the case of assisted reproduction with an egg from an unrelated donor, SNP-based approaches would need to be modified to take into consideration the presence of additional maternally derived fetal alleles not present in the surrogate mother.

The laboratory methods and data analyses used for SNP-based NIPT are substantially different from those used in shotgun and targeted counting approaches. In this Review we therefore consider the emerging clinical trial data for SNP-based testing separately.
Methylated DNA-based approaches

This approach takes advantage of the fact that there will be epigenetic differences throughout the genome which will result in the differential expression of genes, depending on the tissue type\(^5\). These differences can be associated with either hyper- or hypomethylation of the genes. Some genes present in placental cells (specifically, trophoblasts) will therefore differ from maternal cells in the methylation pattern. Methylated DNA can be chemically modified, thus allowing its characterization separate from the unmethylated DNA\(^5\). Alternatively, the hypermethylated DNA can be analyzed separately following restriction enzyme digest of the hypomethylated DNA\(^5\). If, additionally, there are polymorphic differences in the fetal alleles, disturbance from a 1:1 ratio can be used to identify trisomy. This has been demonstrated for detection of Edwards syndrome but the approach has not been tested in clinical trials\(^5\). Differentially methylated regions may be relatively common\(^5\) so the method may also be applied to detect other aneuploidies.

It is also possible to use immunoprecipitation with antibodies specific to 5-methylcytidine to enrich for the methylated DNA. For example, the identification of a set of specific DNA sequences on chromosome 21 that are hypermethylated in placental cells allows the selective enrichment of fetal cfDNA from maternal plasma\(^5\). Following quantitative real-time PCR, there may be measurable differences in the amount of chromosome 21-derived DNA when fetal trisomy 21 is present, compared with in euploid fetuses. One proof-of-principle study has demonstrated the efficacy of such an approach to NIPT\(^5\). A follow-up study using a modified set of differentially methylated chromosome 21 DNA sequences also showed encouraging results\(^5\). This approach is currently the subject of further clinical trials. If an approach such as this can be fully developed, it could be substantially cheaper than sequencing-based methods.

Digital PCR

Another way in which the costs of sequencing can be avoided is through the use of digital PCR. This technology is based on the dilution of test materials such that individual DNA fragments of interest (e.g. chromosome 21) can be amplified separately and counted\(^5\). Theoretical estimates of the number of amplification events have been computed\(^5\) and this approach could become particularly attractive if reliable fetal cfDNA enrichment methods were developed.

RNA-based testing

Identification of genes that are expressed by trophoblasts but not maternal cells should result in the presence of the corresponding cfRNA species in maternal plasma, free of contaminating maternal RNA with a similar sequence. If the cfRNA also carries polymorphisms that distinguish the fetal alleles, the presence of aneuploidy could be deduced from departures from a normal 1:1 ratio in the relative amount of the inherited alleles\(^6\). This approach to NIPT showed some success in the initial proof-of-principle studies, but unpublished clinical trial data were disappointing. Nevertheless, with the discovery of additional RNAs that can be analyzed, this approach may yet play an important role in NIPT\(^6\).

NIPT IN CLINICAL TRIALS: NON-MOSAIC DOWN, EDWARDS AND PATAU SYNDROMES

Proof-of-principle studies demonstrated the feasibility of NIPT using small series of maternal plasma samples from aneuploid and euploid pregnancies\(^6\). However, generally, the samples were not tested ‘blind’ to the outcome of pregnancy, ascertainment criteria were not fully described and some samples were obtained after an invasive diagnostic procedure which could have led to increased transfer of fetal DNA into the maternal circulation. There have now also been several large clinical trials of MPS which have overcome these limitations and potential biases\(^6\). These have established NIPT as a potentially highly effective screening test but not as a test that could replace current invasive prenatal diagnosis. We therefore prefer the term NIPT or cfDNA screening rather than non-invasive prenatal diagnosis, which is misleading.

High-risk studies

There have been seven cfDNA studies involving shotgun or targeted sequencing in women who had invasive prenatal diagnosis because of high risk for: Down syndrome\(^6,65,67\), Down syndrome or another aneuploidy\(^6,68,69\) or any disorder\(^70,71\). In one study, a screen-positive screening test was the only indication for high risk\(^6\), whilst in the four other studies in which the reason for invasive testing was high risk\(^6\), in the four other studies in which the reason for invasive testing was high risk for Down syndrome or another aneuploidy, the indications were mixed. The reason for invasive testing was a screen-positive screening test in 77%\(^6,65\), 43%\(^6\), 30%\(^6\) and 22%\(^6\) of cases; advanced maternal age in 68%\(^6,66\), 37% (only indication in these cases)\(^6\) and 33%\(^6\) of cases; ultrasound in 13%\(^6,66\), 11%\(^6\) and 7%\(^6\) of cases; family history in 5%\(^6,66\), 3%\(^6\) and 3%\(^6\) of cases; and multiple reasons in 22%\(^6\) and 9%\(^6\) of cases. Five studies included results on both Down and Edwards syndromes\(^6\) and three also included Patau syndrome\(^6,65,67\). The study design details for these trials on high-risk women are summarized in Table 1. One study designated as ‘unclassified’ results with z-scores of 2.5–4.0 for autosomal chromosomes and excluded them from the DR and FPR calculations\(^6\). Doing so is not valid\(^6\), particularly when no protocol was suggested for the unclassified group, and in this Review we have reclassified them as negative. However, the unclassified group had a five-fold increased likelihood of aneuploidy (3.5% (5/144) of trisomies unclassified compared with 0.7% (9/1358) of controls) and it could be argued that they should in fact
be reclassified as positive, so those rates are also given in this Review.

Table 2 summarizes the Down and Edwards syndromes results for these seven cfDNA studies carried out in high-risk pregnancies. In total these studies compared 594 samples from pregnancies with Down syndrome with 5745 that did not, and 193 samples from pregnancies with Edwards syndrome with 5459 that did not, although in both cases some had other aneuploidies. The results from the various studies, notwithstanding methodological differences, are consistent with each other and their combined data represent the best estimates of DR and FPR. For Down syndrome, the overall DR of 99.3% has a 95% CI of 98.2–99.8% and FPR of 0.16% (95% CI, 0.08–0.31%). Even with the most extreme CI values this performance exceeds, by far, anything achievable by current Down syndrome screening protocols. However, this performance falls short of that for current diagnostic tests. Furthermore, reclassifying as positive the unclassified results in the study of Bianchi et al. would have increased the overall DR slightly to 99.5%, but almost doubled the FPR to 0.26%. Hence, we can conclude that based on present evidence cfDNA testing is not a diagnostic test of Down syndrome but rather is a very good secondary screening test. A woman with a positive cfDNA test will be at high risk of Down syndrome but depending on her pretest risk it may not be extremely high. This can be illustrated from the combined data in Table 3, by estimating a positive likelihood ratio (LR) using the DR divided by the FPR, i.e. 634. For example, a woman with a pre-test risk of 1 in 270 following a combined test would have a post-test odds of 634:269 or a risk of 2 in 3. This is not sufficiently high as to warrant termination of pregnancy without a confirmatory invasive diagnostic test. Similarly, the negative LR, estimated from the false-negative rate divided by the true negative rate, is 1/148. A woman with a pre-NIPT risk of 1 in 10 would have a 1 in 1333 risk after a negative test, which might be insufficienely reassuring for some women. These LRs are illustrative and cannot be used to counsel individual women because some of the studies had already incorporated prior risk based on maternal age into their algorithms. However, it is reasonable to conclude that using NIPT following a screen-positive test for any of the established screening protocols followed by confirmatory invasive diagnostic testing if the NIPT result is positive will probably hardly affect the overall screening DR but will reduce the FPR about 300-fold.

For Edwards syndrome, the overall DR is 97.4% (95% CI, 93.7–99.0%) and the FPR 0.15% (95% CI, 0.07–0.31%), with positive LR of 665 and negative

Table 2 Down syndrome and Edwards syndrome results in seven cell-free DNA studies carried out in high-risk pregnancies

<table>
<thead>
<tr>
<th>Trial</th>
<th>Design</th>
<th>Average GA (weeks)</th>
<th>Method</th>
<th>Algorithm</th>
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<td>z-score &amp; L-score</td>
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<td>10</td>
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*For autosomal chromosomes. †Includes maternal and gestational age. ‡Results with scores 2.5–4.0 were ‘unclassified’. §Positive if z < 2.5 but L > 1. GA, gestational age at testing.

Table 1 Eleven cell-free DNA studies of shotgun or targeted sequencing: methodological differences

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<td>Dan et al.</td>
<td>Prospective</td>
<td>20</td>
<td>Shotgun</td>
<td>z-score &amp; L-score</td>
<td>2.5 or 1.0§</td>
</tr>
<tr>
<td>Gil et al.</td>
<td>Prospective</td>
<td>10</td>
<td>Targeted</td>
<td>Risk†</td>
<td>1%</td>
</tr>
</tbody>
</table>

*2-plex and 8-plex data were reported but only the more favorable 2-plex results are included here. DR, detection rate; FPR, false-positive rate.
LR 1/38. Including the unclassified results in the study of Bianchi et al. as positive would have increased the DR to 98.4% and the FPR to 0.20%. Since a cfDNA test is unlikely to be aimed at detecting Edwards syndrome alone, these false-positives should be regarded as additional to those generated by cfDNA testing for Down syndrome. There are no data with which to assess whether there is any tendency for those testing falsely positive for one type of aneuploidy to be falsely positive for another. Assuming these are uncorrelated, the FPR for both aneuploidies together is 0.30%, or 0.46% if those unclassified by Bianchi et al. are included.

Combining the results from the three studies which included Patau syndrome gives a DR of 30/38 (79.9%); 95% CI, 65.9–91.9%) and FPR of 17/4112 (0.41%; 95% CI, 0.22–0.61%), with a positive LR of 191 and a negative LR of 1/4.7. Including the unclassified results as positive, the DR increased to 80.0% with no increase in the FPR. On the basis of these results, an MPS test for all three trisomies would have a FPR of 0.7–0.9%, depending on the inclusion of the unclassified results.

Initial prospective clinical experience has been reported for a commercial laboratory, Verinata Inc, offering tests to high-risk women. While follow-up information was incomplete, among 5974 samples there were false positives for Down, Edwards and Patau syndromes and false negatives for Down and Edwards syndromes, with rates compatible with those seen in the trial data.

### Non-high risk

There have been four NIPT studies involving shotgun or targeted sequencing in which the subjects were not specifically selected because they had invasive prenatal diagnosis. Their methods are summarized in Table 1.

Lau et al. reported prospective cfDNA screening results of 567 patients, 49% of whom were tested at 12–13 weeks’ gestation. These women comprised 179 (32%) who were screen-positive by conventional testing, 194 (34%) who were screen-negative or awaiting results and 194 (34%) who were unscreened. There was insufficient follow-up to report DRs reliably, but eight cases of Down syndrome and one of Edwards syndrome were detected and there were no false positives. Seven of the detected cases were among the screen-positive women (1 in 25) and two, both Down syndrome, were among the remainder (1 in 194).

Nicolaides et al. carried out a retrospective study using stored plasma samples from women who had a combined test at 11–13 weeks’ gestation. After excluding non-viable pregnancies, those terminated for reasons other than Down or Edwards syndromes and cases lost to follow-up, 1949 were successfully tested for cfDNA, including eight with Down syndrome and two with Edwards syndrome. All aneuploidies were detected and, while there were no false-positive Down syndrome cases among 1939 tested, there were two (0.05%) false-positive Edwards syndrome cases among 1937 tested. Invasive prenatal diagnosis had been carried out in 4.3% (86/2049) of those selected, including all of the cases with aneuploidy.

A large prospective study of cfDNA screening was carried out in China. A total of 11105 pregnancies were tested successfully, including 4522 (41%) that were screen-positive following conventional screening and 2770 (25%) with risk factors such as advanced maternal age, ultrasound abnormalities and family history. All of the known Down syndrome (n = 140) and Edwards syndrome (n = 42) cases were detected by the cfDNA test. However, approximately one third of the 10915 with negative test results were lost to follow-up; 2818 (26%) had invasive testing, a further 70 (< 1%) were terminated, and follow-up was available for 4524 (41%). The protocol included an insurance scheme whereby the family would be compensated in the event of unpredicted
delivery of either a Down or an Edwards syndrome baby. This is likely to be a considerable incentive to report false-negative results so the lack of follow-up may not be a major problem. There were two proven false-positive cfDNA results, one each for Down and Edwards syndromes. However, a further eight positive cases did not have a karyotype following fetal loss or termination and therefore the possibility of additional false positives cannot be excluded.

Gil et al. carried out a prospective study of cfDNA screening at 10 weeks' gestation in 997 women with median age 37 years; combined tests were also carried out. A total of 15 cases of aneuploidy were detected by cfDNA and confirmed by invasive testing (10 cases of Down syndrome, four of Edwards syndrome and one of Patau syndrome) and there was one false positive (for trisomy 18). One case was positive for trisomy 21 but miscarried before invasive confirmation and no false negatives had emerged at the time of writing.

These studies provide some, albeit limited, evidence to suggest that cfDNA screening may be as effective in the general population as it is in those already scheduled for invasive testing: the FPR is not dissimilar to that in the high-risk studies; all aneuploidies appear to have been detected, although most of them were in pregnancies which were already candidates for invasive testing. Moreover, among high-risk women, performance does not appear to be related to the indication for invasive testing. Palomaki et al. found that the DRs and FPRs were very similar according to indication: screen-positive 99% (96/97) and 0.2% (1/637), respectively; advanced maternal age or family history 100% (24/24) and 0.3% (2/587); ultrasound 100% (51/51) and 0% (0/130); and multiple reasons 95% (37/39) and 0% (0/112). Nevertheless, until much larger general population studies are published, the limited direct evidence of efficacy needs to be supplemented by indirect evidence. The separation in the distributions of z-scores or risks between aneuploid and euploid pregnancies is necessarily dependent on the fetal fraction and it is therefore important to consider whether this might be lower in low-risk women.

Several covariables for fetal fraction have been investigated, including indication for invasive prenatal diagnosis, maternal age, screening marker level and estimated aneuploidy risk. In one published series of pregnancies at high risk of Down syndrome or other aneuploidy, there was no significant difference in mean z-score, in either Down syndrome or euploid pregnancies, according to indication or correlated with maternal age, but there was a significant negative correlation with maternal weight. The latter effect can be explained by there being a fixed mass of fetal DNA, from the placenta, mixing with an increasing mass of maternal DNA; such an effect is also seen in conventional maternal serum markers. In another published series of pregnancies at high risk of Down syndrome, a multivariate regression analysis was carried out which confirmed that maternal weight was a covariable but maternal age was not. In addition it showed that there was a significant positive correlation between fetal fraction and both PAPP-A and free β-hCG, which might be related to placental volume, although there was no association with gestational age in either study. Since in Down syndrome pregnancies PAPP-A is reduced on average and free β-hCG is increased, the correlations with fetal fraction may cancel each other out. In Edwards syndrome pregnancies, both serum analyte markers are reduced, consistent with reduced placental volume. There could therefore be a somewhat increased chance for cfDNA test failure in affected pregnancies. On the other hand, those cases with higher PAPP-A and β-hCG which might not be detected by the combined test may show higher fetal fractions and these may therefore be more amenable to detection by a cfDNA-based NIPT. In a further published series of those having invasive prenatal diagnosis due to high risk generally, the mean fetal fraction was computed for deciles of maternal age, NT and aneuploidy risk. There was no significant difference in means between the highest and lowest deciles. The highest decile for risk comprised 100 women with risk greater than 1 in 10 and a mean fetal fraction of 11.4% compared with 135 women in the lowest decile with risk less than 1 in 6500 and a mean fetal fraction of 10.8%.

Failed results

Several of the MPS studies discussed above specified the number of pregnancies not considered for cfDNA testing because of sample quality. Overall, among 13 260 eligible pregnancies there were 814 (6.1%) untested. This rate ranged from 0.8% to 9.9% between the studies and may reflect the stringent standards required when carrying out a research project. Of more concern is the 2.0% of pregnancies (436/22 222) in which a cfDNA test was performed but failed because of insufficient fetal DNA or other technical reasons (Table 3). The failure rate may be even higher in those resampled after an initial failure: it was 32% (13/40) in one study, and 32% (13/40) in one study, a result was not possible in 2.4% of cases. This is relevant to comparisons with conventional screening, which rarely reports failure to obtain a result.

Three prospective studies of women not specifically selected because they had invasive prenatal diagnosis provide information on the extent to which a successful test result was achieved following an initial failure. In one study, among 567 successful cfDNA tests, four (0.7%) had required a repeat blood sample and no other tests had failed during that period (T.K. Lau, pers. comm.). The time from the initial blood draw to the cfDNA report was greater than 14 days for 16 (2.8%) tests, including those requiring a repeat sample. In another study, among 11 015 tests, 97 (0.9%) needed resampling. In this study as a whole, the turnaround time was within 10 working days for 95% of pregnancies and within 15 for 99%; those requiring resampling took a further 10–15 days to
produce a report. In the third study, a request for a patient redraw was made in 1.9% of referrals and among those who did undergo a redraw there was sufficient fetal cfDNA in the second sample in 56% of cases. The success rate was strongly dependent on maternal weight.

SNP studies

Following the initial proof-of-concept study using polymorphisms and maximum LRs for test interpretation, the results of one small clinical trial have been published and additional information has been presented at scientific meetings in the form of an abstract and a poster. The study of Nicolaides et al. involved 242 singleton pregnancies with results available for 229 (95%). All 32 aneuploid and 210 euploid cases were identified correctly. The abstract provided information on 407 pregnancies including 44 with aneuploidies and the results were 100% correct among samples that passed quality control. In addition to the maternal plasma, paternal blood or buccal mucosa samples were genotyped. Whilst this was not an absolute requirement (paternally inherited alleles in the fetus can be deduced), the failure rate was lower when a paternal sample was available. In the poster, there were 763 samples, of which 45 (5.9%) failed to pass quality control, and among the remaining 717 (including 47 Down, 15 Edwards, 7 Patau and 12 Turner syndromes) samples all except one result, for Turner syndrome, was correct.

Multiple pregnancies

There is a potential problem with using cfDNA to screen for aneuploidy in multiple pregnancies. As with biochemical screening, in twins discordant for trisomy the excess in fragments from a specific chromosome in the affected twin may be masked by the normal proportion of those fragments in the euploid cotwin. This would also be the case for higher order multiple pregnancies. This problem might be overcome by using SNPs to measure the variations in the fetal fraction between genomic regions. The method allows determination of zygosity and, in dizygous twins, examination of the distribution of fetal fragments for each fetus.

At present, only two small multiple-pregnancy cfDNA series have been published, using the same method as for singletons. The first series was derived from one of the high-risk studies and included three discordant twins (two with Down and one Patau syndrome), five discordant Down syndrome twins, 17 euploid twins and two euploid triplets; there were no false-positive or false-negative cfDNA test results. The second series was from one of the studies of women not selected because they were at high risk and included only one discordant twin with Down syndrome and 11 euploid twins; all pregnancies were correctly classified by the cfDNA test.

In a singleton pregnancy that had had a vanishing twin, it is theoretically possible that apoptosis of cells from the fetoplacental remains of the non-viable fetus could interfere with the cfDNA result; there is a relatively high chance that the fetal loss would have been associated with aneuploidy and this could lead to a false-positive result.

COST OF NIPT

Currently, the cost of a cfDNA test is high, ranging from about $800 to $2000 in the USA, and from $500 to $1500 elsewhere. Whilst this may not necessarily deter individuals from being tested, it is likely to determine whether public health planners provide the testing and will be the basis for whether health insurance schemes reimburse those tested. We have published a sensitivity analysis in which the factors contributing to these overall costs are elucidated for four cfDNA screening policies: (1) cfDNA screening for those screen-positive on combined test; (2) universal cfDNA screening, replacing all current screening modalities; (3) contingent cfDNA screening, for the 10–20% of women with the highest risk based on a combined test; (4) cfDNA screening for women of advanced maternal age and younger women with a positive combined test.

Restricting cfDNA testing to screen-positive cases leads to a small reduction in detection, a massive reduction in false positives and is the least expensive way in which to utilize cfDNA testing. With this policy the average cost per Down syndrome birth avoided is dependent only on the difference in unit cost of a cfDNA test and of invasive prenatal diagnosis. For a combined test with FPR of 3%, the average cost would be reduced by using secondary cfDNA testing, if the unit cost was less than or equal to three-quarters that of invasive testing. Even at a higher unit cost of cfDNA, the average cost per fetal loss prevented would be relatively low.

For other policies in which the aim of cfDNA testing is to increase detection, the most important public health consideration will be not the total or average cost but the ‘marginal’ cost compared with the existing screening provision. The marginal cost is the average cost per Down syndrome birth avoided or loss prevented over and beyond that which would have been avoided by the existing service. This is shown in Table 4 for fixed unit costs for the combined test and invasive testing and with variable unit costs of cfDNA. The table also shows screening performance.

Universal cfDNA screening yields both a very high DR and a small FPR but the marginal cost is very high, exceeding the lifetime cost to the system of a Down syndrome birth. With universal screening the cost per fetal loss prevented when replacing conventional screening is also very high. Contingent protocols, in which all women receive the combined test and the 10–20% with the highest risk receive cfDNA testing, are efficient and can reduce massively the marginal cost compared with universal screening. The average cost of preventing a fetal loss is also several times lower than that for universal cfDNA testing. A policy of cfDNA testing only for older...
women was not substantially more effective than was the combined test and is more costly than contingent screening. A hybrid policy of offering cfDNA testing to all those of advanced maternal age plus all combined test screen-positive women was better as it reduced the FPR massively but it was less effective and more expensive than was a contingent testing policy.

The detection rate for a contingent policy can be increased by including additional serum and ultrasound markers. Nicolaides et al. suggested markers which are also of value in screening for pre-eclampsia and fetal cardiac abnormalities. Cost-benefit analyses for such variants have not yet been carried out.

Two other published studies have estimated costs when cfDNA is applied to women with positive conventional screening results, using the estimated NIPT DR and FPR from their own single study. Palomaki et al. considered total costs following screening in 100 000 screen-positive women, with an average risk of 1 in 33 for a Down syndrome fetus. They concluded that if the unit cost of cfDNA was less than 96% of the unit cost of invasive testing, there could be a net saving (4% of costs need to be assigned to test failures and confirmatory invasive testing following cfDNA positive results). The second study also considered 100 000 screen-positive women, modeled as a mixture of combined test or second-trimester serum screening. They estimated total costs including both the conventional screen and subsequent testing and concluded that with a unit cost of cfDNA $1200, CVS $1700 and amniocenteses $1400, there would be a 1% cost reduction. One other study has estimated costs for cfDNA screening in older women and in younger women with screen-positive conventional screening tests, using DR and FPR estimates from the literature. The authors claimed that this policy, in the USA, would have lower total cost compared with conventional screening. However, this conclusion appears to have been reached following incorrect assumptions about the DR and FPR in younger women and a very low uptake of invasive testing for women screen-positive by conventional screening compared with cfDNA screening.

**FUTURE PROSPECTS: NIPT FOR ADDITIONAL CYTOGENETIC ABNORMALITIES**

**Other autosomal abnormalities**

With the widely anticipated rapid improvements in sequencing, it may soon be possible to screen for other autosomal trisomies in situations in which the fetal fraction is very low, thereby allowing detection of aneuploidy even earlier in pregnancy. Early pregnancy is associated with very high levels of chromosomal abnormality and although nearly all abnormal cases will spontaneously abort, early identification could be beneficial to women. First-trimester autosomal trisomy can involve any chromosome, but some occur much more frequently than others. Detection of these through NIPT will be facilitated by the larger size of the additional chromosome in the non-viable trisomies. Early studies suggested that there was greater variability in the fragment counts for some of the larger chromosomes, but this could be overcome by GC correction and selective use of the most consistent regions of chromosomes.

Screening for a large number of potential abnormalities together does require very high specificity for each component so that the aggregate FPR for all chromosomes combined is low.

**Sex-chromosome abnormality**

Establishing NIPT for sex-chromosome imbalances is desirable because some specific sex-chromosomal abnormalities are associated with a sufficiently severe phenotype as to fully justify prenatal diagnosis: for example, many cases of Turner syndrome, most of which will not survive to full term, gain of multiple X chromosomes and some unbalanced structural rearrangements of the X-chromosome. Collectively, unbalanced sex-chromosomal abnormalities constitute over a third of the unbalanced karyotypes seen in amniocenteses performed in women under 35 years of age and nearly a quarter of those in older women. There is mosaicism in approximately one

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Table 4 Four cell-free (cf)DNA policies† performance, marginal cost per Down syndrome (DS) birth avoided and average cost per fetal loss prevented, according to unit cost of cfDNA testing†

<table>
<thead>
<tr>
<th>Policy</th>
<th>Performance</th>
<th>Marginal cost per DS birth avoided ($ million) for unit cost cfDNA of:</th>
<th>Average cost per fetal loss prevented ($ million) for unit cost cfDNA of:</th>
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<tr>
<td>Combined cfDNA</td>
<td>DR (%)</td>
<td>FPR (%)</td>
<td>OAPR</td>
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<tr>
<td>None</td>
<td>99.3</td>
<td>0.16</td>
<td>1:1</td>
</tr>
<tr>
<td>All Contingent:</td>
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<td></td>
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<tr>
<td>10%</td>
<td>90.4</td>
<td>0.016</td>
<td>7:1</td>
</tr>
<tr>
<td>15%</td>
<td>92.8</td>
<td>0.024</td>
<td>5:1</td>
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<tr>
<td>20%</td>
<td>94.4</td>
<td>0.032</td>
<td>4:1</td>
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<tr>
<td>&lt; 35 years ≥ 35 years</td>
<td>85.0</td>
<td>2.1</td>
<td>1:19</td>
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<tr>
<td>&lt; 35 years ≥ 35 years or positive on combined test</td>
<td>84.7</td>
<td>0.017</td>
<td>6:1</td>
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</table>

*Three reported in a modeling analysis and the fourth, cfDNA restricted to older women, using the same model. †Combined test, false-positive rate (FPR) = 3%, unit cost = $150; invasive prenatal diagnosis, unit cost = $1000, iatrogenic fetal loss rate = 0.5%. DR, detection rate; OAPR, odds of being affected given a positive result.

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third of sex chromosomal aneuploidies detected through amniocentesis.

One commercial laboratory that provides NIPT, Sequenom Inc, was routinely providing information on the presence or absence of Y-chromosome DNA in their reporting. Based on a series of 1639 samples, the DR for a Y-chromosome was 828/833 (99.4%), and the accuracy for the prediction of a female fetus was 803/806 (99.6%)\(^9\). More recently, they have been providing NIPT for Turner syndrome, Klinefelter syndrome, 47,XXX and 47,XXY based on a study by Mazloom et al.\(^ {100} \), with DRs of 83% (25/30), 85% (11/13), 83% (5/6) and 75% (3/4), respectively, for each of the these abnormalities. The FPR for Turner syndrome was 0.2% (4/1945) and there was one other false positive (47,XXX) in the series.\(^ {100} \) Of the normal cases in which gender was interpreted, it was inconsistent with the karyotype result in 0.7% (13/1814). Bianchi et al.\(^ {88} \) developed a complex algorithm for evaluating sex-chromosome-derived cfDNA in plasma. Their study derived z-scores for the X-chromosome (zx) and Y-chromosome (zy). A result was classified as positive for Turner syndrome if zx < −4.0 and zy < −2.5; it was classified as 46,XX or 46,XY or 47,XXX or 47,XXY or 47,XYY using different cut-offs, and all others were unclassified. Among the non-mosaic Turner syndrome cases the DR was 75% (15/20) and the FPR was 0.2% (1/462), with 49 remaining unclassified (including four affected and 45 controls). Among the mosaic cases with a 45,X cell line, the DR for the monosomy was 29% (2/7). For the other non-mosaic sex chromosomal abnormalities the DRs were: XXX, 75% (3/4); XYY, 100% (1/1); and Klinefelter syndrome, 67% (2/3). Robust estimates for the efficacy of sex chromosomal aneuploidy detection are therefore not provided, although Verinata Inc. does offer testing on the basis of this study.

There are several factors that could confound the development of highly effective NIPT for X- and Y-chromosomal imbalances. First, many fetal sex chromosomal aneuploidies are mosaic, with either a normal cell line or an abnormal normal cell line. This includes situations in which one abnormal cell line may mask another when cfDNA is analyzed. For example, fetal 45,X/46,XXX mosaicism may show approximately normal proportions of X-chromosome cfDNA if the proportions of the two cell types are similar. Second, although the detection of Y-chromosome-specific DNA is relatively simple and reliable in establishing gender,\(^ {101} \) finding sufficient Y-chromosome loci that are informative for copy number quantification may be difficult. The potentially informative euchromatic part of the Y-chromosome is relatively small, it contains many sequences that show polymorphic CNVs, and some segments show partial homology with X-chromosomal sequences.\(^ {102} \) A third difficulty is that normal adult females show an age-related loss of X-chromosomes.\(^ {103} \) In women at normal reproductive age, the proportion of maternal cells with a missing X-chromosome can be comparable to the proportion of cfDNA in a maternal plasma specimen and there may be some women with unusually high levels of 45,X cells.\(^ {104} \)

The age-related loss of the X-chromosome is thought to involve preferentially the inactive X-chromosome.\(^ {105} \) Some women may also show cells that have gained an X-chromosome.\(^ {106} \) Clonal populations of maternal cells with X-chromosome gain or loss in hematological cells (which are believed to be the source maternal cfDNA in plasma), maternal constitutional mosaicism or even a non-mosaic X-chromosomal abnormality such as 47,XXX are also possible.\(^ {107} \) The fetal X-chromosomal dosage therefore has to be identified against a background of maternal X-chromosomal DNA that is likely to be quantitatively more variable than is seen for the autosomes. These considerations suggest that NIPT based on simply counting the total X- and Y-chromosomal sequences in maternal plasma and comparing them with the expected normal karyotype values may be less effective than that which is achievable for autosomal gain or loss. Furthermore, it may be misleading to quote a DR that is based on all sex chromosomal abnormalities combined for genetic counseling.

Approaches based on quantification of SNPs present in the plasma DNA may be informative for at least some fetal sex chromosomal aneuploidies. Y-chromosome gain, most cases of Turner syndrome,\(^ {108} \) approximately half of cases with Klinefelter syndrome\(^ {109} \) and some other imbalances are attributable to erroneous segregation of a paternally derived sex chromosome. These cases could therefore be identified in a maternal plasma specimen through the over- or under-representation of paternal X- and Y-chromosomal SNPs that differ from the mother’s SNPs. On the other hand, most cases of 47,XXX\(^ {110} \) and the remaining cases of Turner syndrome, Klinefelter syndrome and others are attributed to erroneous segregation of a maternally derived chromosome. The reliable quantification of cfDNA with these fetal X-chromosomal SNPs, against a background of the identical maternal SNPs, is more difficult.

**Mosaicism**

Two of the NIPT clinical trials have specifically addressed the ability to detect mosaic cytogenetic abnormalities in the fetus.\(^ {68,111} \) In one study\(^ {68} \), of 11 cases in which a non-complex mosaicism was known to be present based on karyotyping, five were called affected and six unaffected. In additional studies involving deeper sequencing, four mosaic abnormalities recognized by conventional chromosomal analysis were not detected by NIPT.\(^ {112} \) In the second study involving five cases,\(^ {111} \) the abnormal line was detected in one case and not detected in four cases.

Approximately 14% of all cytogenetic abnormalities identified through karyotyping of amniotic fluid cells are mosaic.\(^ {28} \) For CVS, the proportion showing mosaicism is much higher; approximately 45%\(^ {23} \). This excludes cases in which there is pseudomosaicism (possible cell culture artifact) and includes cases in which there is a discrepancy between CVS direct or short-term culture preparation results (cytotrophoblasts) and longer term cell culture results (mesenchymal tissue). Discrepancy usually involves abnormal cells in cytotrophoblasts with normal
mesenchyme, but the reverse situation can also be present. An abnormal cell line can also be present in both direct and long-term cultures but be undetectable in the fetus. Longer term cell culture is usually considered to be a more reliable indicator of the true karyotype of the fetus compared with direct preparations. Overall, approximately 1% of all CVS specimens will show a second cell line that is not detectable in the fetus, a situation termed ‘confined placental mosaicism’ (CPM). This may sometimes result in spontaneous loss, low birth weight or preterm delivery, particularly when it involves both trophoblastic and mesenchymal cell lineages and/or when it involves specific karyotype abnormalities such as trisomy 16. However, in most pregnancies in which CPM is detected the pregnancy outcome is normal.

The frequency and significance of CPM is relevant to NIPT because cfDNA is believed to originate primarily from non-viable trophoblasts. CPM could result in either false-positive or false-negative NIPT results. It has been suggested that viable pregnancies with Edwards or Patau syndrome may have a substantial euploid cell line in the trophoblasts and therefore NIPT could fail to detect some viable trisomic cases in which there is a substantial population of abnormal cells in the fetus but not in the placenta. This would lead to an apparent false-negative result by NIPT. Conversely, the trophoblasts may be mostly abnormal and the fetal cells normal, leading to an apparent false-positive result. Moreover, if chromosomally abnormal trophoblasts were more likely to undergo apoptosis, NIPT could preferentially identify cases in which the proportion of abnormal cells in the placenta is low.

A number of cases have already been described which illustrate how differences in the distribution of normal and abnormal cells may lead to test result discrepancies between NIPT and karyotyping. Faas et al. described the identification of Turner syndrome based on NIPT when the abnormal cell line was confirmed by cytogenetic analysis of short-term CVS cultures but the abnormality was not seen in the long-term CVS cultures or in the newborn blood. Choi et al. described a case involving possible trisomy 22 detected by cfDNA testing. The trisomy 22 cell line was confirmed by analyzing the term placenta but the abnormality was not detected in a blood specimen from the dysmorphic baby. Hall et al. describe a case with a positive Patau syndrome cfDNA test, mosaic 47,XY,+13/46,XY result for CVS, normal result for amniotic fluid cells, normal result for cord blood, but trisomy 13 mosaicism confirmed in the placenta. These examples can each be interpreted as CPM although the presence of low-level true fetal mosaicism cannot be excluded.

Early trials on chromosomal analysis following CVS provide an indication as to how often CPM might lead to an apparent false-positive or false-negative result. Because milder abnormalities such as sex chromosomal aneuploidy or mosaicism involving abnormalities not typically seen in livebirths may not have been fully pursued in follow-up studies, this analysis needs to be confined to Down, Edwards and Patau syndrome. Ledbetter et al. noted six cases (four involving chromosome 13, one chromosome 18 and one chromosome 21) interpreted as a false-positive CPM result and one false-negative result (chromosome 18) among 6560 cases that had analysis of a direct CVS preparation. Similarly, Smith et al. reported nine cases (two involving chromosome 13, four chromosome 18 and three chromosome 21) interpreted as a false-positive CPM result and five false-negative results (two chromosome 18, three chromosome 21) in approximately 18 195 cases that had direct preparation analysis. Combining these data, for chromosome 21, 18 or 13, the incidence of false positives was 1 in 1650 and the incidence of false negatives was 1 in 4126. These estimates are crude because the number of cells analyzed on direct CVS preparation is often small, some women receiving an abnormal result terminated their pregnancy without confirmation, and low-level true fetal mosaicism may have gone undetected. It should be noted that most cases of CPM do not involve chromosomes 21, 18 and 13. Therefore, as NIPT tests are expanded to include additional chromosomes or chromosomal regions, the issue of erroneous results due to CPM is likely to become increasingly important.

For those cases that do show true fetal mosaicism, the phenotype can be highly variable but will generally be less severe than that present with the non-mosaic abnormal karyotype. Although the proportion of abnormal cells in amniotic fluid or chorionic villi can be a poor guide to phenotype in individual cases, in general, a high proportion of abnormal cells is associated with adverse pregnancy outcome. NIPT tests that only present a result categorically as either ‘positive’ or ‘negative’ obviously cannot provide any information about the proportion of abnormal cells or clinical severity. Furthermore, since some of the clinical trials have specifically excluded mosaic cases and optimized their z-score cut-off on the basis of non-mosaic cases, testing is presumably biased towards not detecting mosaic cases. It is not yet clear whether patients with z-scores close to the cut-off should be offered invasive testing to help rule out mosaic trisomy. To present this problem appropriately to clinicians, and also to allow better integration of the results with other screening and diagnostic techniques, we recommend that NIPT reports should be formatted so that they provide patient-specific risks for each non-mosaic trisomy, the z-score and the fetal fraction, rather than just a categorical positive or negative finding.

The possibility of maternal mosaicism also needs to be considered. This includes the rare situation in which the abnormal maternal cfDNA is derived from a malignant cell population. Maternal mosaicism could potentially be recognized using SNP-based NIPT methodology.

Translocations

Unbalanced translocations, in theory, should be identifiable in NIPT as a result of the partial trisomy and monosomy that is present. In a retrospective analysis of six plasma specimens from pregnant women with known fetal imbalances, Srinivasan et al. were able to identify
the imbalances in maternal plasma in all six cases. In two of their cases the karyotype showed additional material of unknown origin and the NIPT analysis was able to provide a more precise characterization of the extra chromosomal material. The ability to detect routinely smaller imbalances would require deeper sequencing and improvements in bioinformatic analysis of sequencing data. For SNP-based approaches to NIPT, detection of small imbalances requires identification of sufficient informative polymorphisms in the region of interest. Conventional chromosomal analysis does allow detection of most apparently balanced translocations and large inversions. Prenatally identified de-novo rearrangements are associated with an increased risk for abnormality. It would therefore be desirable to detect these non-invasively. Paired-end sequencing of DNA fragments that span a chromosomal breakpoint can identify at least some translocations through the analysis of cellular DNA. For example, Schluth-Bolard et al. used a whole-genome paired-end protocol to identify the specific sequence disruptions in four cases with apparently balanced translocations and abnormal phenotypes. Breakpoints were mostly in repeat sequences and most breakpoints were associated with gain or loss of some basepairs. Talkowski et al. used sequencing to refine breakpoints and establish a diagnosis of CHARGE syndrome based on disruption of the CHD7 gene by analyzing amniotic fluid cells. These studies involved the sequencing of larger pieces of DNA and were carried out in cases in which the karyotype information was used to help identify candidate DNA fragment sequences that spanned the breakpoints.

Although the routine detection of de-novo rearrangements using cfDNA, without knowledge of where to look, would not seem to be technically achievable at this time, it is potentially possible. The challenge is similar to the detection of other mutations, but is made simpler with the presence of two related reciprocal events. By sequencing cfDNA it should be possible to construct the haplotype sequence in which one end matches perfectly to a chromosome, the other end matches perfectly to a second chromosomal location and there are similar hybrid sequences present that correspond to the reciprocal translocation product(s). Improved sequencing fidelity, deeper sequencing and the development of advanced bioinformatic algorithms that specifically look for the hybrid sequences would be needed.

Inherited rearrangements are generally considered benign, but their detection through karyotyping following CVS or amniocentesis is considered advantageous because it identifies a risk factor for future pregnancies. When a balanced translocation is already known to be segregating in a family and the phase of SNPs on the chromosomes in the region of interest can be established, the presence or absence of the breakpoint can be inferred from the SNPs in the cfDNA in a plasma specimen. In the case of a paternally inherited translocation, the presence of the translocation chromosomal SNPs and absence of the normal homolog SNPs identifies the translocation in the conceptus. In maternal inheritance, detection of the translocation relies on the identification of a relative excess of the translocation chromosomal SNPs and relative deficiency of the normal homolog SNPs in the plasma sample.

Triploidy

Triploid pregnancies can arise as a result of an additional set of maternal (digynic) or paternal (diandric) chromosomes. Most recognized cases show a 69,XXX or 69,XY karyotype; 69,XYY forms are thought to undergo early first-trimester miscarriage. Most cases of triploidy will be identified by ultrasound abnormality and characteristic first- or second trimester-maternal serum marker levels. It is exceptional for these pregnancies to survive into the third trimester.

Since all chromosomes are proportionately represented in a 69,XXX pregnancy, NIPT based on the counting of all chromosomal sequences would yield a normal result. Pregnancies with either a 69,XXX or a 69,XY karyotype might be identifiable through unexpected sex chromosomal sequence ratios. Digynic triploidy might be particularly difficult to detect by NIPT because the placenta is very small and the fetal fraction may therefore be reduced. Consistent with this, Bianchi et al. reported that of nine cases of fetal 69,XXX triploidy, there was insufficient fetal cfDNA for analysis in three (33%).

Using the approach that takes into consideration the SNPs present in the cfDNA, diandric triploidy could be identified through the presence of two different paternally inherited SNPs at some loci. Diganic cases with sufficient fetal cfDNA for analysis might be recognized through the quantitative excess of maternally inherited fetal DNA fragments relative to paternally inherited fragments.

CNVs

Small duplications and deletions have the potential to be detected through NIPT. Examples that have been detected include a 4-Mb deletion on chromosome 12, and two cases in which 3-Mb 22q11.2 deletions were present. In a series of 11 prenatal plasma specimens, Srinivasan et al. identified small gains or losses not reported in the clinical karyotypes in eight cases, including four with two such variations. However, it is not clear whether they were true or false positives.

A variety of strategies have been proposed to detect the small deletions, insertions, inversions, and duplications that are common in the human genome. Much of the analysis is contingent on the ability to identify departures in the location and orientation of sequenced DNA fragments from where they are expected to be found in reference mapped genomes. The ability to do this type of analysis in a non-invasive test is a long-term goal in molecular cytogenetics.

Overview of future testing

Currently, only methods based on MPS have been the subject of clinical trials and can be viewed as being
suitable for clinical use. An optimal NIPT for fetal aneuploidy will be accurate, simple, cheap, applicable early in pregnancy and fully compatible with existing prenatal screening methods so that risks developed using the different approaches can be combined. Many of the methods under consideration can be refined to allow for the detection of other chromosomal imbalances. MPS refinements that will aid this process include selection of optimal sequences for analysis, adjustments for base composition and the use of sequencing platforms that provide higher read accuracy.

Sequencing also provides opportunities to detect the presence or absence of single gene disorders. The methods can even be applied to the construction of an entire fetal genotype. On the other hand, methods that do not require sequence information from the parents or which do not generate unwanted additional genotypic status of the parents (for example resulting in information about paternity or other disease risks) should also be considered advantageous. The optimal technologies may ultimately be those which are most amenable to answering only those clinical questions that are being asked, rather than those which provide the most information.

COUNSELING

The internationally accepted approach to dealing with patients’ diverse ethical, religious and cultural values is to provide individual non-directive counseling, testing and other clinical information at each step in the screening and diagnosis process. This model, which maximizes patient autonomy in reproductive decision making, has been adopted by diverse populations throughout the world.

Counseling of women who are considering NIPT is challenging. The range of cytogenetic abnormalities currently detectable through cfDNA testing is smaller than that detectable by conventional karyotyping and substantially less than that achievable through microarray testing. In the absence of public health policies that define testing strategies, each high-risk woman who previously would have made a decision about amniocentesis or CVS and karyotyping, will now need to choose between no testing, NIPT, invasive testing with conventional cytogenetics or invasive testing with microarray testing. These women will need to be counseled carefully regarding the complex benefits, hazards and trade-offs associated with each option.

In part, these decisions will be determined by the indication for testing and perceived risk, but they will also be determined by the available alternative testing services. For example, in a situation in which routine invasive testing is based on RAT for a limited set of disorders, the choice of NIPT that detects the same range of aneuploidies might seem preferable. On the other hand, offering array technology may provide much more valuable information concerning fetal wellbeing than can current NIPT. This benefit has to be weighed against the risk of losing a normal pregnancy following an invasive procedure or the heightened anxiety caused by the presence of a CNV of unknown significance, poorly defined penetrance and/or variable expressivity. In addition, the testing could expose consanguinity or non-paternity. Given these realities, the challenge now facing those providing pretest counseling cannot be understated. The National Society of Genetic Counselors recognizes that due to limited resources, pretest counseling cannot always be provided by a genetic counselor and this information will therefore also need to be communicated by other qualified healthcare providers.

The availability of professional guidelines, such those from the American College of Obstetricians and Gynecologists, together with the Society for Maternal-Fetal Medicine, the Society of Obstetricians and Gynaecologists of Canada, the National Society of Genetic Counselors, the American College of Medical Genetics and Genomics and the International Society for Prenatal Diagnosis (ISPD), can greatly facilitate the process by clarifying which processes are scientifically valid and clinically acceptable standards of care.

ETHICAL ISSUES

The promise of NIPT is that it will reduce the number of women with an indication for invasive testing and only those women who are at very high risk for defined disorders such as Down syndrome will then need to deal with the ethical challenges associated with procedure-related loss and pregnancy termination. However, the ease of providing NIPT will potentially result in substantially more prenatal diagnoses and terminations of affected pregnancies. The issue is confounded by an inability to provide adequate counseling for all women who might be considering NIPT. There may also be a shift in patients’ and/or healthcare providers’ views on prenatal diagnosis, with a greater expectation for pregnancy termination as the usual response to an abnormal diagnosis. This has been referred to as ‘normalization’ of testing and termination. Potential consequences could include a sharply reduced incidence of Down syndrome neonates, greater emphasis on prevention and less on support and accommodation of their disabilities, and altered attitudes to individuals with Down syndrome and their parents who chose not to test or terminate an affected pregnancy.

The currently available NIPT for aneuploidy is the consequence of a massive investment by private companies and venture capital, and so far is only provided by private, for-profit companies. Aspects of the testing are the subject of contested patents and other proprietary ownership. Claims of exclusive intellectual property ownership can inflate costs, restrict access to test protocols and limit further test development. Although there is currently no direct-to-consumer testing, there are press releases, patient information materials and extensive marketing to healthcare providers, many of whom may have a poor understanding of the true performance of the tests.

In the future, NIPT may also be encouraged by governments or insurance carriers who view genetic disorders as an economic burden. The concern is that these financial pressures will be the dominating factor in determining...
who has access to testing, without adequately considering medical need. Changes in financial support for testing, counseling and clinical services can also indirectly compromise individual patient autonomy when deciding whether to continue or terminate an affected pregnancy.

It is also worth noting that the early history of NIPT shows how easily test quality can be compromised when development and introduction is left entirely to unregulated free market forces.


DISCLOSURES

H. Cuckle is a consultant to PerkinElmer Inc, Ariosa Diagnostics Inc, Natera Inc, and director of Genome Ltd; E. Pergament is a consultant to PerkinElmer Inc and Natera Inc, and director of Northwestern Reproductive Genetics Inc.

REFERENCES


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