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Review

# Cell-free fetal DNA-based noninvasive prenatal testing of aneuploidy

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**Key content**

- Noninvasive prenatal testing (NIPT) uses cell-free fetal DNA (cffDNA) to test for aneuploidy, as opposed to noninvasive prenatal diagnosis (NIPD), which uses cffDNA to diagnose fetal sex, Rhesus D status and monogenic disorders. This classic review focuses on screening for aneuploidy.
- NIPT is a screening test and needs confirmatory invasive testing in cases of a high-risk (positive) result.
- NIPT demonstrates high sensitivities and specificities according to our recent meta-analysis, although it is less accurate for Trisomy 18, Trisomy 13, Monosomy X and sex chromosomal aneuploidies than for Trisomy 21.
- It is imperative that the implications of false positive and false negative results are investigated and considered in a clinical context.

**Learning objectives**

- To be able to discuss NIPT with patients, including test accuracy and disadvantages.
- To be up to date with the implementation of NIPT in the National Health Service (NHS).

**Ethical issues**

- NIPT requires careful counselling: patients may consider it a 'trivial' or routine blood test and may not fully understand the implications of a high-risk (positive) result.
- There are issues surrounding other diagnoses that NIPT can potentially reveal, including maternal cancers, maternal sex chromosome aneuploidies and milder fetal phenotypes.

**Keywords:** aneuploidy / antenatal screening / cell-free fetal DNA / noninvasive prenatal testing

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## Introduction

Cell-free fetal DNA-based (cffDNA) noninvasive prenatal testing (NIPT) is heralded as one of the biggest advances in antenatal care since the invention of ultrasound scanning. NIPT is a screening test for aneuploidy (an abnormal number of chromosomes), and therefore requires confirmatory invasive testing in cases of high-risk results, also known as positive results. NIPT is not to be confused with non-invasive prenatal diagnosis (NIPD), which although also based on cffDNA, is considered diagnostic and does not, therefore, require further testing. NIPD is used to determine fetal sex, fetal Rhesus status and monogenic disorders. This review focuses on aneuploidy and aims to provide clinicians with sufficient information to counsel women for NIPT. Here we present test accuracy data, highlight the limitations of NIPT, discuss the ethical issues surrounding this relatively new test, outline current guidance, and describe its likely future role in the antenatal care pathway.

## Basis of the NIPT technique

cffDNA comprises small fragments of fetal DNA, thought to originate from trophoblast. These fragments circulate in maternal plasma and form approximately 10% of the DNA fragments in maternal plasma (Figure 1).[1] It is present in reliably measurable levels for aneuploidy screening from 10 weeks of gestation and is cleared quickly from the

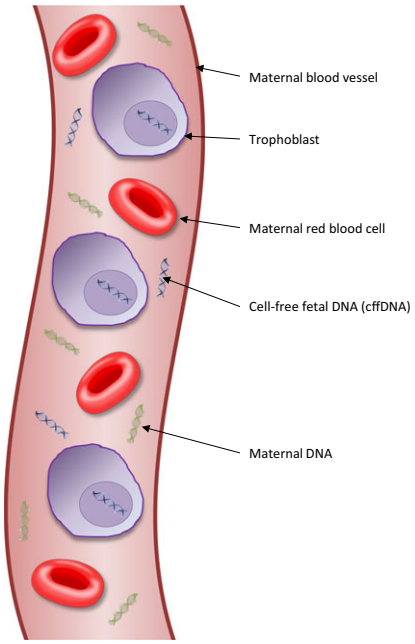


**Figure 1.** Fragments of cell-free fetal DNA in maternal blood used in noninvasive prenatal testing. Adapted from figure provided by Illumina.

maternal circulation hours after delivery, making it specific to that pregnancy. The commercial sector has shown particular interest in NIPT, thus enabling the rapid development of the technology, but potential commercialisation is not without consequence.

The ability to identify fetal chromosomal anomalies (principally aneuploidy) has been possible since 2011 with the introduction of massively parallel sequencing (MPS) for this purpose. The premise of aneuploidy testing is different to that of NIPD. In aneuploidy, DNA from each chromosome is quantified and common autosomal trisomies are detected based on a difference in the proportion of each chromosome (e.g., chromosome 21 in the case of Trisomy 21, compared to the other chromosomes from that fetus). Following complex biostatistical analysis, a result of 'low risk' or 'high risk' is given. MPS technology has continued to advance and two different subtypes are now recognised: (a) massively parallel shotgun sequencing, whereby the whole genome is randomly sequenced, and (b) 'targeted' MPS in which only specific genomic regions known to contain the chromosome (or single nucleotide polymorphism [SNP]) of interest are sequenced and compared to reference regions.

## Test accuracy

The authors recently published a systematic review and meta-analysis that informs data on test accuracy of NIPT and NIPD in singleton pregnancies.[2] This review is different from another recent review by Gil et al. published in 2015[3] as it included only cohort studies, thus reducing risk of bias compared to case–control studies that do not represent the true incidence of a condition in the population. The authors also performed bivariate meta-analysis, which is considered superior to univariate meta-analysis since it allows for the correlation between the sensitivity and specificity within the same study and is therefore more representative of the true population. The review comprised 117 papers and explored all possible conditions, although only the results relating to aneuploidy are presented in this article (Table 1).

NIPT for Trisomy 21 and Trisomy 18 demonstrated high sensitivity and specificity (Table 1). Results for Trisomy 13 revealed a lower sensitivity; although the exact reasons for this remain unclear, it is thought to be associated with the low guanine–cytosine (GC) content known to exist in chromosome 13. Monosomy X demonstrated reduced sensitivity compared to Trisomy 21 and 18, although it was evaluated by fewer studies, which equated to many fewer tests (146 344 Trisomy 21 tests versus 6712 Monosomy X tests).

The authors also performed a sensitivity analysis (results not shown) to evaluate the effect of population risk on Trisomy 21 test accuracy by removing five studies that assessed accuracy in women with an average pre-test risk of aneuploidy. This demonstrated no significant difference in test accuracy between high and average risk populations. Unfortunately there were insufficient eligible studies to meta-analyse 47XXX, 47XXY, 47XYY and Trisomy 16. Because of the very low prevalence of sex chromosome aneuploidy (SCA), the 95% confidence intervals (CI) were very wide.[2] Gil et al.[3] pooled all the SCA results (n = 56/6755 tests in singleton pregnancies with SCA, excluding Monosomy X) to perform a meta-analysis and reported a detection rate of 93.0% (95% CI 85.8–97.8%) and false positive rate of 0.14% (0.06–0.24%). Maternal SCA is believed to contribute to reduced SCA test accuracy, as often these conditions have a mild phenotype if the fetus survives. Mosaicism (maternal, placental and fetal) has also been reported as a contributing factor to false results. The ethical implications of testing for SCA are discussed below.

### Multiple pregnancy

A dearth of appropriate NIPT data means that the authors' meta-analysis did not include multiple pregnancies. In the meta-analysis by Gil et al.,[3] Trisomy 21 was detected at a rate of 93.7% (95% CI 83.6–99.2%) and had a false positive rate of 0.23% (95% CI 0.00–0.92%) in twin pregnancies (n = 430 pregnancies, five studies) demonstrating lower sensitivity than testing in singleton pregnancies.

One may hypothesise that the larger placental mass in multiple pregnancies, which presents a higher fraction of circulating cffDNA compared to singletons[4,5] would lead to more accurate NIPT results. However, testing in multiple pregnancies presents unique challenges. In dizygotic twins,

**Table 1.** Summary results of test accuracy of cell-free fetal DNA noninvasive prenatal testing[2]

| Condition | Number of studies (tests) | Sensitivity (95% CI) | Specificity (95% CI) | Diagnostic odds ratio (95% CI) | Positive likelihood ratio (95% CI) | Negative likelihood ratio (95% CI) |
|---|---|---|---|---|---|---|
| Trisomy 21 | 31 (148 344) | 0.994 (0.983–0.998) | 0.999 (0.999–1.00) | 285 903 (124 215–658 053) | 1720 (1111–2662) | 0.006 (0.002–0.017) |
| Trisomy 18 | 24 (146 940) | 0.977 (0.952–0.989) | 0.999 (0.998–1.00) | 68110 (29 137–159 209) | 1569 (810–3149) | 0.023 (0.011–0.048) |
| Trisomy 13* | 16 (134 691) | 0.906 (0.823–0.958) | 1.00 (0.999–1.00) | 2788 (285–27 252) | 453 (26–7864) | 0.188 (0.080–0.44039) |
| Monosomy X | 8 (6712) | 0.929 (0.741–0.984) | 0.999 (0.995–0.999) | 18849 (2277–156 069) | 1337 (213–8407) | 0.071 (0.017–0.292) |

NB. All analyses performed by bivariate meta-analysis, apart from *, which indicates that univariate analysis was performed. CI = confidence interval

aneuploidy discordance is a significant issue and there can be nearly a two-fold intertwin difference in cffDNA fraction. This means that the affected fetus may have a cffDNA fraction below the threshold of 4% required for testing, while the unaffected twin may contribute a high cffDNA fraction; therefore, the total cffDNA fraction may appear sufficient and produce a false negative (low-risk) result.[6,7]

Testing in monozygotic twins theoretically should be easier as they produce identical DNA molecules, but chorionicity must be certain. Another problem is that of single twin demise, as the effect that cffDNA from the demised twin has on the NIPT result is unknown. Because of these factors, various professional bodies do not currently recommend NIPT for aneuploidy in twin pregnancies, including the Royal College of Obstetricians and Gynaecologists (RCOG)[8] and the American College of Obstetricians and Gynecologists (ACOG).[9] However, it is available privately in the UK, which causes dilemmas when a high-risk result is reported. More clinical studies are needed to investigate the unique challenges that these pregnancies present for NIPT.

## Benefits of NIPT

There are many benefits of NIPT as reflected by its rapid progress. It is a noninvasive test and thus does not pose the risks of chorionic villus sampling (CVS) or amniocentesis, such as pain, small risk of infection and the 0.22% (95% CI –0.71 to 1.16%) and 0.11% (95% CI –0.04 to 0.26%) procedure-related risk of miscarriage associated with CVS and amniocentesis, respectively.[10] Since cffDNA is cleared quickly from the maternal circulation, it is specific to that pregnancy. The test has a quick processing time, with the potential for results to be reported in 3–5 working days, equivalent to quantitative fluorescence-polymerase chain reaction (QF-PCR) testing for invasive samples. However, in the clinical setting, processing time depends on the demand for NIPT.

## Disadvantages of NIPT

### Technical: false, inconclusive and failed results

Test accuracy is not 100% as there are false negative and false positive results, and occasions when the test will not produce a result (an inconclusive test result). It is therefore important to reiterate that it is a sensitive 'screening test'. The authors' review highlighted the fact that false and inconclusive results were poorly reported for all indications in the published data, although the rate of inconclusive results has been quoted as 1.9–6.4% of samples.[11] This information is vital as some studies have shown that those who have an inconclusive result are more likely to have a chromosomal aberration, and of those who have a first inconclusive result, 20% will have a failed repeat NIPT sample.[12]

One particular issue was the different quality control (QC) standards, which meant that less stringent studies reported a false negative or false positive result in a low quality sample, whereas others with more stringent criteria report it as inconclusive or a 'failed' sample. The lack of guidance on QC standards was recently acknowledged by the International Society for Prenatal Diagnosis (ISPD), who advised the development of specific guidelines.[11] When evaluating test accuracy data, false and inconclusive result rates are vital, particularly when the test is potentially to be offered to the entire obstetric population, irrespective of background risk. The most common reasons offered by authors for false and inconclusive results were:

- A low fetal DNA fraction in the blood sample, which is measured by specific markers of fetal DNA or algorithms applied to the sequencing data.
- A 'vanishing' twin that has disappeared prior to the woman's dating ultrasound scan, which if non-identical may cause a false positive result. This is likely to remain an issue even as technology advances.
- Confined placental mosaicism, whereby the fetus and placenta have two different lineages. As the fetal DNA fragments originate from the placenta, NIPT is unable to distinguish between the two. This is also something that is unlikely to be overcome, despite continued advances in test technology, but it should be noted that this is an issue for invasive placental sampling (e.g., CVS) as well.
- NIPT can detect maternal cancers and maternal copy number variants, which result in false positives and have ethical implications (see below).

### Effects on medical training

NIPT has an effect on fetal medicine specialists. The number of invasive tests performed since the introduction of NIPT in the USA has decreased by as much as 53% for amniocentesis and 77% for CVS, based on clinical data.[13] This pattern is believed to be replicated in the UK's public healthcare system as well.[14] Therefore, doctors will potentially become de-skilled or have insufficient training opportunities.[15] This will not only affect doctors' performance in invasive testing, but also has implications on their ability to perform other invasive fetal procedures such as fetoscopic laser ablation, which require similar entry techniques.

### Financial cost at present

In the UK, NIPT is currently available only on a private basis in some areas; with tests costing £300–900, it is therefore dividing populations, as only those patients with a higher socioeconomic status are able to undergo testing. However, it is likely that the cost of NIPT will fall as the technology becomes cheaper.[16]

## Ethical Issues

### Informed consent

NIPT raises many ethical issues, which are under intense debate. Testing for Trisomy 21, 18 and 13 has been commercially available since 2011, but some believe that its introduction into clinical practice has been too fast and the ethical implications not fully explored. A major concern is that women and their families do not understand the potential sequelae of the test – there are fears that it is viewed it as 'just another routine antenatal blood test,'[17] whereas in reality, the results may lead to the difficult decision of either terminating the pregnancy, or continuing with a pregnancy in the knowledge that the baby could be born with a condition on a wide spectrum of severity. The importance of adequate pre-test counselling is thus paramount, with clinicians understanding that their priority (test accuracy) is different to the patient's priority (test safety for their fetus).[18] Clinicians must also understand that a substantial proportion of couples will undergo testing so that they can better plan for the arrival of a baby with a chromosomal abnormality.[14] Similar concerns exist for any screening test in pregnancy, e.g. combined screening for Down syndrome – there is an online NHS patient decision aid for 'Diagnostic testing for Down syndrome'; however, this does not include NIPT at present.[19] There are many written materials and online e-learning packages being developed for parents considering NIPT to enable fully informed consent (in the authors' anecdotal experience).

### Sex chromosome aneuploidy (SCA)

Screening for SCA is considered less accurate than screening for autosomal aneuploidy. One important reason for this is the presence of maternal SCAs,[20] which are often unknown because the phenotype may appear normal. SCA screening using NIPT is not conventional in the UK, but it is offered in the private sector. Consequently, if a maternal SCA is diagnosed, this can create a problem since it can be associated with learning difficulties or reduced fertility. There is also the question of what to do with the result. Often, if an SCA is severe the pregnancy will miscarry; however, if the fetus survives then the offspring may be mildly affected, but may then have the stigma of a genetic abnormality that might otherwise have remained undetected.

### Detection of maternal health problems

Another matter that has recently come to light is the ability of NIPT to detect maternal cancer – a distressing and anxiety-inducing result, perhaps even more so in the context of antenatal testing. Some may also view this as a benefit of NIPT, since earlier diagnosis allows earlier treatment. There have also been cases in which previously unknown maternal

genetic abnormalities have been detected as a consequence of abnormal NIPT results. This adds another layer of complexity to obtaining women's consent for NIPT. It also creates more issues that need careful consideration, such as the effect on the mother's future insurance policies, as highlighted by Bianchi et al.[21]

## Current guidance

At present, there is no official guidance in the UK regarding the use of cffDNA for aneuploidy. The RCOG Scientific Impact Paper published in March 2014, stated that, 'while the [NIPT] result is much more accurate than existing screening strategies, it is still not a diagnostic assay'. However, the authors believed that, 'in time, this technology [NIPT] is likely to become the primary screen for chromosomal abnormalities in pregnancy' in the NHS, and that 'all obstetricians should have knowledge of the counselling issues involved'.[8] In January 2016 the UK National Screening Committee (UKNSC) published a press release recommending the evaluative implementation of NIPT as a contingent screening test (i.e., a second-line screening test) for women with a risk higher than 1:150 on conventional screening (either nuchal translucency [NT] ultrasound scan, serum beta-human chorionic gonadotrophin [β-hCG] and pregnancy-associated plasma protein-A [PAPP-A]; or serum β-hCG, alpha-fetal protein [AFP], estriol and inhibin-A), which – in the case of a high-risk result on NIPT – would then require diagnostic invasive testing.[22] This implementation will be rolled-out gradually alongside a programme of staff training, and it is expected that the first test will be offered in the NHS in 2018/2019. In 2015, ACOG[9] recommended conventional combined testing as first-line screening for women in the general obstetric population, and although it stated that any woman may undergo NIPT provided she is appropriately counselled, a positive (high-risk) NIPT result should not be the basis of a decision for termination, and the result should be confirmed by invasive testing. In the case of an inconclusive/failed test result, ACOG advocates invasive testing and a detailed ultrasound scan.

## The future of NIPT

Many healthcare professionals believe that NIPT will be implemented in routine NHS antenatal care. How it will be implemented in the NHS, however, is being determined.

For a screening test to be considered appropriate to implement, it must satisfy various criteria as outlined by Wilson and Jugner.[23] Many papers have been published on models of NIPT screening implementation for Down syndrome, with different cut-offs, costs and clinical pathways.[14,16,24–30] When evaluating these models, the prevalence of the disease in the test population should be considered, as this will influence the positive-predictive value of the test; i.e., if a woman has a positive NIPT result, what is the likelihood that the result is a true positive in her case? In a low risk population with low disease prevalence, there will be a greater proportion of positive results that are in fact false positives. Therefore, models based on women above the age of 35 years, for example, may not be applicable to the general NHS obstetric population. Morris et al.[16] created a robust model based on the UK screening population and calculated that by using NIPT in the NHS as a contingent screening test, following a combined screening risk cut-off of >1:150, fewer Down syndrome cases are detected compared to combined screening (11.26 versus 13.24, respectively, equating to missing 2/10 000 hypothetical cases).[16] However, contingent screening has fewer procedure-related miscarriages (0.06 versus 0.80/10 000 cases, respectively) and costs the same as current Down syndrome testing when NIPT is priced at £500 (see Table 2 and Table 3). If NIPT were to be introduced as a first-line screening test compared to combined screening, this would produce more favourable outcomes (16.49 versus 13.24/10 000 cases detected, respectively; 0.11 versus 0.80 procedure-related miscarriages), but at a higher financial cost (£50 more per NIPT).[16]

The 5-year Reliable Accurate Prenatal non-Invasive Diagnosis (RAPID) project is the first study to evaluate the use of NIPT in the NHS.[31] Women with a combined screening risk of ≥1:1000 for Down syndrome (n = 1164 women) were offered NIPT. Results were available for 91% of participants with sensitivity 1.00 (95% CI 0.88–1.00) (32/32 cases) for Trisomy 21 and no false negatives. The use of NIPT as a contingent test afforded a reduction in invasive tests from 10 to 2.8 per Trisomy 21 case diagnosed. A major benefit of the RAPID study was that it assessed the performance of NIPT in an NHS setting (clinical and laboratory), with standardisation of technique and transparency of reporting of false and inconclusive results. The RAPID study reported eight (0.7%) failed or inconclusive tests – much lower than previously reported.

Despite RAPID's positive findings, there are still several issues to be considered. One unknown at present is NIPT uptake in the general population and high-risk population, compared to current screening and invasive testing uptake. A recent paper by Chitty et al.[14] used the results of the RAPID study to evaluate this for the UKNSC and found that uptake of further testing (NIPT or invasive testing) after a conventional screening result of >1:150 increased from 54% to >90%. In those with a high-risk NIPT result, approximately one third decided to continue with the pregnancy. This suggests that NIPT may not affect the rate of infants born with Down syndrome, which has also been shown in US studies.[13] However, there are some people who believe women with a very high risk, and/or who have

**Table 2.** Modelled outcomes of testing strategies in a screening population of 10 000 women (taken from Morris et al., 2014).[16] Assumed 69% uptake of Down syndrome (DS) screening using the combined test, 80% uptake of noninvasive prenatal testing (NIPT) as contingent screening for unaffected pregnancies, and 90% for affected pregnancies. Sixty-nine percent uptake of NIPT as first-line screening

| Testing strategy | Screening risk cut-off (1 in) | Number undergoing screening | Number undergoing NIPT | Number with a positive NIPT result | Number having an invasive diagnostic test | Number of procedure-related miscarriages | Number of DS cases detected |
|---|---|---|---|---|---|---|---|
| DS screening using the combined test | 150 | 6881.66 | 0 | | 160.59 | 0.80 | 13.24 |
| NIPT as contingent testing | 150 | 6881.66 | 153.75 | 13.30 | 11.48 | 0.06 | 11.26 |
| | 500 | 6881.66 | 361.43 | 14.75 | 12.71 | 0.06 | 12.31 |
| | 1000 | 6881.66 | 591.02 | 15.26 | 13.13 | 0.07 | 12.55 |
| | 2000 | 6881.66 | 912.32 | 15.85 | 13.63 | 0.07 | 12.78 |
| NIPT as first-line screening | - | 0 | 6881.66 | 28.02 | 22.03 | 0.11 | 16.49 |

**Table 3.** Modelled costs of testing strategies in a screening population of 10 000 women (taken from Morris et al., 2014).[16] Assumed 69% uptake of Down syndrome (DS) screening using the combined test, 80% uptake of noninvasive prenatal testing (NIPT) as contingent screening for unaffected pregnancies, and 90% for affected pregnancies. Sixty-nine percent uptake of NIPT as first-line screening

| Testing strategy | Screening risk cut-off (1 in) | Cost per NIPT test (£) | (A) Cost of screening (£000s) | (B) Cost of NIPT (£000s) | (C) Cost of invasive diagnostic tests (£000s)* | (A) + (B) + (C) (£000s) |
|---|---|---|---|---|---|---|
| DS screening using the combined test | 150 | | 200 | 0 | 79 | 279 |
| NIPT as contingent screening | 150 | 50 | 200 | 8 | 6 | 213 |
| | 150 | 250 | 200 | 39 | 6 | 244 |
| | 150 | 500 | 200 | 78 | 6 | 283 |
| | 150 | 750 | 200 | 116 | 6 | 322 |
| | 500 | 50 | 200 | 18 | 6 | 225 |
| | 500 | 250 | 200 | 91 | 6 | 298 |
| | 500 | 500 | 200 | 183 | 6 | 389 |
| | 500 | 750 | 200 | 274 | 6 | 480 |
| | 1000 | 50 | 200 | 30 | 6 | 237 |
| | 1000 | 250 | 200 | 149 | 6 | 356 |
| | 1000 | 500 | 200 | 298 | 6 | 505 |
| | 1000 | 750 | 200 | 448 | 6 | 655 |
| | 2000 | 50 | 200 | 46 | 7 | 253 |
| | 2000 | 250 | 200 | 230 | 7 | 438 |
| | 2000 | 500 | 200 | 461 | 7 | 668 |
| | 2000 | 750 | 200 | 691 | 7 | 898 |
| NIPT as first-line screening | | 50 | 0 | 438 | 11 | 449 |
| | | 250 | 0 | 1642 | 11 | 1825 |
| | | 500 | 0 | 3535 | 11 | 3546 |
| | | 750 | 0 | 5255 | 11 | 5266 |

*Including procedural miscarriages.

abnormal ultrasound findings, should have direct access to invasive testing.[9] In the study by Chitty et al., 54% of women with a risk of >1:150 underwent invasive testing before the NIPT results were known.

Another issue, as demonstrated by the example of Morris et al. – and as with many screening programmes – is that a balance must be made between detection rates, false negative rates and cost-effectiveness. Although the increased uptake of further testing improves the detection rate, the presently high cost of NIPT means it is not cost-effective to introduce it as the first-line screening test; indeed, studies favour contingent NIPT screening.[14,16,27,28,30] However, as the authors highlight, the cost of NIPT will probably decrease over time.

How NIPT affects ultrasound scan usage must also be considered. Although the NT measurement is involved in the combined screening test, it also provides other useful information, for example, on the risk of cardiac defects. The dating scan provides valuable information, for example, about the number of fetuses, chorionicity in multiple pregnancy, or the presence of a molar pregnancy, which NIPT cannot provide. A recent study exploring the utility of first-line NIPT in 251 pregnancies with a variety of anomalies on ultrasound did not advocate first-line NIPT in this scenario, although the authors did not comment on whether the women underwent conventional screening.[32]

As well as deciding the role that NIPT will play in the antenatal care pathway, other challenges must be met in terms of the logistics of procurement, and running these tests on a national level, in a quality assured way, to satisfy UK National External Quality Assessment Services (NEQAS). Support will also be needed from the Down Syndrome Screening Quality Assurance Support Service (DQASS).

## Conclusion

NIPT demonstrates high sensitivity and specificity for Trisomy 21, 18 and 13. Commercial interest in NIPT means that its development has been rapid, which may have contributed to the poor reporting of false and inconclusive results. Some advise caution with its use, particularly given the ethical implications, and the potential this technology has to reveal unexpected diagnoses in the mother. NIPT will change the face of prenatal testing: it is important that healthcare professionals counselling women on NIPT provide all the information required for them to make an informed decision regarding antenatal testing, and keep up with the rapid advances being made in this exciting area.

## Further reading

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### Disclosure of interests

There are no conflicts of interest.

### Contribution to authorship

MDK conceived the article and helped write the article. FLM and RKM researched and drafted the article. SA assisted with research. All authors approved the final version.

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### Declarations

With the knowledge of the editors of *BJOG: An International Journal of Obstetrics and Gynaecology* and *The Obstetrician & Gynaecologist* (*TOG*), there are reproductions of data in the original *BJOG* article within this *TOG* review.

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