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Green-top Guideline (new): Cell-Free Fetal DNA Screening for Chromosomal Anomalies

Karim J | Kilby MD | Mone F | Nanda S | Nicolaides K | Fisher J | Shakespeare T | Pandya P | Papageorghiou AT
On behalf of the Royal College of Obstetricians and Gynaecologists

Correspondence: Royal College of Obstetricians and Gynaecologists, 10–18 Union Street, London SE1 1SZ. Email: clinicaleffectiveness@rcog.org.uk

This is the first edition of this guideline.

Key recommendations

Cell-free fetal DNA testing for chromosomal conditions (T21, T18, T13)

- Cell-free fetal DNA (cffDNA) testing can be safely and feasibly offered to pregnant women and people either as a first line screening test in unselected populations, or within a contingent model for the accurate identification of fetuses with T21, T18 and T13. [Grade A]

- In the setting of raised nuchal translucency (≥3.5 mm) or the finding of a fetal anomaly, women should be referred to a fetal medicine unit and, where indicated, offered invasive diagnostic testing. In a setting where invasive testing has been declined, cffDNA testing may be offered. [Grade A]

- For pregnant women who have a higher chance for T21, T18 or T13 due to a previous affected pregnancy, cffDNA testing may be offered as a first line screening test. [GPP]

- Pregnant women must be informed that cffDNA testing is a form of prenatal screening as opposed to a diagnostic test. [Grade A]

- In test failure due to low cffDNA fetal fraction, the presence of T18 and T13 should be considered. [Grade B]

- When cffDNA fails to yield a result, a detailed review of the pregnant woman's or pregnant person's initial a-priori chance and indications for cffDNA testing should be undertaken. [GPP]

- Pregnant women and people who want to avoid a diagnostic test and would wish to continue a pregnancy with T21, T18 or T13 may still value the information a cffDNA test can give them. This is a valid choice and must be respected. [GPP]

Expanding the use of cffDNA testing beyond the detection of T21, T18 and T13

- cffDNA testing for detection of copy number variants is not currently recommended. [Grade B]

- cffDNA testing for rare autosomal trisomies (RATs) is not currently recommended. [Grade B]

- Offering cffDNA testing for single gene disorders is recommended in high-risk pregnancies such as those where parent(s) are known carriers for a condition, when there has been advanced work-up by a clinical geneticist and the relevant genomic laboratory. [Grade B]

cffDNA testing in the setting of multiple pregnancy

- cffDNA testing may be offered to women or pregnant people with a twin pregnancy. [Grade A]

- There is a higher chance of a false positive result with a vanishing twin, and therefore cffDNA testing in the presence of a vanishing twin is not recommended. [Grade C]

- cffDNA testing cannot currently be recommended for aneuploidy screening in triplet or higher order multiple pregnancy. [Grade D]

Follow-up care after a high chance cffDNA test result and post-test counselling

- A confirmatory invasive diagnostic test should be offered where there is a high chance result on cffDNA testing for T21, T18 or T13. [Grade A]

- Where there is a high chance non-invasive prenatal testing (NIPT) result for T21, a chorionic villous sample can be performed with careful interpretation of the result. [Grade D, GPP]

- Where there is a high chance result for T18 or T13 and a significant fetal anomaly is present, a chorionic villous sample can be performed with careful interpretation of the result. [Grade D, GPP]

- Where there is a high chance result for T18 or T13 and no significant structural anomaly is evident, the recommended diagnostic test of choice is an amniocentesis. [Grade D, GPP]

- Both pre- and post-test counselling for cffDNA testing should include signposting to relevant support organisations and resources. [Grade C]

1. Purpose and scope

This guideline will provide current evidence-based recommendations for the provision of cell-free fetal DNA (cffDNA) testing in pregnancy. Guiding clinical practice, it is intended for use by healthcare professionals to aid informed discussions and the provision of clear, non-directive information to

pregnant women, which is essential to ensure they can make informed choices regarding cffDNA testing.

This guideline has been developed by the Royal College of Obstetricians and Gynaecologists (RCOG). It is not intended to replace or supersede screening pathways, which vary between the devolved nations of the UK. Healthcare professionals are encouraged to consider this guideline alongside national screening policies and adapt recommendations as appropriate to their setting.

Within this document we use the terms woman and women's health. However, it is important to acknowledge that it is not only women for whom it is necessary to access women's health and reproductive services in order to maintain their gynaecological health and reproductive wellbeing. Gynaecological and obstetric services and delivery of care must therefore be appropriate, inclusive and sensitive to the needs of those individuals whose gender identity does not align with the sex they were assigned at birth.

2. Introduction and background epidemiology

2.1 Antenatal screening in the first trimester

Chromosomal conditions are a significant cause of perinatal mortality and childhood disability. Trisomy 21 or Down syndrome (T21), Trisomy 18 or Edwards syndrome (T18) and Trisomy 13 or Patau syndrome (T13) represent the most common autosomal aneuploidies at birth. Of these, T21 is the most common and a cause of congenital intellectual disability.[1,2]

In England, data from the National Congenital Anomalies Disease Registry (NCARDRS) report the prevalence of T21 ,T18 and T13 per 10 000 total births (Table 1).[3] However, the overall incidence is difficult to estimate, and may be higher than indicated due to the lack of reporting in cases of natural pregnancy loss and the termination of affected pregnancies.

Table 1: Data on prevalence of trisomies, derived from the National Congenital Anomalies and Rare Diseases Registration Service (NCARDRS). Prevalence may underestimate true incidence due to unreported cases of pregnancy loss or terminations following diagnosis.

Chromosomal condition	**Prevalence (per 10,000 total births)**	**95% Confidence interval**	**Proportion (1 in X births)**
Trisomy 21 (Down syndrome)	26.5	25.2 to 27.9	1 in 377
Trisomy 18 (Edwards syndrome)	7.4	6.7 to 8.1	1 in 1,351
Trisomy 13 (Patau syndrome)	2.7	2.3 to 3.2	1 in 3,703

Antenatal screening for T21 has been used in clinical practice since the late 1960s. Screening provision and pathways vary between the devolved nations of the UK, currently all pregnant women and people are offered screening for T21, T18 and T13 in England, Scotland and Wales.[4,5] The important characteristic of any screening test is the ability to distinguish between individuals with and without the condition. This is measured by the detection rate (sensitivity) and false positive rate (FPR, 1 - specificity), and these depend on the test used and the population tested.

2.2 Screening tests for T21, T18 and T13

Historically, screening for T21 was based on maternal age alone, with women aged 35 or older considered to be at high risk. In the 1970s, this included approximately 5% of pregnant women and

identified 30% of fetuses with T21.[6] While the rate of chromosomal aneuploidy increases significantly with maternal age,[7] the majority of babies with these conditions are born to women under the age of 35 years, which accounts for the relatively poor screening performance of this approach. It should also be noted that recent trends in delaying childbirth have led to more than 20% of pregnant women now being 35 or older meaning such an approach also has a high FPR.[1]

In the 1980s and 1990s, screening using a combination of maternal age and various serum biomarkers increased detection rates. At a FPR of 5%, detection rates increased from 30% with maternal age alone to 70–75% using the quadruple (QUAD) test, comprising maternal age, serum alpha fetoprotein (AFP), free beta human chorionic gonadotrophin (β-hCG), unconjugated estriol (uE3) and inhibin A.[8] In the 1990s, screening using the combined screening test (CST) was developed in the first trimester, achieving a detection rate of approximately 90% at the same FPR of 5%. This is based on maternal age, ultrasound measurement of fetal nuchal translucency (NT) and maternal serum biochemistry (β-hCG and pregnancy associated plasma-protein A [PAPP-A]).[6] Additional ultrasound markers, such as absence of the nasal bone, increased resistance in the ductus venosus, and tricuspid regurgitation, have been described to increase the accuracy of the CST further (achieving detection rates of over 95% and decreasing the FPR to fewer than 3%).[9-11] However, these markers have not been widely implemented in national screening programmes due to training challenges.

2.3 Diagnostic testing with chorionic villous sampling (CVS) and/or amniocentesis

The most common reason for invasive testing, amniocentesis or chorionic villus sampling (CVS), is to diagnose chromosomal aneuploidies.[6] However, due to the risk of miscarriage associated with invasive testing (the additional risk of miscarriage following amniocentesis or CVS performed by an appropriately trained operator is likely to be below 0.5%-), this is usually only offered in pregnancies that have a high chance of T21, T18 or T13 following a screening test. Details on invasive diagnostic testing are available in Green-top Guideline no. 8 *Amniocentesis and Chorionic Villus Sampling* (2021).[12]

2.4 Current policy and performance of aneuploidy screening in the NHS

Screening policy is devolved to the four nations in the UK. The policy in England, Scotland and Wales is to offer all pregnant women and people an assessment for T21, T18 and T13 using the CST in the first trimester where the fetus has a crown rump length (CRL) of 45.0–84.0 mm.[13] For pregnancies where the CRL measurement is greater than 84.0 mm, or when sonographers have been unable to obtain an NT measurement, the maternal serum QUAD test can be offered. This is offered between 14^{+2} and 20^{+0} weeks or when the head circumference (HC) measurement is between 101.0 mm and 172.0 mm. With this screening policy, data in England show a combined standardised screen positive rate (SPR) for T21, T18 and T13 of 2.8%.[14] In 2020 to 2021, the age-adjusted detection rate for T21 was 81.9% (95% CI 79.5–84.3) for the CST, and 75.2% (95% CI 67.9–82.6) for the QUAD test. The CST detection rate for T18 was 89.4 (95% CI 77.0–86.2) and for T13, 68.9% (95% CI 61.3–76.5) (data provided via personal communication with the Fetal Anomaly Screening Programme [FASP]). In Northern Ireland there is currently no availability of routine first trimester screening, except on a self-funded basis.

In the past, women that received a higher chance result (between 1 in 2 and 1 in 150) for birth of a baby with T21 from CST or QUAD screening, were offered the option of diagnostic testing with CVS or amniocentesis. However, since June 2021, cffDNA testing (also referred to as non-invasive prenatal testing, NIPT) for T21, T18 and T13 is offered to women with a higher chance result as part of a three-year evaluative roll-out by the NHS in England.

As a result, the uptake of cffDNA testing for trisomies is becoming widespread within the NHS in women with a higher chance and in the private sector, often as a primary screening method.[15-17] This guideline summarises the evidence and provides an overview of different clinical scenarios and care pathways for healthcare providers offering such screening.[18]

3. Identification and assessment of evidence

The Cochrane Database of Systematic Reviews and electronic databases (CINAHL, EMBASE, MEDLINE and PubMed) were searched looking for systematic reviews and meta-analyses with the following terms in the title or abstract: 'non-invasive prenatal testing', 'NIPT, NIPD, NIPS', '*prenatal diagnosis', 'prenatal screen*', 'antenatal test/diagnosis*/screen*', 'cell-free nucleic acids' 'screen*', 'cell free', 'DNA', 'fetal or foetal', 'fetus or foetus', 'maternal' and 'diagnostic procedure'. The search was restricted to articles published until March 2023. The full search strategy is available to view online as supporting information. The recommendations given in this guideline have been graded according to the RCOG guidance, *Developing a Green-top Guideline: Guidance for developers*. Where possible, recommendations are based on available evidence. Areas lacking evidence are highlighted and annotated as 'good practice points' (GPP). Further information about the assessment of evidence and the grading of recommendations may be found in Appendix A.

4. cffDNA testing for chromosomal conditions (T21, T13, T18)

4.1 Background

Placental cells undergo cycles of fusion and apoptosis, releasing short fragments of cffDNA into the maternal bloodstream.[19-21] In 1997, Lo et al. first reported fetoplacental cffDNA in maternal plasma.[22] A landmark discovery in 2008 showed higher relative amounts of fetoplacental DNA in maternal plasma in T21 pregnancies compared with euploid pregnancies.[23,24] This paved the way for the development of cffDNA-based tests for fetal aneuploidy. In 2011, the first commercial cffDNA-based test, primarily for T21, was introduced to the market. Since then, cffDNA testing has gained widespread popularity due to its non-invasive nature and high accuracy.

cffDNA fragments, derived from the placenta and considered as representative of the fetal genotype,[25] are detectable in maternal plasma as early as the fifth week of gestation. With increasing gestational age there is an increasing fraction of cffDNA compared with the total maternal plasma cell-free DNA pool.[26-29] Levels of cffDNA are typically sufficient for reliable analysis from 9–10 weeks of gestation depending on assay type; following birth, cffDNA is rapidly cleared from the maternal circulation.[30,31]

cffDNA analysis is performed on maternal plasma containing both maternal and placental cell-free DNA. The test counts DNA fragments from a chromosome of interest (for example, chromosome 21) and compares this with the reference set of chromosomes. By analysing the relative abundance of specific chromosomal sequences, cffDNA testing can detect excess genomic material due to the presence of a trisomic fetus.[23,32] This is the basis of testing for T21, T18 and T13, although theoretically other aneuploidies could also be detected.

Common testing platforms use three main methods: massively parallel shotgun sequencing (MPSS), chromosome selective sequencing (CSS) and single nucleotide polymorphism (SNP) based approaches (see glossary, Appendix B).[15,33,34] All rely on a chromosome dosage approach detecting over-representation of sequences from the affected chromosome by comparison to unaffected reference chromosomes.[33]

The field of prenatal testing using cffDNA is rapidly expanding to include the detection of genetic conditions beyond aneuploidies, but testing for these disorders is much less accurate. They include copy number variations (CNVs) and single-gene disorders.[34] Such expansion of cffDNA testing applications raises ethical and implementation implications, which are the subjects of ongoing discussion.

Regardless of the method used, it is important to note that cffDNA is a screening test with high detection rate and low FPR, but it is not a diagnostic test. Thus, positive results require confirmation through diagnostic testing such as amniocentesis or CVS; while negative results do not exclude the presence of T21, T18 or T13.

4.2 What are the different approaches to screening with cffDNA?

Recommendation	Evidence level	Strength	Rationale for the recommendation
cffDNA testing can be safely and feasibly offered to women either as a first line screening test in unselected populations, or within a contingent model for the accurate identification of fetuses with T21, T18 and T13	**1++**	**A**	Large scale studies and meta-analyses demonstrate that cffDNA testing has a high detection rate and low FPR
cffDNA testing should be offered after a detailed ultrasound scan at 11^{+2} to 14^{+1} weeks of gestation (equivalent to a fetal crown rump length of 45–84 mm) to confirm a viable intrauterine pregnancy, estimate gestational age, identify multiple pregnancy, exclude signs of a vanishing twin pregnancy, raised NT (≥3.5 mm) and major fetal anomalies	**1++**	**A**	cffDNA results must be interpreted in light of multiple pregnancy and vanishing twin. In the presence of raised NT and/or fetal anomalies, counselling for invasive diagnostic testing for chromosomal conditions beyond T21, T18 and T13 should be undertaken
For women opting for cffDNA testing prior to 11^{+2} weeks, a pre-test ultrasound scan should be offered to confirm a viable intrauterine pregnancy, estimate gestational age, identify multiple pregnancy and exclude signs of a vanishing twin pregnancy; a subsequent detailed ultrasound should be offered at 11^{+2} to 14^{+1} weeks	**1++**	**A**	cffDNA results must be interpreted in light of multiple pregnancy and vanishing twin. The presence of raised NT and/or fetal anomalies should trigger counselling for invasive diagnostic testing
In the setting of raised nuchal translucency (≥3.5 mm) or the finding of a fetal anomaly, women should be referred to a fetal medicine unit and, where indicated, offered invasive diagnostic testing. In a setting where invasive testing has been declined, cffDNA testing may be offered	**1++**	**A**	In the presence of raised NT or fetal anomalies, counselling for invasive diagnostic testing should be offered

For pregnant women who have a higher chance for T21, T18 or T13 due to a previous affected pregnancy, cffDNA testing may be offered as a first line screening test	**D**	**GPP**	Women with a previous pregnancy with T21, T18 or T13 may benefit from cffDNA testing as this is the most accurate screening test currently available

Implementation of cffDNA screening has been undertaken successfully in many countries. Models of cffDNA screening include: (i) a first line screening policy where cffDNA testing is offered to all pregnant women and people; (ii) part of a contingent model, where either the CST or the QUAD test is first offered in order to identify women with a higher chance for T21, T18 or T13 (defined by different cut-offs in different countries) who are then offered second-line cffDNA testing. Issues to screening in twins are discussed in further detail in **Section 8**.

4.2.1 Universal cffDNA testing for T21, T18 and T13

A number of studies have evaluated the routine use of cffDNA testing as a first line screening methodology in all pregnant women and have demonstrated this to be feasible, with high detection rates and low FPRs.[35-41] *[Evidence level 2+]*

For example, universal cffDNA testing programmes have been implemented in Belgium and the Netherlands for T21, T18 and T13.[42,43] Population level data demonstrate the feasibility of implementation, high detection rates for T21, T18 and T13 and low FPRs with a resultant decrease in the rate of invasive diagnostic testing. These studies also confirm the need for the offer of diagnostic testing and clinical follow-up after a high chance cffDNA result as cffDNA testing does not meet the standards required for a diagnostic test. *[Evidence level 2+]*

These unique data deserve more detailed review. The programme in Belgium reported on cffDNA tests from over 150,000 singleton pregnancies[42]. A test result was reported for 99.3% of cases, with 0.7% having inconclusive results even after repeated testing. There were 494 cases of T21, 115 of T18 and 91 of T13. The detection rate for T21 was 98.91% (97.23–99.58), with a FPR of 0.02% (0.01–0.03) and a PPV of 92.39% (89.34–94.61). For T18 the detection rate was 97.47% (91.23–99.30), FPR 0.01% (0.01–0.02) and PPV 84.62% (75.82–90.61), while for T13 the respective figures were 100.00% (90.36–100.00), 0.03% (0.02–0.04) and 43.90% (33.67–54.68). Considerable efforts were made to test placentas, so that the authors were able to show that at least half of the false-positive cases could be explained by confirmed confined placental mosaicism (CPM), as opposed to technical concerns (see **section 4.6** for a detailed discussion on mosaicism). There were six false negative cases: four cases of T21 and two cases of T18 (no cases of T13). One of the false negative cases of T21 and one of T18 were demonstrated to have mosaicism, with placental follow up for the remaining cases unavailable. As a result of primary cffDNA testing, there was a 52% decrease in invasive procedures. *[Evidence level 2++]*

The Dutch TRIDENT-2 implementation study also evaluated routine first-line cffDNA testing for all pregnant women.[43] The uptake was 42% and the authors report on over 70 000 women. In total, 1.5% of cases tested did not receive a reported result after first blood draw, but a result became available in 99.7% of all women once repeat testing was included. There were 239 cases of T21 and the detection rate was 98% (95–99), FPR was 0.01% and PPV of 96%. For T18 (n=49) it was 91% (79–97%), 0.001% and 98% and for T13 (n=55) it was 100% (87–100%), 0.03% and 53%, respectively.

4.2.2 Contingent screening for T21, T18 and T13

cffDNA testing is clearly superior to the CST or QUAD test in the identification of fetuses with T21, T18 or T13.[44-47] However, in many settings, costs and logistic demands remain too high for first line universal cffDNA testing.[48,49] A contingent model, where either the CST or the QUAD test is first offered in order to identify women with a higher chance, offers a number of advantages. [46,50,51] Firstly, it gives pregnant women and people with a higher chance result after CST the opportunity to avoid an invasive test (when no increased NT or fetal abnormalities are identified). Secondly, identifying those pregnancies that have the highest chance of T21, T18, and T13 means that the total number of cffDNA tests undertaken is lower than if universal cffDNA screening was offered; and thirdly, it identifies an 'intermediate' chance group of pregnancies (for example, those with a chance of 1 in 151 to 1 in 1000 who under most policies would not be offered further testing)[51] meaning the FPR of CST screening alone is reduced. *[Evidence level 1++]*

Using published data from the National Down Syndrome Cytogenetic Register,[52] Mackie et al. have modelled the estimated outcomes expected at population level in the UK if a policy of cffDNA testing was offered to (and accepted by) women contingent on a CST result of greater than 1:1000 for T21 resulting in a DR of 96% for a SPR of 12%.[46] The modelling suggests that such a policy for contingent screening would decrease the invasive testing rate (from 2000 to 222 per 100 000 women), and the associated pregnancy loss rate compared with the current policy of CST, and also compared with a first line cffDNA approach. This is because the PPV and NPV are higher when cffDNA testing is applied to an intermediate chance group, compared with an unselected or low a-priori chance population. The authors suggested that such a model would reduce the undiagnosed trisomy live birth rate from 32 in 100 000 (CST screening alone) to 10 in 100 000 (contingent model), but this remains less effective than first line cffDNA screening where the rate is expected to be 1 in 100 000. *[Evidence level 2–]*

Two important UK based studies have demonstrated the feasibility, safety and diagnostic performance of a contingent screening policy.[50,51] Gil et al. (2016) examined over 11 000 pregnancies where women were offered CST at 11^{+2} to 14^{+1} weeks of gestation[50]. Those with a high chance result (≥1 in 100, equivalent to 1 in 150 at term) were offered the option of invasive testing, cffDNA testing or no further investigations. Those with an intermediate chance (between 1 in 101 and 1 in 2500) were offered cffDNA or no further investigations. This policy resulted in diagnosis of 91.5% of cases with T21 (43/47) and 100% of cases with T13 or T18 (28/28). One case of T21 was in the low chance group (and therefore not offered further testing). In two cases the mothers were offered, but opted against, further testing; while in one case, the mother had an intermediate chance and received a false negative result from cffDNA testing. Results were highly impacted by parental decision making: within the high chance group, 38% opted for invasive testing while 60% of parents requested cffDNA; in the intermediate chance group, 92% opted for cffDNA. Overall, cffDNA provided a result at first attempt in 97.3% of women, with repeat testing successful in 63% of initial failed results, such that cffDNA results were available in 98.7% of women. The introduction of cffDNA resulted in a 43% reduction in the rate of invasive testing in the high chance group. *[Evidence level 2+]*

Chitty et al. examined over 30 000 women in the multi-centre RAPID (Reliable, Accurate, Prenatal non-Invasive Diagnosis) study.[51] Women with a high chance (≥1 in 150) after CST or QUAD testing were offered the option of invasive testing, cffDNA testing or no further investigations. Implementation of this contingent model was shown to be feasible and also significantly decreased the FPR associated with CST or QUAD screening. Modelling suggested that, for an estimated 698 500 births in England, the policy would decrease invasive tests undertaken (3368 fewer investigations; 95% CI 2279–4027) and fewer procedure related miscarriages (n = 17; 95% CI 7–30), while detecting an additional 195 pregnancies with T21 (95% CI 34–480), at no additional cost to the NHS. Extensive qualitative work conducted as part of the RAPID study showed that women considered to be at a higher chance of

carrying an aneuploid fetus are often motivated to proceed with cffDNA testing as they consider it to be a safe test, comparing favourably to the miscarriage risk associated with diagnostic testing.[53] *[Evidence level 2+]*

A contingent model offering cffDNA testing within the NHS in England is currently being implemented as part of an evaluative roll-out undertaken by the NHS Fetal Anomaly Screening Programme (FASP).[18] cffDNA testing is offered to all pregnant women and people with a higher chance result (between 1 in 2 and 1 in 150) from either CST or QUAD testing in singleton and twin pregnancies. The evaluative roll out ended in June 2024 and the findings are expected to further inform NHS policy in the future.

4.2.3 Women with a previous history of T21, T18 or T13

The availability of cffDNA testing is of relevance to pregnant women who have a previous pregnancy with T21, T18 or T13. These women may be sufficiently reassured with a negative result of a cffDNA screen, without having to undergo an invasive test, and are offered this as a first line option (National Genomic Test Directory's R445 pathway). *[Evidence level 4]*

4.2.4 The importance of first trimester ultrasound

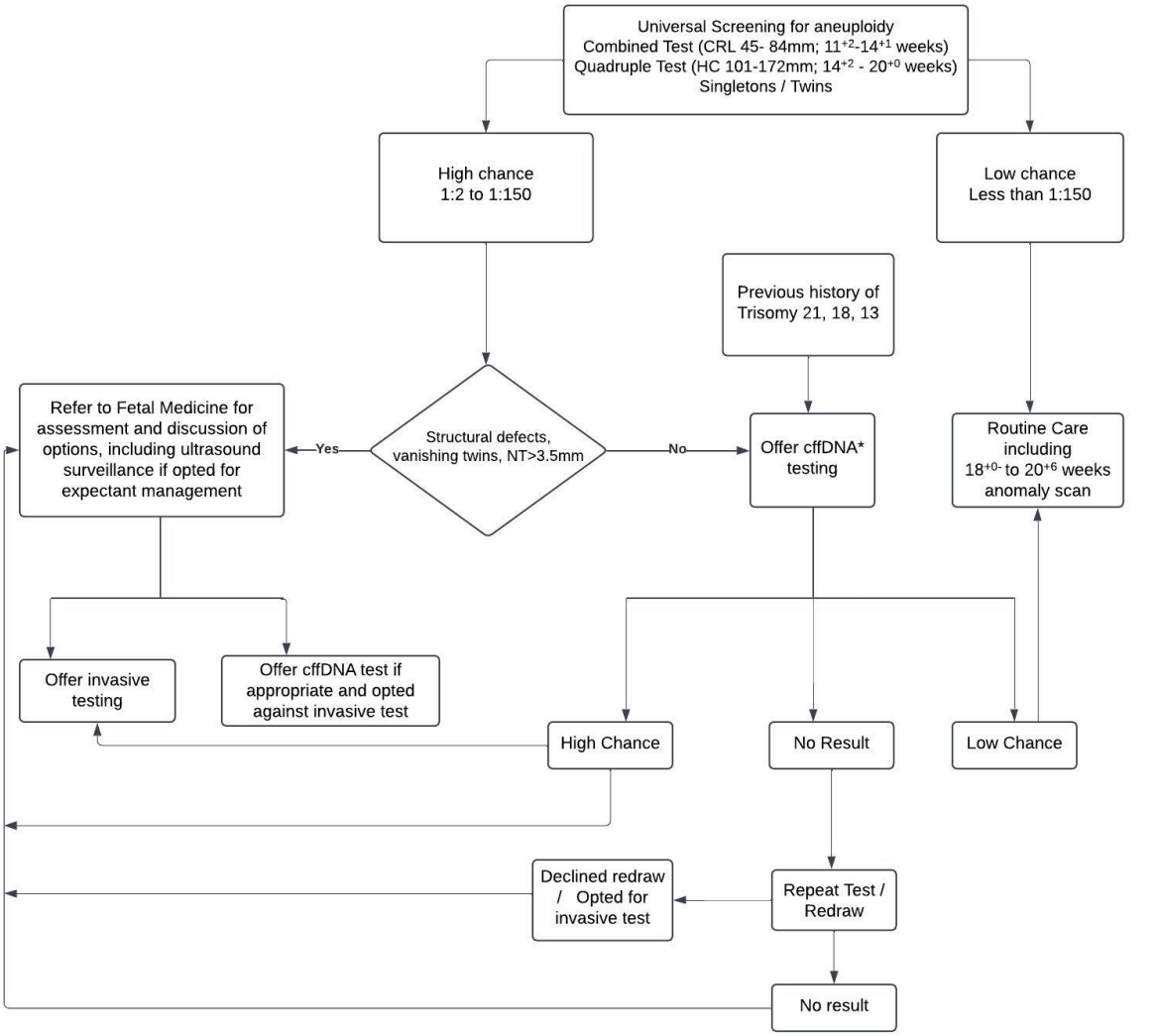
Ultrasound assessment prior to undertaking cffDNA testing is essential to ensure screening is appropriate. Thus, findings of a vanishing twin, multiple pregnancy, fetal anomalies, a fetal nuchal translucency of ≥3.5 mm or incorrect dating all impact the accuracy and choice of testing. In a retrospective cohort study of 2337 women with advanced maternal age, Vora et al. demonstrated that an ultrasound offered prior to cffDNA testing between 10 and 14 weeks of gestation would have altered management in 16.1% of women because of incorrect dating or findings of a fetal anomaly, multiple gestation or non-viable pregnancy.[31] Similarly, in an unselected population (n = 6250), Brown et al. reported that 9.6% of pregnancies undergoing cffDNA testing had pre-test ultrasound findings that would alter management for similar reasons.[54] *[Evidence level 2+]*

Current screening for first trimester CST includes first trimester ultrasound with measurement of NT. In many centres this also includes an anatomical survey undertaken at 11^{+2} to 14^{+1} weeks of gestation. It is important to ensure that a universal cffDNA screening policy does not remove the benefit of such ultrasound. Findings such as raised fetal NT (≥3.5 mm) or a major fetal anomaly (in particular, holoprosencephaly, exomphalos, major cardiac anomaly or megacystis) are associated with fetal aneuploidy and such women should be offered the option of invasive diagnostic testing.[55,56] This is because invasive testing is not only diagnostic for T21, T18 and T13, but also allows for more detailed genetic analysis. Benachi et al. studied women at high chance of fetal aneuploidy who obtained negative results with cffDNA testing and found that the presence of a fetal anomaly or raised NT was associated with an 8% chance a chromosomal anomaly other than T21, T18, T13.[57] Bardi et al. examined fetuses with NT above the 99th centile and found that 30% had a chromosomal condition, 2% with single gene disorders and 2% with submicroscopic conditions.[58] *[Evidence level 2+]*

Useful data in this regard also come from Kagan et al. who conducted a randomised controlled trial including 1518 women receiving either (i) CST or (ii) cffDNA testing in combination with first trimester detailed ultrasound examination and showed that women in group (ii) had a significantly lower FPR than those screened with CST only (0% *vs* 2.5%).[59] *[Evidence level 1+]*

For pregnant women and people undergoing cffDNA testing at early gestations (prior to 11^{+2} weeks), the information available from a pre-test ultrasound assessment is often limited to fetal viability, evaluation of multiple pregnancies and exclusion of vanishing twin pregnancy. For these women, a

second ultrasound should be offered later in the first trimester for the reasons highlighted above. *[Evidence level 4]*



4.3 What do women need to know before choosing to have screening with cffDNA?

Recommendation	Evidence level	Strength	Rationale for the recommendation
All pregnant women should be offered prenatal screening for T21, T18 and T13 and be made aware that the choice to undertake this testing is optional	**D**	**GPP**	Decision making for pregnant women should be based on their understanding of the benefits and risks of screening

Pregnant women should receive pre-test counselling that provides clear information on test objectives, potential benefits and harms of screening, possible test outcomes and screening performance characteristics of cffDNA testing	**D**	**GPP**	Non-directive pre-test counselling is part of best practice
Pregnant women must be informed that cffDNA testing is a form of prenatal screening as opposed to a diagnostic test	**1++**	**A**	Despite the high levels of accuracy, cffDNA testing is associated with false positive and false negative diagnoses

All pregnant women should be offered prenatal screening for T21, T18 and T13 and be made aware that this testing is optional.[60-62] Pregnant women should understand the conditions for which they are having screening and their clinical variability. [62,63] *[Evidence level 4]*

Pregnant women should also understand the tests available to them, what test they are having and what it is for. In many countries, cffDNA testing is offered either as a primary test of universal screening, whereas in others, it is contingent on the results of another prior test such as CST or QUAD test. While cffDNA testing is highly accurate and non-invasive, it requires careful consideration. Pregnant women should have a clear understanding of the test objectives, possible test outcomes and performance characteristics of cffDNA testing.[61-63] The provision of accurate, balanced information by healthcare providers that supports pregnant women in informed decision-making is imperative. [60-62] *[Evidence level 4]*

Pregnant women should understand that they may choose to opt against cffDNA testing and opt for no further testing or invasive prenatal diagnosis. The expected detection rates and FPR should be provided.

Pregnant women should be given a clear understanding of the possible outcomes (and their likelihoods) that may follow cffDNA testing for T21, T18 and T13. These are:

(1) Low chance result
(2) High chance result
(3) No result (for example: test failure, inconclusive or indeterminate result).

Pregnant women must be informed that cffDNA testing is a form of prenatal screening as opposed to a diagnostic test, which means that:

- a low chance result does not exclude the possibility of T21, T18 or T13
- a high chance result after cffDNA testing will lead to an offer of prenatal diagnostic testing (CVS or amniocentesis)
- there are limitations of this test, in particular that screening for T21, T18 and T13 cannot rule out the presence of other severe or lethal genetic conditions or structural anomalies.[61,62]

In some countries, tests based on whole genome sequencing may report incidental findings such as maternal malignancy. In such settings women should be made aware that in a minority of cases, incidental findings regarding maternal health may be reported.

Table 2. Salient points in pre-test counselling.[13,32]

- Give all available options including no further investigations, cffDNA testing or invasive testing.
- Clarify that cffDNA is a screening test and not a diagnostic test (see **table 3** for accuracy rates).
- Describe limitations: testing is only for T21, T18 and T13, possibility of a 'no result' or need for a second sample.
- Review the ultrasound findings and maternal history to ensure it is suitable to perform cffDNA (see **table 4** for exclusions).
- Explain test method.
- Explain how results will be reported.
- Explain that a positive screening test should be confirmed with a diagnostic test.
- Discuss that a 'low chance' result does not exclude all chromosomal conditions or genetic syndromes.
- Explain that if cffDNA has been undertaken, subsequent CST and QUAD testing is not recommended.
- Explain the possibility of incidental findings regarding maternal health.

Adapted from Bianchi and Chiu 2018; FASP 2016;2021.

4.4 What is the screening performance of cffDNA?

Recommendation	Evidence level	Strength	Rationale for the recommendation
Pregnant women and people can be informed that cffDNA has the highest detection rate and lowest FPR among all non-invasive tests available for T21, T18 and T13	**1++**	**A**	Large studies and meta-analyses show that cffDNA testing has high detection rates and low FPR for the detection of T21, T18 and T13
Healthcare professionals should be aware that cffDNA testing using the following methodologies have been validated within the literature and have similar screening performance: massively parallel shotgun sequencing, chromosome selective sequencing and single nucleotide polymorphism	**1++**	**A**	Large studies and meta-analyses show that these different methods have similar performance of screening
Pregnant women and people having cffDNA testing prior to 10^{+0} weeks of gestation and those with increased body mass index (BMI), should be informed that screening test failures are more common	**1++**	**B**	Screening undertaken at early gestational ages and in women with increased BMI have higher failure rates

There is clear evidence from multiple systematic reviews that cffDNA testing has the highest detection rate and lowest FPR available for T21, T18 and T13 among non-invasive tests in singleton pregnancies.[44-47,64] Findings from a meta-analysis, which only included studies with pregnancy outcome data for greater than 85% of their study populations, can be found in **Table 3**.[44] Detection rates and FPR of any test should not be impacted by the prevalence of a condition, and the meta-analysis showed no differences in detection rates and FPR between high chance, mixed chance and unselected populations.[44,46] *[Evidence level 1++]*

Many of the studies included within existing systematic reviews, in particular those without consecutive recruitment strategies, are deemed to have a high risk of bias, and the test performance of cffDNA may not be achievable in the setting of routine population based screening.

There are no studies performing a head-to-head comparison of cffDNA testing using massively parallel shotgun sequencing (MPSS), chromosome selective sequencing (CSS) and single nucleotide polymorphism (SNP) methodologies within the same population cohort. However, sub-group meta-analysis from several independent groups suggests similar screening performance between those studies performing cffDNA with these different laboratory methods.[44-47] *[Evidence level 1++]*

Despite high accuracy, cffDNA must be considered a screening test for T21, T18, and T13, as the circulating cell-free DNA assessed from maternal serum reflects both fetoplacental and maternal DNA (See **section 4.6**). Therefore, all pregnancies with a high chance cffDNA result should be referred for consideration of diagnostic testing to further investigate fetal karyotype (See **section 9**).

4.4.1 Test failures

A test failure occurs when no result is produced after cffDNA testing. This may be secondary to a number of factors, including human error in collection and/or transport of the blood sample, low fetal fraction (typically below 2–4%),[65] assay failure due to failed DNA extraction, amplification or sequencing, or failure of the test to meet laboratory quality assurance standards.[44] The occurrence of indeterminate test results and test failures is poorly reported. Taylor-Phillips et al. determined that rates of test failure range from 0% to 12.7% in studies and that among 5789 women who underwent a second test, this was reported as a repeat test failure in 13.9%.[45] Based on their meta-analysis, Gil et al. found that the most common underlying reason for test failure is a low fetal fraction.[44] Fetal fraction has been found to be reduced in women with raised maternal body mass index (BMI), increased maternal age, assisted conception and gestational age less than 10 weeks.[28,66] *[Evidence level 1++]*

cffDNA test failure is more likely when it is undertaken at less than 10 weeks of gestation due to lower fetal fractions seen with earlier pregnancy.[30] In an analysis of over 22 000 pregnancies, Wang et al. demonstrated that between 10 and 21 weeks of gestation, fetal cffDNA increased by 0.1% per week and beyond 21 weeks, by 1% per week.[29] It should, however, be noted that Norton et al. found no association between test failure and gestational age in their cohort of women tested between 10 and 14 weeks of gestation.[36] In their meta-analysis, Gil et al. reported that there was insufficient published data to explore a relationship between test failure rates and gestational age.[44] *[Evidence level 2+]*

There has been obvious concern regarding the suggestion that the cffDNA failure rate may be higher in aneuploid fetuses compared with euploid fetuses. Evidence has shown that test failures are no more likely to occur in fetuses with T21 than euploid pregnancies (meta-analysis, consensus odds ratio of 0.98, 95% CI 0.62–1.55)[67] *[Evidence level 1+]*

In contrast, test failure rates in fetuses with T18 (8.0%) and T13 (6.3%) were found to be considerably higher than in unaffected pregnancies (2.9%) and in those with T21 (1.9%) in a study of 10,698 fetuses by Revello et al.[66] Evidence from Rava et al. supports this finding, and suggests that fetal fraction (and therefore test failures) varies with fetal karyotype: it is higher in T21, but lower in T18 and T13 when compared with euploid fetuses.[68] *[Evidence level 2+]*

Pregnancies with T18 and T13 (but not T21) have been shown to have smaller placental mass with consequently lower cffDNA fetal fractions, which may explain the higher incidence of test failure.[69] Care for those with a failed test results is discussed in **Section 4.7**. *[Evidence level 4]*

Table 3: Results from systematic review and meta-analysis on screening characteristics for cffDNA testing in singleton pregnancies (Gil et al. 2017).[44]

Condition	Studies Included (n)	Fetuses with the condition (n)	Pooled Detection Rate (%, 95% CI)	Pooled False Positive Rate (%, 95% CI)
Trisomy 21	30	1963	99.7 (99.1–99.9)	0.04 (0.02–0.07)
Trisomy 18	25	563	97.9 (94.9–99.1)	0.04 (0.03–0.07)
Trisomy 13	23	119	99.0 (65.8–100)	0.04 (0.02–0.07)

4.5 What is fetal fraction and what is its significance?

Recommendation	Evidence level	Strength	Rationale for the recommendation
In test failure due to low cffDNA fetal fraction, the presence of T18 and T13 should be considered	**2+**	**B**	Pregnancies with T18 and T13, but not T21, have been shown to have smaller placental mass and lower cffDNA fetal fractions, and a higher incidence of test failure compared with euploid fetuses

Circulating DNA fragments in maternal serum originate from the placenta, the fetus and the mother[32] with cffDNA testing performed on a mixed sample. The proportion of fetoplacental cffDNA to total plasma cell-free DNA is referred to as the fetal fraction. Factors influencing cell-free DNA contributions affect this fetal fraction ratio,[65] which is crucial as insufficient placental DNA cannot provide the information required for an accurate test result.[32,70] Thus, very low fetal fractions are associated with less accurate test results.[44] Some laboratories will measure the fetal fraction and report an inconclusive result if the fraction does not meet a required pre-set threshold (often set at 2–4%) but cut-offs and methodologies vary between laboratories, and measurement can be imprecise. There is little consensus as to whether, or how, fetal fraction should be reported, and how this should be clinically interpreted.[15,65,71-73]

The fetal fraction is influenced by a number of factors including gestational age, maternal weight, assisted reproduction, fetal karyotype and maternal conditions such as autoimmune conditions, use of heparin, and vitamin B12 deficiency. [28,29,66,74-79] *[Evidence level 2+]*

Fetal fraction peaks at 10–20% between 10 and 21 weeks of gestation.[19] While cffDNA is possible from 9 weeks of gestation, the lower limit is determined by individual laboratories as fetal fractions prior to this time may be too low.[30] *[Evidence level 2+]*

Higher BMI is associated with lower fetal fraction due to increased maternal cffDNA release from adipose tissue and dilutional effects due to higher maternal blood volume.[66,74,80] A systematic review found that obese women had higher cffDNA test failure rates compared with normal weight pregnant women.[80] *[Evidence level 2++]*

Ashoor et al. reported fetal fractions below 4% increased from 0.7% at 60 kg to 7.1% at 100 kg and 51.1% at 160 kg.[28] *[Evidence level 2+]*

IVF pregnancies show lower fetal fraction and increased chance of test failure compared with naturally conceived pregnancies[75], although the underlying mechanism (placental *versus* maternal) remain unclear.[28,66] *[Evidence level 2+]*

As explained above, fetal fraction may also be lower in fetuses with T18 and T13 resulting in a greater likelihood of test failure in this population compared with euploid fetuses and fetuses with T21.[66,68,74,81] *[Evidence level 2+]*

The available evidence associating reduced fetal fraction with maternal autoimmune conditions, thromboembolic disease, vitamin B12 deficiency and use of heparin consists mainly of case study reports and while the biological plausibility of these associations has been demonstrated, they have not been studied or reported in larger scale prospective studies.[76-79,82] *[Evidence level 3]*

4.6 What are the patient factors known to impact the performance of cffDNA testing?

Recommendation	Evidence level	Strength	Rationale for the recommendation
Prior to undertaking cffDNA screening, pregnant women and people should be asked about their personal medical history and should not be offered the test if they meet exclusion criteria (see table 4)	**2+**	**B**	Certain maternal conditions can potentially affect the accuracy of cffDNA screening
Women should be counselled regarding the possibility of confined placental mosaicism, which may influence screening results	**2+**	**B**	Confined placental mosaicism can potentially affect the accuracy of cffDNA screening for T18 and T13

Despite high accuracy, there are factors that impact the test accuracy of cffDNA testing. The influence of biological factors such as CPM, true fetal mosaicism, vanishing twin syndrome, maternal copy number variations (CNVs) and maternal pathology can lead to false positive and false negative results.[32,83] Understanding these is essential for ensuring appropriate candidate selection and providing women with comprehensive pre-test and post-test counselling.[60-62] Important maternal conditions that need to be considered or where cffDNA testing should not be performed are listed in **Table 4**. Most common causes of false positive and false negative results after cffDNA testing are summarised in **Table 5**.

4.6.1 Maternal factors

Most cell-free DNA in the maternal plasma is maternal in origin, derived from maternal apoptotic haematopoietic cells (70–90%).[19,84] Therefore, maternal conditions impacting the release and the quality of circulating cell-free DNA into maternal plasma have the potential to influence cffDNA test results leading to false positive or false negative results, or suggest an abnormality that is not fetal in origin.[19,83] *[Evidence level 3]*

Maternal medical conditions can impact cffDNA test results either due to the condition itself or secondary to indicated treatments that could impact quantity, metabolism and quality of circulating maternal DNA fragments. Thus, abnormal cffDNA profiles have been reported in association with severe maternal vitamin B12 deficiency, autoimmune conditions such as systemic lupus erythematosus, and intrahepatic cholestasis of pregnancy. Maternal treatment with low molecular weight heparin is associated with decreased placental apoptosis and may increase the likelihood of receiving a failed test result secondary to low fetal fraction.

Other maternal factors, such as obesity, assisted conception and increased maternal age have also been shown to impact fetal fraction and likelihood of test failure[28,29,66,74-79,82,85] (see **Section 4.5**). *[Evidence level 2+]*

Although maternal malignancy during pregnancy is rare, the release of apoptotic cell-free tumour DNA has been reported in the setting of haematologic, gynaecological and solid organ malignancies.[86-91] In this context, whole genome based sequencing methods may detect a genome-wide imbalance that is misinterpreted as fetal aneuploidy, leading to a false positive cffDNA testing result. *[Evidence level 2+]*

Similarly, benign uterine leiomyomas have been associated with discordant results, although most are small with limited blood supply and therefore are unlikely to impact the majority of sequencing methods targeting T21, T18 and T13.[83,92] *[Evidence level 3]*

Maternal mosaicism for autosomal aneuploidies may cause a discordant cffDNA result. In the setting of genome-wide, as opposed to targeted sequencing methodologies, CNVs of maternal origin may also impact cffDNA testing results.[83,93-95] *[Evidence level 2+]*

NHS FASP has produced guidance for England as part of their cffDNA evaluative roll-out highlighting the maternal conditions that need to be considered, or where cffDNA testing should not be performed, due to the higher risk of erroneous results (exclusion criteria checklist), described in **Table 4**.[13]

4.6.2 Fetal factors

Screening results of cffDNA can be impacted by the death of a fetus in a multiple pregnancy (referred to as vanishing twin), which is estimated to occur in 0.42% of pregnancies.[32] A dichorionic twin placenta releases two individual components of cffDNA into the maternal circulation, both of which are analysed as part of cffDNA testing. Fetoplacental DNA originating from an aneuploid vanishing twin could therefore cause a false positive screening result for a live euploid fetus. Few data are available to understand how long after demise a vanishing twin placenta might contribute to maternal plasma cffDNA.[96] However, an aneuploid fraction that is significantly lower than fetal fraction may point to this condition and SNP technologies now allow for the detection of an extra fetal haplotype, which is discussed in more detail in **Section 8**.[97] Given the impact of vanishing twin syndrome on cffDNA testing results, a fetal ultrasound should always be conducted prior to the offer of cffDNA testing in order to exclude this condition. *[Evidence level 2+]*

4.6.3 Placental factors and confined placental mosaicism

The placenta is composed of the outer cytotrophoblast layer and the inner mesenchyme.[19,20] In cases of confined placental mosaicism the cytotrophoblast may contain an abnormal cell line that is not present in the fetus causing a false positive cffDNA result.[98,99] *[Evidence level 2+]*

Confined placental mosaicism affects 1–2% of pregnancies.[100] occurring less frequently for T21 than for T18 and T13 (2%, 4% and 22% of all cases).[101] This in part explains why the PPV for cffDNA testing for T21 is higher than for T18 and T13. *[Evidence level 2+]*

Where QF-PCR is performed on chorionic villi, results are interpreted by the relevant accredited laboratory in line with the Association for Clinical Genomic Science (ACGS) guidance.[102] In addition, a karyotype analysis should be undertaken following a diagnosis of aneuploidy to identify structural rearrangements; if identified, parental bloods should be taken to assess recurrence risk. *[Evidence level 4]*

Given that some of the false positive results from cffDNA testing will be due to CPM, amniocentesis is a superior diagnostic test than CVS in this setting as this tests fetal rather than placental cells. However, amniocentesis is considered safe to perform only after 15 weeks of gestation, whereas CVS can typically be performed from 11 weeks of gestation. The risk of a false positive result from CVS in the setting of CPM occurs if only the cytotrophoblast is analysed. Given the evidence described above, CVS for high chance T21 screening results with analysis of both cytotrophoblast cells and mesenchymal cells is appropriate and should be offered (see ACGS guidelines).[98, 101] In cases with high chance screening results for T18 and T13, a detailed ultrasound assessment is essential to look for fetal anomalies. If anomalies are seen, CVS can be offered, but in the absence of anomalies, amniocentesis is the most appropriate diagnostic test. See **Section 9** for further details and recommendations. *[Evidence level 4]*

Table 4: Exclusion criteria for cffDNA testing based on maternal medical history.[13]

Exclusion Criteria	Rationale
Active cancer (unless in remission)	A cancerous tumour may release cell-free DNA into maternal plasma affecting the screening result
History of blood transfusion received in the previous four months prior to testing	Donor DNA received as part of a prior blood transfusion may be present in the plasma of the recipient
Previous bone marrow or organ transplant	Donor DNA may be present in the plasma of the recipient
Immunotherapy in the current pregnancy (excluding intravenous immunoglobulin)	Possible effect on cffDNA fragmentation patterns
Stem cell therapy	Dependent on whether mother has received her own stem cell or transfusion from a donor
Down syndrome or a balanced translocation or mosaicism of T21, T18 or T13 in the pregnant woman	Impacts the relative proportion of maternal and cffDNA

Table 5: Common causes of false positive and false negative cffDNA testing results (Adapted from Bianchi et al. 2018).

False Positive NIPT results	**False Negative NIPT results**
• Confined placental mosaicism (placenta aneuploid, fetus euploid) • Vanishing Twin • Maternal incidental findings - Copy-number variant - Chromosome abnormality - Monosomy X (Turner syndrome, 45,X) or trisomy X (triple X syndrome, 47,XXX) - Mosaic autosomal trisomy • Benign tumours, e.g. uterine leiomyoma • Malignancy - Hodgkin's or non-Hodgkin's lymphoma - Other lymphomas (follicular, cutaneous T cell) - Breast cancer - Colorectal cancer - Chronic myelogenous leukemia - Multiple myeloma - Other cancers: neuroendocrine,	• Confined placental mosaicism (placenta euploid, fetus aneuploid, or mosaic) • Low fetal fraction - Maternal obesity - Multiple gestation causing low fetal fraction per fetus - Maternal medical condition or treatment affecting quality of circulating DNA - Certain fetal chromosomal aneuploidies (e.g. triploidy)

angiosarcoma, small-cell carcinoma. • Previous organ or bone marrow transplant • Recent blood transfusion (less than four months) • Medical condition or treatment affecting quality of circulating DNA - Autoimmune disease (some) - Immunotherapy (excluding intravenous immunoglobulins) - Vitamin B12 deficiency - Intrahepatic cholestasis of pregnancy (severe)	

4.7 What is the recommended care pathway in cases where cffDNA testing fails to yield a result?

Recommendation	Evidence level	Strength	Rationale for the recommendation
When cffDNA fails to yield a result, a detailed review of the pregnant woman's or pregnant person's initial a-priori chance and indications for cffDNA testing should be undertaken	**4**	**GPP**	Review of the a-priori chance will allow for a detailed discussion about further testing options
The possible care options and their advantages and disadvantages should be discussed in detail with the woman. A repeat blood test for cffDNA analysis will yield a result in about two-thirds of cases	**1++**	**A**	Repeat cffDNA testing will yield a result in the majority of cases
If there is a repeat test failure, or if the pregnant woman opts against repeat testing, referral should be offered to fetal medicine for assessment of fetal anatomy and further detailed discussion. Guided by the fetal anatomical assessment, an individualised plan should be made and options include (i) CST or QUAD test depending on gestational age if not previously performed; (ii) no further testing; (iii) diagnostic invasive testing	**4**	**GPP**	Repeat cffDNA testing will yield a result in the majority of cases. Repeated test failure may indicate higher chance of abnormalities in the pregnancy. Alternative management strategies are appropriate in this situation and should be discussed with the woman

Evidence from a number of studies demonstrates that test failure happens relatively infrequently and, in this setting, a repeat blood test will yield a reliable result in the majority of cases. In the TRIDENT-2 study no result was available in 1.5% of cases in the first instance.[43] The test was repeated with a new sample in 90% of these women with 0.02% requiring a second re-draw. Of the women with an initial report of 'test failure', a conclusive result was issued in 86% of cases. *[Evidence level 2++]*

This is consistent with analysis from a large systematic review from Gil et al. whose findings suggest that when cffDNA fails to yield a result, a repeat test will provide a result in ≥60% of cases[44]. In a systematic review of 18 cohort studies focused only on T21, Palomaki et al. demonstrated that 83% of women with an initial failure submitted a subsequent sample and of these, 79% returned a usable

result suggesting that two-thirds of initial failed tests will be rectified with repeat sampling.[67] *[Evidence level 1++]*

5. Expanding the use of cffDNA testing beyond the detection of T21, T18 and T13

5.1 Fetal sex

Recommendation	Evidence level	Strength	Rationale for the recommendation
Offering cffDNA testing to determine the fetal sex is recommended when there is a medical indication	**1++**	**A**	cffDNA testing has a high PPV regarding fetal sex determination and can mitigate the need for an invasive procedure
Reporting of fetal sex is not currently recommended outside of a medical indication	**4**	**GPP**	There are potential ethical, societal and cultural challenges

cffDNA can be used to screen for fetal sex with a detection rate of 96.6–98.9% and FPR of 0.4–1.1%, using PCR technology to assess presence of Y chromosome sequences (**Table 6**).[33,46,103-105] In sex-linked conditions such as Duchenne Muscular Dystrophy or Haemophilia, cffDNA is used clinically to reduce the need for invasive testing.[105,106] cffDNA testing for sex selection is anecdotal[103] and the Nuffield Council on Bioethics recommends that non-medical NIPT sex determination is not recommended.[60,107] Ultimately the full impact of routine prenatal sex determination is yet to be fully elucidated with potential societal, cultural and ethical impacts.[15,108,109] *[Evidence level 1++]*

5.2 Sex chromosome aneuploidies

Recommendation	Evidence level	Strength	Rationale for the recommendation
cffDNA testing for sex chromosome aneuploidy is not recommended	**2–**	**C**	The clinical requirements of a screening test are not fulfilled
Where a result suggests a high chance of a sex chromosome aneuploidy, referral to a fetal medicine specialist and subsequent clinical geneticist are recommended	**4**	**GPP**	Due to high FPRs, detailed ultrasound assessment and careful choice of diagnostic invasive test are advisable as well as detailed post-test counselling based on cohorts that are prenatally diagnosed

Sex chromosome aneuploidies (SCA) are relatively common. They occur in about 1 in 400 newborns, although most remain undiagnosed.[110] Sex chromosomes are more vulnerable to aneuploidy and mosaicism than the autosomes.[110] The PPV for SCA using cffDNA testing is approximately 50% with similar results across meta-analyses of general risk populations: monosomy X, Klinefelter syndrome (XXY), trisomy X (XXX) and XYY syndrome are 29.5%–32.0%, 67.6%–74.5%, 53.9%–57.5% and 70.9%–74.5%, respectively (**Table 6**).[103,111] This is predominantly secondary due to a higher FPR than for the common aneuploidies, which may be associated with an undiagnosed maternal SCA or CPM.[46,112,113] *[Evidence level 2–]*

Guidance and expert opinion regarding SCA screening is inconsistent: it is currently supported in guidelines of the American College of Obstetricians and Gynecologists, the American College of Medical Genetics and Genomics, the International Society for Prenatal Diagnosis and the Royal Australian and New Zealand College of Obstetricians and Gynecologists.[16,11,112,114] *[Evidence level 2–]* However, there are also important challenges around SCA screening, including:

(i) There is no clear benefit to diagnosis of a SCA prenatally over postnatally. While there is an argument that advanced knowledge allows early multi-disciplinary intervention to improve long-term outcome, at present this is speculative.[15,112,115-120]

(ii) There is often no detection of a corresponding phenotype on ultrasound with the exception of monosomy X with a cystic hygroma.[15]

(iii) The postnatal phenotype can be highly variable in relation to physical and developmental issues, making prognosis challenging.[121]

(iv) There are ethical implications in relation to indirect sex selection, the potential for unnecessary invasive testing based on an uncertain result, and challenges as to whether grounds for termination of pregnancy are met. It has been proposed, however, that prenatal knowledge of SCA does not impact on rates of termination of pregnancy.[122] *[Evidence level 2–]*

The phenotype in individuals diagnosed prenatally may be milder than those diagnosed postnatally, hence counselling should be based on studies of similar populations.[115] Counselling should be provided by a team including a clinical geneticist and fetal medicine subspecialist. Mosaicism and structural rearrangements are common in SCA, hence confirming a diagnosis and follow-up are also key, with the need for a detailed first trimester anatomy scan, offering a diagnostic test (an amniocentesis is preferable where there is no identifiable fetal structural anomaly, to overcome the issue of CPM) and appropriate follow-up.[115,123] *[Evidence level 2–]*

5.3 Copy Number Variants

Recommendation	Evidence level	Strength	Rationale for the recommendation
cffDNA testing for detection of copy number variants is not currently recommended	**1–**	**B**	The PPV is low and there is a paucity of robust evidence regarding clinical utility

Expanded cffDNA testing has the ability to assess all chromosomes genome-wide, detecting sub-chromosomal imbalances such as CNVs, including microdeletions, microduplications and unbalanced translocations.[33] These imbalances affect 1–1.7% of the population, independently of maternal age. While individually rare they are collectively common; the most prevalent is 22q11.2 microdeletion (DiGeorge) syndrome, seen in up to 1 in 3000 livebirths.[124] While most commercially available platforms will detect pathogenic CNVs at a resolution of greater than 7Mb [125], most clinically relevant syndromes present with pathogenic CNVs of less than 5Mb.[126] Many test panels target the five commonest microdeletion syndromes using SNP analysis.[127] CNV syndromes often have variable penetrance, which makes prognosis counselling challenging.[33] *[Evidence level 1–]*

The pooled PPV for fetal segmental CNVs ranges from 37.5%–44.1% (95% CI 30.6–44.8) with high heterogeneity (I^2=93.9%–98.9%, **Table 6**).[128,129] Meta-analyses are limited by pooled PPV that combine all CNVs of variable prevalence, and do not account for low or high prior risk or testing approach. Additionally, delayed postnatal diagnosis may underestimate the false negative rate. In combination with the rarity of CNVs individually, current evidence remains insufficient to recommend screening. *[Evidence level 1–]*

In studies offering expanded cffDNA as a first-tier screening test, a Dutch study reported 41 (1.6%) chromosome aberrations identified in 2527 screened pregnancies; 33 of these had follow-up, and 10 of the 33 were confirmed postnatally (PPV 30.3%).[131] In a Belgian study, a segmental imbalance was detected in 109 (0.07%) of 153,575 screened pregnancies. Of the 93 with postnatal follow-up, 43 were confirmed true positives (PPV 46.2%).[42] *[Evidence level 1–]*

Most individual CNV studies focus on 22q11.2 microdeletion.[126,132,133] The largest, the SMART study, assessed over 20,000 women using a SNP-based cffDNA approach and found a prevalence of 1 in 1524 pregnancies; a PPV of 52.6% (95% CI 28.9–75.6%) with a detection rate of 83% (95% CI 51.6–97.9%) for a 0.05% FPR.[124] *[Evidence level 1–]*

5.4 Rare autosomal trisomies

Recommendation	Evidence level	Strength	Rationale for the recommendation
cffDNA testing for rare autosomal trisomies (RATs) is not currently recommended	**1–**	**B**	There is a paucity of evidence regarding test performance with a pooled PPV of <10%
Where there is a high chance result for a RAT, a detailed fetal anatomy scan, amniocentesis +/- extended methylation studies are recommended with counselling provided by a clinical geneticist	**4**	**GPP**	There is a high risk of CPM. If autosomes 6, 7, 11, 14, 15 or 20 are affected there is a risk of an imprinting disorder

Full trisomies of autosomes outside 21, 18 or 13 are termed rare autosomal trisomies (RATs) and are lethal in most cases, with a high risk of missed miscarriage. The prevalence in unselected pregnancies ranges from 0.12–0.36%.[33,113,134] Those pregnancies that continue are predominantly mosaic, with the majority associated with CPM.[100,135] In over 90% of cases, RATs affect chromosomes 2, 3, 7, 8, 9, 12, 14, 15, 16, 20 and 22, with trisomy 7 the most commonly detected RAT using cffDNA testing.[136] Where mosaicism occurs, affecting an imprinting region in chromosomes 6, 7, 11, 14, 15 or 20, there can be subsequent uniparental disomy (both chromosomes inherited from one parent), which can lead to phenotypic effects and autosomal recessive condition in the fetus.[112,137] All mosaic RATs have variable clinical impact dependent on a range of molecular findings. Where there is true fetal mosaicism, counselling is challenging due to a wide range of potential phenotypes of variable severity.[137,138] *[Evidence level 1–]*

RATs can be detected using genome wide cffDNA testing with a frequency of 0.12–0.95% dependent on the population screened.[134] The pooled PPV for RAT detection using cffDNA testing is 9% (95% CI 2.5–18.8%) based upon a meta-analysis of five studies.[139] There is a paucity of high quality evidence to accurately ascertain detection rate and FPR (**Table 6**).[139,140] As each mosaic RAT is so heterogeneous, and due to challenges with quantifying the degree of clinical impact, reporting of RAT and subsequent invasive testing can only be justified if there is sufficient evidence that this has the potential to improve care during pregnancy and its outcome.[136,137] Where there is a high chance result of a RAT using cffDNA and no fetal structural anomaly on scan, the recommended diagnostic invasive test is an amniocentesis, as over 97% of cases detected are due to CPM.[100] Where there is a trisomy of chromosomes 6, 7, 11, 14, 15 or 20, further methylation studies should be performed to assess for the presence of uniparental disomy.[15,138] *[Evidence level 1–]*

5.5 Single gene disorders

Recommendation	Evidence level	Strength	Rationale for the recommendation
Offering cffDNA testing for single gene disorders is recommended in high-risk pregnancies such as those where parent(s) are known carriers for a condition, when there has been advanced work-up by a clinical geneticist and the relevant genomic laboratory	**2++**	**B**	Robust evidence to demonstrate the effectiveness of this form of testing. The result is regarded as diagnostic, negating the need for an invasive test
Offering cffDNA testing for single gene disorders in low-risk pregnancies is not currently recommended	**2–**	**C**	There is no evidence to demonstrate the PPV in low-risk pregnancy

Using technology similar to NIPT, two primary technological approaches are available based on the inheritance pattern of a condition: bespoke-PCR and relative haplotype or mutation dosage analysis (see **Glossary**).[141] This has been demonstrated to be effective in those at high risk e.g. where there is a suspected phenotype of a skeletal dysplasia on ultrasound when the *FGFR* (fibroblast growth factor receptor) NIPD panel can be applied, or where parents are carriers for a known genetic condition.[141-143] In these cases the technique is regarded as diagnostic and confirmatory invasive testing is not required while reproductive choices and opportunities for fetal therapy are optimised. This has been an accredited service within two laboratories in the UK for over a decade and requires prospective clinical genetic approval.[33,141,144,145] *[Evidence level 2++]*

With the discovery of long cffDNA fragments, commercial tests are now being developed to screen for single gene disorders in low-risk pregnancies, most notably a selection of conditions of an autosomal dominant nature, with or without prior carrier screening with reflex single gene cffDNA testing.[33,144,146-148] There are technical and analytical challenges related to gene coverage and detection of maternal somatic mosaicism. Due to the rarity of single gene disorders, compounded by the limited follow-up of prenatally screened cases and combination of high and low-risk populations when using a cffDNA testing, the PPV in a truly low-risk population is as yet unknown (**Table 6**).[148] In contrast to high-risk populations, where the performance is known, a single gene diagnosis suspected on cffDNA testing in low risk populations makes the offer of a diagnostic invasive test essential to facilitate counselling.[144,149,150] *[Evidence level 2++]*

Table 6: Detection rate, specificity and positive predictive value of expanded use of cffDNA testing in unselected populations.

	Cases (n)	Detection rate % (95% CI)	Specificity % (95% CI)	Positive predictive value % (95% CI)
Fetal sex[104]	6541	94.4% (94.7–96.1%)	98.6% (98.1–99.0%)	98.8%
Sex chromosome aneuploidy[111]	1,531,240	94.1% (90.8–96.3%)	99.5% (99.0–99.7%)	49.4% (45.8–53.1%)
Copy number variants[128]	1,591,459	77.4% (65.7–86.0%)	99.4% (98.0–99.8%)	37.5% (30.6–44.8%)

Rare autosomal trisomies[139]	1703	87.2–100%	90.7–99%	11.46% (7.80–15.65%)
Single gene disorders	Unknown			

Based on the largest meta-analyses to date reporting pooled sensitivity, sensitivity and PPV. [CI, confidence interval provided where available.]

6. Clinical scenarios and advised tests for further screening and/or diagnosis

Clinical Scenario	Recommendation	Evidence level	Strength
Previous history of T21, T18 or T13	Offer cffDNA testing	4	GPP
Assisted conception after preimplantation genetic testing for aneuploidy (PGT-A)	Offer cffDNA testing as for all other pregnant women (i.e. via a contingent or universal approach).[16,179-181] Where there is a high chance result for T21, T18 or T13 following cffDNA testing, the PPV is reduced following a normal PGT-A result[179]	2+	C
CST High chance result (between 1 in 2 and 1 in 150) for T21, T18 or T13 with a normal NT	For T21 offer either no further testing, or cffDNA testing, or diagnostic invasive testing (CVS) For T18 or T13, refer to fetal medicine for detailed anatomical assessment. The majority of fetuses with T18 or T13 will have a structural anomaly, and pregnant women may wish to opt directly for invasive testing to confirm the diagnosis, rather than having cffDNA. In addition, no further testing or cffDNA testing are appropriate if the pregnant woman wishes to avoid diagnostic invasive testing, or would not consider a termination of pregnancy. If a structural anomaly is not identified, offer: - No further testing - cffDNA in accordance with NHS FASP NIPT operational guidance. However, if this shows a high chance result for T18 or T13, the correct diagnostic test is an amniocentesis because of the risk of CPM. This recommendation is not part of the NHS FASP pathway at present, and will be reviewed when the three year evaluative roll out has been completed and data analysed - Diagnostic testing. CVS or amniocentesis following ACGS recommendations.[102] If associated with a PAPP-A <0.415 MoM, consider 150mg once daily aspirin and fetal growth monitoring [51,162-170]	2++	B
QUAD test high chance result (between 1 in 2 and 1 in 150) for T21	Offer the options of no further testing, cffDNA testing or a diagnostic invasive test (amniocentesis)[13,35,171-174]	1++	A

Fetal anomalies detected by ultrasound at any gestation, irrespective of the first trimester screening result		Refer to fetal medicine for anatomical assessment. Offer a diagnostic invasive test if appropriate.[175-177] cffDNA is not a recommended first line test where there is a fetal anomaly[178] but in the presence of anomalies associated with a high chance of T21, T18 or T13 where invasive testing has been declined, cffDNA testing may be discussed with careful counselling to give information to pregnant women and clinicians	2++	B, D, GPP
Women who have had cffDNA testing with a low chance result and a subsequent finding of:	Increased NT ≥3.5 mm	Refer to fetal medicine for anatomical assessment and offer invasive testing (QF-PCR +/- chromosomal microarray analysis +/- exome sequencing (dependent on ultrasound findings)[155,156]	1++	A
	Fetal anomalies detected by ultrasound	Refer to fetal medicine for anatomical assessment. Offer a diagnostic invasive test if appropriate[175-177]	2++	B
	High chance result from CST or QUAD test	If due to biochemistry, reassure as test performance of cffDNA testing for fetal aneuploidy is superior to CST and QUAD.[64] However, very low PAPP-A and low or high beta-hCG can be associated with an increased chance of triploidy (undetectable by most cffDNA tests) and referral to fetal medicine should be considered.[182] If PAPP-A <0.415 MoM consider 150mg once daily aspirin and fetal growth monitoring[46,51,162-170]	1++	A
		If PAPP-A or hCG MoM <0.1 offer referral to fetal medicine to assess for potential digynic triploidy, which is not detectable by cffDNA		GPP

7. Caring for women with a high chance cffDNA test result who choose not to have further testing

Recommendation	Evidence level	Strength	Rationale for the recommendation
Pregnant women and people who want to avoid a diagnostic test and would wish to continue a pregnancy with T21, T18 or T13 may still value the information a cffDNA test can give them. This is a valid choice and must be respected	**4**	**GPP**	Detailed counselling and personalised care should be offered to women and their wishes respected

Evidence suggests that many women undertake cffDNA testing to aid with preparations with caring for a child with T21, T18 or T13. There are already recommendations based on a consensus statement on pregnancy screening from the Royal College of Obstetricians and Gynaecologists, Royal College of Midwives, Society and College of Radiographers *Supporting women and their partners through prenatal screening for Down's syndrome, Edwards' syndrome and Patau's syndrome*.[183] These explain in detail that women and their partners with a higher chance cffDNA result may not want an amniocentesis or CVS because of the risk of miscarriage, as they would not want to terminate pregnancies found to be affected, or other reasons. They should be signposted to additional support and information about continuing their pregnancy and receive the enhanced scans and multi-

disciplinary team support that a confirmed diagnosis pregnancy would warrant. Good documentation should record informed decisions on further testing decisions. In addition, pregnant women should be able to change their minds, but not be pressured into doing so, with decisions accepted and respected at all times.[60,61] *[Evidence level 4]*

8. cffDNA in the setting of multiple pregnancy

8.1 How to interpret NIPT results for twin pregnancies

Recommendation	Evidence level	Strength	Rationale for the recommendation
cffDNA testing may be offered to women or pregnant people with twin pregnancy	**1++**	**A**	cffDNA testing performance for T21 in twin pregnancies is good. Data are less robust for T18 and T13
cffDNA testing for T21 may be offered in women with twin pregnancies via a contingent [chance on CST or QUAD screening between 1 in 2 and 1 in 150] or a first-line screening approach	**4**	**GPP**	There is no evidence to support one approach over the other; clinical limitations of biomarker and ultrasound screening in twin pregnancy may strengthen the argument for first-line cffDNA testing
As with singleton pregnancy, presence of a structural fetal abnormality or a high chance cffDNA test result should be followed by a referral to fetal medicine for detailed anatomical ultrasound assessment, and appropriate prenatal testing should be considered, depending on the gestation and the wishes of the woman	**2++**	**B**	Where an anomaly is detected, diagnostic invasive testing is preferable
As with singleton pregnancy, when the cffDNA gives 'no result', a redraw should be considered	**2++**	**B**	Test failure rates are greater in twin pregnancy cffDNA testing than in singletons. This is commonly due to inadequate fetal fraction. Redraw successfully yields a result in over 50% of cases

There is growing evidence that the performance of cffDNA as a screening tool for T21 in twin pregnancies is similar to singleton pregnancies (**Table 7**),[198] and superior to first trimester CST and second trimester QUAD testing. Theoretically, in monochorionic twins (which are always monozygotic and therefore genetically identical), cffDNA testing will perform equal to or better than in singletons.[184] In dichorionic twins, most of which are dizygotic, fetal fractions between twins are not equal, particularly where one placental mass is smaller such as in T18, T13 or triploidy.[185,186] This may account for false negatives, or a higher failure rate or 'no result'. *[Evidence level 2++]*

Various factors affect the test performance of cffDNA in twin pregnancy. The fetal fraction of cffDNA in maternal plasma for women with twin pregnancies is higher, but less than two-fold, compared with singleton pregnancies.[187,188] For monozygotic twins, the same fetal fraction cut-offs as for singleton

pregnancies are often adopted. Studies have shown variable failure rates in twins from 1.6–13.2%, with a median of 3.6%, higher than for singletons. The median success rate on redraw are approximately 50% (range 14.3–83.3%)[187,189-196] *[Evidence level 2++]*

Meta-analyses of the screening performance of cell-free DNA in twin pregnancies at 10^{+0}–14^{+1} weeks are summarised in **Table 7** and demonstrate high detection rates and low FPR for T21.[197] *[Evidence level 1++]*

Since this meta-analysis, a multicentre blinded study in the UK evaluated the screening performance of cffDNA in twin pregnancies using NGS.[190] Data were available from 961 twin pregnancies including 276 monochorionic and 685 dichorionic. The mean fetal fraction was 12.2% (range, 3–36%) and the detection rate for T21 was 100% (95% CI 75–100). For T18 the detection rate was 82% (n=22; 95% CI 66–93) with a FPR of 0.08% (n=4869; 95% CI 0.02–0.18%). There was a single pregnancy affected by T13, which was detected. Using pooled data from 11 other studies, the authors concluded that the detection rate for T21 was 95% (n=74; 95% CI 90–99) with a FPR was 0.09% (n=5598; 95% CI 0.03–0.19). A number of more recent studies in twin pregnancies have shown similar results. *[191,196,199] [Evidence level 1++]*

While the performance for cffDNA testing for T21 in twins is well established, the performance of cffDNA testing in T18 and T13 is more difficult to ascertain owing to the low number of cases of T18 and T13 reported in the literature.[44,190,197] This is particularly the case for T13: there are just 11 cases of T13 in the literature, meaning that the pooled weighted detection rate (94.7%) is associated with wide confidence intervals (95% CI 9.14–99.97%).[197] *[Evidence level 2++]*

cffDNA test failure in twin pregnancies is higher than in singleton pregnancies. Khalil et al. reported a test failure rate of 0.31%.[190] Another study by the Fetal Medicine Foundation assessing 928 twin pregnancies reported a higher chance of test failure or 'no result' in dichorionic compared with monochorionic twins or singleton pregnancies (OR, 1.75; 95% CI 1.34–2.26). They also noted a higher test failure in pregnancies conceived by in-vitro fertilisation than in those conceived naturally (OR, 3.82; 95% CI 3.19–4.55). The authors concluded that the risk of test failure is higher in dichorionic twin than in singleton pregnancies, mainly because of the higher proportion of twins being conceived by in-vitro fertilisation and more nulliparity in this cohort.[198] *[Evidence level 1+]*

Table 7: Results from systematic review and meta-analysis on screening characteristics for cffDNA testing in singleton pregnancies (Gil et al. 2019).[198]

Condition	Studies included (n)	Fetuses with the condition (n)	Pooled detection rate (%, 95% CI)	Pooled false positive rate (%, 95% CI)
Trisomy 21	8	56	98.2 (83.2–99.8)	0.05 (0.01–0.26)
Trisomy 18	5	18	88.9 (64.8–97.2)	0.03 (0.00–0.33)
Trisomy 13	3	3	66.7%	0.19

8.2 Can cffDNA be offered in a twin pregnancy in the setting of a single empty sac or vanishing twin syndrome?

Recommendation	Evidence level	Strength	Rationale for the recommendation
There is a higher chance of a false positive result with a vanishing twin, and therefore cffDNA testing in the presence of a vanishing twin is not recommended	**2+**	**C**	Insufficient evidence to recommend an optimal gestation where fetal DNA from the demised twin is no longer detectable
Where a vanishing twin is suspected prior to or following the time of cffDNA sampling the laboratory should be notified	**4**	**GPP**	Such clinical information is important for reporting and interpretation

A vanishing twin refers to an empty second gestational sac with or without a fetal pole that has no cardiac activity, typically in the first trimester.[200,201] It is estimated to occur in up to 39% of IVF twin pregnancies.[196,200,202,203] As the demised fetus is more likely to be aneuploid it may lead to a false positive cffDNA result for the remaining singleton.[200] *[Evidence level 2+]*

In instances of vanishing twin in the first trimester there is a higher FPR of (2.6% versus 0.3% in singleton pregnancy). [200,201,202] Fetal DNA from the vanished twin can still be detected within the maternal circulation from 8–15 weeks following demise.[200,201] Resampling after 15 weeks of gestation improved the accuracy of test results in the presence of a vanishing twin in another study, with the FPR falling from 5.2% to 0.8% either side of 14 weeks of gestation identified. There is, however, insufficient evidence to recommend cffDNA in clinical practice in this scenario.[202] *[Evidence level 2+]*

Given cffDNA is not recommended, in this instance aneuploidy screening is usually performed using NT measurements and maternal age, as serum biomarker screening results can be impacted leading to a falsely high PAPP-A result and potential false negative result (https://www.gov.uk/government/publications/fetal-anomaly-screening-programme-handbook/289099d7-ded0-43be-a901-600b78fb727e#screening-in-twin-pregnancies).[200, 202]

8.3 How should a high chance cffDNA result be managed in twin pregnancies?

For further guidance on selecting the most appropriate diagnostic test in twin pregnancies, including considerations for chorionicity, invasive testing techniques, and post-test counselling, please refer to **Section *9.2***: *What is the optimal diagnostic test in twin pregnancies?*

8.4 Is there a role for cffDNA testing in triplets and higher order pregnancies?

Recommendation	Evidence level	Strength	Rationale for the recommendation
cffDNA testing cannot currently be recommended for aneuploidy screening in triplet or higher order multiple pregnancy	**2–**	**D**	There is limited evidence demonstrating cffDNA testing test performance with high failure rates

There is limited evidence available regarding cffDNA performance in triplet or higher order multiple pregnancy due to the rarity of such pregnancies compounded by low rates of aneuploidy, with many studies including triplets failing to include any cases of aneuploidy.[195] Failure rates have been reported

at 16.5–21.3% primarily due to an insufficient fetal fraction.[203,204] *[Evidence level 2–]*

8.5 Is there a role for cffDNA testing in multiple pregnancy discordant for NT (≥3.5 mm) or structural fetal anomaly?

Recommendation	Evidence level	Strength	Rationale for the recommendation
cffDNA testing is not recommended in multiple pregnancies when there is increased NT (≥3.5 mm), or affected by a fetal structural anomaly, whether concordant or discordant	**4**	**GPP**	Such cases should be referred to fetal medicine for pre-test counselling, and individualised care (usually depending on chorionicity)

NT aneuploidy screening in monochorionic twins has a higher FPR than in dichorionic twins, as elevated NT can indicate early twin-twin transfusion syndrome, and discordance in NT affects about 20% of twin pregnancies.[205,206] In monochorionic twin pregnancy, discordant karyotypes (heterokaryotypia) are rare and the prevalence of discordant genotype beyond aneuploidy is unknown.[207] Invasive testing offers the option of chromosome microarray, which should be offered in the presence of an increased NT measurement.[15,153-155] *[GPP]*

In a twin pregnancy the quoted risk of miscarriage with invasive testing is higher than in singleton pregnancy (around 1%), although more recent evidence is conflicting around this statistic. Women with twin pregnancy who request cffDNA testing in the first instance to mitigate the risk of miscarriage must be counselled regarding the limitations. [12] No studies have assessed the cffDNA performance in discordant NT in twins; individualised pre-test counselling with a fetal medicine specialist with expertise in twin pregnancy is required. *[GPP]*

Twin pregnancies have a higher incidence of fetal anomaly, up to five times greater in monochorionic than dichorionic twins.[207] Structural anomaly in twins should prompt referral to a regional fetal medicine unit for counselling regarding the options, which will include the appropriate gestation for invasive testing and a discussion around selective reduction. While some structural anomalies are associated with a greater incidence of aneuploidy, ultrasound does not reliably detect all anomalies.[208] Women with discordant anomaly who opt against invasive testing can consider cffDNA testing with detailed counselling on limitations.[115] *[GPP]*

9. Follow-up care after a high chance cffDNA test result and post-test counselling

9.1 What is the optimal diagnostic test for singleton pregnancies?

Recommendation	Evidence level	Strength	Rationale for the recommendation
A confirmatory invasive diagnostic test should be offered where there is a high chance result on cffDNA testing for T21, T18 or T13	**1++**	**A**	Due to the potential for false positive results with cffDNA testing, post-test counselling should be performed, and an invasive test should be offered

Where there is a high chance NIPT result for T21 a CVS can be performed with careful interpretation of the result	**4**	**D, GPP**	Interpretation by an accredited laboratory (UK accreditation service (UKAS) to ISO 15189: 2022 standards) of the QF-PCR normal or triallelic abnormal result is diagnostic in this instance
Where there is a high chance result for T18 or T13 and a significant fetal anomaly is present a CVS can be performed with careful interpretation of the result	**4**	**D, GPP**	Accredited laboratory interpretation of the QF-PCR triallelic abnormal result is diagnostic in this instance; the detection of fetal anomalies on ultrasound would be expected in these conditions. Interpretation by an accredited laboratory to ISO 15189: 2022 standards) of the QF-PCR normal or triallelic abnormal result is diagnostic in this instance
Where presence of a suspected confined placental mosaicism is the cause of a discrepant CVS result, clinical genetics should be consulted	**4**	**D, GPP**	Clinical genetics will aid in interpretation of the findings and mitigate relating a falsely positive diagnostic test result
Where there is a high chance result for T18 or T13 and no significant structural anomaly is evident, the recommended diagnostic test of choice is an amniocentesis	**4**	**D, GPP**	Structural fetal anomalies are typically evident where there is T18 or T13 hence for a high chance result, there is a suspicion of CPM and amniocentesis is preferred over CVS
Where the presence of confined placental mosaicism is suspected, fetal growth surveillance is recommended	**2++**	**B**	CPM is associated with an increased risk of fetal growth restriction

High chance cffDNA results should be interpreted in combination with a first trimester anatomy scan to determine the optimal choice of confirmatory invasive testing (CVS or amniocentesis [see below]). This is particularly important where women are considering termination of pregnancy but less so if results are for 'information only'.[15] *[Evidence level 1+]*

Where a high chance cffDNA result has been detected, this should prompt referral to a fetal medicine centre or specialist with expertise in detailed fetal anatomical assessment and facilities for invasive testing. The benefits and risks of invasive testing should be discussed as well as the procedure and turnaround time. [15,115,210] *[Evidence level 4]*

Where correct laboratory protocols adhering to ACGS guidance are used, CVS can be relied upon for confirmatory diagnosis of T21, T18 and T13 although recommendations regarding the choice of confirmatory invasive test are conflicting.[15,211,215,216] *[Evidence level 4]*

In T21 the risk of mosaicism in a CVS following a high chance cffDNA screen is 2% and a triallelic pattern on QF-PCR with or without the presence of a fetal anomaly is regarded as diagnostic.

Fetal structural anomalies are common in T18 and T13. [217] In addition, in T18, T13 (as well as 45,X) the risk of CPM is much greater with rates of mosaicism in CVS following a high chance cffDNA testing result of 4%, 22% and 59%, respectively. [101,112] Therefore in instances of no structural anomaly, amniocentesis after 15 weeks of gestation should be considered as opposed to CVS. [12,101,112, 217]

Where CPM is suspected, awaiting the long term culture or karyotype (turnaround time 2–3 weeks) assessing both cytotrophoblast and mesenchymal core is recommended as the QF-PCR result (turnaround 2–3 days) may be representative of cytotrophoblast only, essentially the same source as the cffDNA.[5,101, 102,112,138, 211,--214] *[Evidence level 4]*

Where CPM is suspected, regular monitoring of fetal growth is recommended due to the risk of associated fetal growth restriction and fetal loss, and this is most notable in CPM affecting chromosomes 2, 3, 7, 13, 15, 16 and 22.[215,216,218,219] *[Evidence level 4]*

9.2 What is the optimal diagnostic test in twin pregnancies?

Recommendation	Evidence level	Strength	Rationale for the recommendation
When a result indicates a high chance for T21 in a twin pregnancy, even without the presence of a fetal abnormality, it is reasonable to proceed to CVS with careful interpretation of the result and post-test counselling awaiting a long-term culture result where feasible	**4**	**D, GPP**	A triallelic pattern on QF-PCR analysis for T21 should be evident on the electropherogram
Where there is a high chance result for T18 or T13 and no significant structural anomaly is evident, the recommended diagnostic test of choice is an amniocentesis	**4**	**D, GPP**	Structural fetal anomalies are typically evident where there is T18 or T13 hence for a high chance result, there is a suspicion of CPM and amniocentesis is preferred over CVS

Performance of invasive testing in multiple pregnancy should be in keeping with RCOG Green-top Guideline no. 8.[12] Counselling and subsequent care should be undertaken by a fetal medicine specialist with expertise in managing complex multiple pregnancies, including invasive testing and selective fetal reduction. *[Evidence level 4]*

The approach may differ dependent upon chorionicity. Due to the 1% risk of contamination, where there is discordancy between ultrasound findings and cffDNA testing results, it is reasonable to wait and perform an amniocentesis of both sacs at 15 weeks of gestation.[189,220] Discordant genotypes are more likely in dichorionic twins and are rare in monochorionic twins (the risk of heterokaryotypia in monochorionic twin pregnancy is rare and in the majority of scenarios both or either fetus are aneuploid).[221] CVS can be considered where there is a high chance T21 result or T18 or T13 result in the presence of structural anomalies, ensuring both placentas are sampled via a double uterine entry technique. A one pass technique traversing the level of the intertwin membrane can be considered in

monochorionic twins, although it is not apparent that the number of needle insertions increase the risk of fetal loss.[222] Ultimately operators should use the technique with which they are most comfortable.[12] Awaiting a long-term culture from CVS is preferable although if this will lead to challenges regarding risk of a later selective reduction, careful counselling is required to facilitate decision making.[220,223,224] *[Evidence level 4]*

9.3 What is the role of support organisations in this setting?

Recommendation	Evidence level	Strength	Rationale for the recommendation
Both pre- and post-test counselling for cffDNA testing should include signposting to relevant support organisations and resources	**2+**	**C**	Accurate, timely, non-directive information can optimise autonomous decision making

At the time of pre- and post-test counselling, pregnant women and people should be signposted to relevant support organisations, as well as the option of information delivery via a range of techniques such as web-based education videos, telehealth and computerised decision aids.[15] This is particularly important in the setting of a high chance cffDNA testing result where couples may retain little information from the consultation and need reliable, accurate aids in a timely fashion to review to optimise informed decision making regarding further prenatal testing.[225,226] The Nuffield Council on Bioethics presents three primary principles around their ethical approach to NIPT, one being that: *'Pregnant women and couples should have access to NIPT within an environment that enables them to make autonomous, informed choices'*.[60] This is particularly important in women with inadequate health literacy or a previous history of T21, T18 or T13.[227] It is important that counselling offered is non-directive and accurate emphasising communication and commitment to patient values.[228,229] It has been demonstrated that optimising knowledge in relation to prenatal testing has been shown to reduce decisional conflict, without increasing the level of worries or anxiety.[226,227] This is a particularly important in relation to counselling where there is the potential of a false positive result where uncertainty can significantly impact on decision making and where in instances where there has been poor-quality pre-test counselling, women may not recognise the possibility of a false positive result.[225,227] *[Evidence level 2+]*

Pregnant women and people with a high risk result following cffDNA testing or a confirmed diagnosis of T21, T18 or T13, may benefit from consultation with organisations such as Antenatal Results and Choices (ARC), Down's Syndrome Association, Support Organisation for Trisomy 18, 13 and Related Disorders (SOFT UK) and Twins Trust (where appropriate, in multiple pregnancies) (see **Section 12**). *[Evidence level 4]*

10. Recommendations for future research

- Further studies are required to understand the performance of cffDNA aneuploidy screening in twin, triplet and higher order multiple pregnancies, as well as in the setting of a diagnosed vanishing twin pregnancy.
- Studies performing head-to-head comparisons of cffDNA aneuploidy screening test approaches (e.g. MPSS versus targeted SNP and microarray) in relation to test failure and performance would be of benefit.
- The test performance of cffDNA screening for monogenic conditions requires further investigation.

- Larger cohort studies are required to investigate the sensitivity and specificity in the detection of submicroscopic chromosomal rearrangements (i) as 'panels' (i.e. a list of specific microdeletions/rearrangements) or (ii) agnostic.
- Further studies exploring parental views on the rare event of false positives and false negatives results would be of benefit.

11. Auditable topics

In order to evaluate the provision of services and support the undertaking of high-level of care, the following auditable standards have been developed to align with those of the FASP cffDNA screening roll out (NHS England)[18] and include:

- The proportion of pregnant women and people eligible for cffDNA testing for whom a conclusive screening result is available.
- The proportion of all cffDNA samples received in the genomic laboratory ≤2 working days.
- The proportion of cffDNA test results reported within 5 working days of sample receipt.
- The proportion of pregnant women and people with higher chance or 'no result' results attending an appointment within 3 working days to discuss their results.
- The proportion of invasive prenatal diagnostic procedures offered within 3 working days to women receiving a higher chance or 'no result' NIPT screening results.

12. Useful links and support groups

Antenatal Results and Choices (ARC): www.arc-uk.org
Down's Syndrome Association: www.downs-syndrome.org.uk
Support Organisation for Trisomy (SOFT UK): www.trisomy.org
The Twins Trust: www.twinstrust.org

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Please note the references will be formatted later in the guideline's development.

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Appendix A: Explanation of grades and evidence levels

Classification of evidence levels

1++	High-quality meta-analyses, systematic reviews of randomised controlled trials or randomised controlled trials with a very low risk of bias
1+	Well-conducted meta-analyses, systematic reviews of randomised controlled trials or randomised controlled trials with a low risk of bias
1–	Meta-analyses, systematic reviews of randomised controlled trials or randomised controlled trials with a high risk of bias
2++	High-quality systematic reviews of case–control or cohort studies or high-quality case–control or cohort studies with a very low risk of confounding, bias or chance and a high probability that the relationship is causal
2+	Well-conducted case–control or cohort studies with a low risk of confounding, bias or chance and a moderate probability that the relationship is causal
2–	Case–control or cohort studies with a high risk of confounding, bias or chance and a significant risk that the relationship is not causal
3	Non-analytical studies, e.g. case reports, case series
4	Expert opinion

Grades of Recommendation	
A	At least one meta-analysis, systematic reviews or RCT rated as 1++, and directly applicable to the target population; or a systematic review of RCTs or a body of evidence consisting principally of studies rated as 1+, directly applicable to the target population and demonstrating overall consistency of results
B	A body of evidence including studies rated as 2++ directly applicable to the target population, and demonstrating overall consistency of results; or Extrapolated evidence from studies rated as 1++ or 1+
C	A body of evidence including studies rated as 2+ directly applicable to the target population, and demonstrating overall consistency of results; or Extrapolated evidence from studies rated as 2++
D	Evidence level 3 or 4; or Extrapolated evidence from studies rated as 2+
Good Practice Points	
GPP	Recommended best practice based on the clinical experience of the guideline development group.*

*on the occasion when the guideline development group find there is an important practical point that they wish to emphasise but for which there is not, nor is there likely to be any research evidence. This will typically be where some aspect of treatment is regarded as such sound clinical practice that nobody is likely to question it. These are marked in the guideline, and are indicated by GPP. It must be emphasised that these are NOT an alternative to evidence-based recommendations, and should only be used where there is no alternative means of highlighting the issue.

Appendix B. Glossary of Terms

Amplification A technical process prior to sequencing to produce multiple copies of a specific DNA sequence

Aneuploidy The occurrence of one or more extra or missing chromosomes within the organism

Bespoke cffDNA testing This platform uses polymerase chain reaction – next generation sequencing. It can be used where the fetus is at risk of an autosomal dominant condition which is *de novo* or paternally inherited (e.g. achondroplasia) or an autosomal recessive condition where parents carry different variants i.e. compound heterozygous (e.g. cystic fibrosis). Here, bespoke cffDNA testing can be applied with sequencing using a PCR-based targeted amplicon enrichment for paternal exclusion testing. The method is based on the fact that such variants are absent from the maternal fraction of cffDNA. This requires workup prior to a pregnancy and knowledge of previous fetal and parental genotypes

Chorionicity The number of placentas in a multiple gestation e.g. dichorionic

Chromosome selective sequencing Next generation sequencing (see below) of specific chromosomes

Confined placental mosaicism Where the placenta and fetus have a different genetic makeup to each other

Copy number variations A variation in the number of copies of a particular sequence of DNA present in the genome of an individual e.g. microdeletion or microduplication

Euploid An organism with an exact multiple of the haploid number of chromosomes; 23 pairs in the case of humans (22 pairs of autosomes and one pair of sex chromosomes)

Fetal fraction The ratio of cffDNA to all circulating cell-free DNA in the maternal plasma

Genotype The genetic constitution of an individual organism

G-banding karyotype A cytogenetic test involving Giemsa staining of chromosomes during metaphase and subsequent identification of each chromosome by its characteristic banding pattern

Long cell-free DNA fragments These fragments are typically greater than 500bp and with developing technologies their identification and sequencing could facilitate a more optimal fetal fraction as well as an extended scope for cffDNA screening over and above the standard approach[230]

Long term culture The cultivation of chorionic villi or amniocytes so that all cell lineages are expressed, which is important in the detection of mosaicism

Massive parallel shotgun sequencing A high-throughput method used to determine a portion of the nucleotide sequence of an individual's genome

Mb A megabase is a unit of measurement used to help designate the length of DNA. One megabase is equal to 1 million bases

Microarray hybridisation approach to cffDNA testing This applies a targeted approach with amplification of cffDNA fragments using polymerase chain reaction and subsequent hybridisation to a slide containing reference DNA regions and subsequent measurement of fluorescent probes

Next generation sequencing approach to cffDNA testing Using massive parallel sequencing millions of cell free fetal DNA fragments undergo reading of their DNA code and are mapped to the loci on the

chromosomes where they should be located. This can be targeted assessing chromosomes 13, 18 and 21 or genome-wide assessing all chromosomes including imbalances to the resolution of 7Mb. This approach can also detect maternal chromosomal imbalances and potentially maternal malignancies

QF-PCR Quantitative fluorescence polymerase chain reaction is a rapid test used most commonly in the prenatal setting to detect common aneuplodies

Relative haplotype dosage and relative mutation dosage analysis A haplotype refers to a set of genetic markers (in this case single nucleotide polymorphisms) along a chromosome that tend to be inherited together. Where a maternally inherited variant may be present in the proband causing an autosomal recessive disorder where both parents carry the same variant (homozygous) or the disorder is X-lined, a linkage based approach using sequencing to determine which haplotypes the fetus has inherited can be utilised via Bayesian statistical modelling. The same assay can be utilised by all families at risk of a specific disorder without need for bespoke work-up but utility can be limited in consanguineous unions. Relative mutation dosage uses a similar approach with direct measurement of the relative abundance of the pathogenic variant of interest in the cffDNA sample relative to fetal fraction, but negates the need for a paternal or proband sample and remains under evaluation. Examples of where these approaches have been used include spinal muscular atrophy and Duchenne muscular dystrophy

Rare autosomal trisomy Trisomies (three copies of a chromosome) other than those involving the chromosomes 13, 18, 21, X and Y

Sex chromosome aneuploidy A group of chromosome disorders characterised by the loss or gain of one or more sex chromosomes e.g. monosomy X or Turner syndrome

Single gene disorder A disease caused by a known alteration or mutation in one or more genes in nearly every cell in the body

Single nucleotide polymorphism (SNP) approach to cffDNA testing SNPs represent regions in the DNA which vary within the population. This approach can compare the difference between maternal and fetal DNA and the relative dosage differences are determined. This approach has the advantage of being able to determine zygosity in twin pregnancy, screen for triploidy and certain chromosomal imbalances

Triallelic Interpretational term used to describe the appearance of three peaks representing the detection of three markers for a given chromosome on the electropherogram following QF-PCR analysis

True fetal mosaicism A biological phenomenon that indicates the presence of two or more chromosomally different cell lines within a fetus arising from a single zygote

Uniparental disomy A phenomenon where both members of a chromosome pair are inherited from one parent, and the other parent's chromosome for that pair is missing. This is associated with genetic diseases known as imprinting disorders e.g. Angelman syndrome

Vanishing twin syndrome When one or multiple embryos demise in utero and become resorbed partially or entirely, with an outcome of a spontaneous reduction of a multiple to a singleton pregnancy

This guideline was produced on behalf of the Royal College of Obstetricians and Gynaecologists by: **Dr J Karim, Oxford; Professor MD Kilby, Birmingham; Dr F Mone, Belfast; Dr S Nanda, London; Professor K Nicolaides, London; Ms J Fisher, ARC; Professor T Shakespeare, London; Professor P Pandya, London and Professor AT Papageorghiou, Oxford.**

Peer reviewers: XX

Committee lead reviewers were: Dr M Savvidou, Dr K Navaratnam, Dr MA Ledingham MRCOG, Glasgow;[1] Dr SJ Pierce MRCOG, Leeds;[2] Dr R Bahl MRCOG, Bristol[3]

[1]until December 2021; [2]until May 2023; [3]from January 2022; [4]from June 2023.

The Co-Chairs of the Guidelines Committee were: Dr. A Campbell FRCOG, Edinburgh;[1] Miss L Knight MRCOG, Devon;[1] Dr. N Potdar FRCOG, Leicester;[2] Mr. A McKelvey MRCOG, Norwich;[2] Dr. MA Ledingham MRCOG, Glasgow;[3] and Dr. B Magowan FRCOG, Melrose.[4]

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The final version is the responsibility of the Guidelines Committee of the RCOG.

The guideline will be considered for update 3 years after publication, with an intermediate assessment of the need to update 2 years after publication.