

Implementation of maternal blood cell-free DNA testing in early screening for aneuploidies

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ABSTRACT

Objective To explore the feasibility of routine maternal blood cell-free (cf) DNA testing in screening for trisomies 21, 18 and 13 at 10 weeks' gestation.

Method In this prospective study, women attending The Fetal Medicine Centre in London, UK, between October 2012 and April 2013, with singleton pregnancy and live fetus with CRL 32–45 mm, were screened for trisomies 21, 18 and 13 by cfDNA testing at 10 weeks and the combined test at 12 weeks.

Results cfDNA testing was performed in 1005 singleton pregnancies with a median maternal age of 37 (range, 20–49) years. Risks for trisomies were provided for 957 (95.2%) cases and in 98.0% these were available within 14 days from sampling. In 48 (4.8%) cases no result was provided due to problems with delivery to the laboratory, low fetal fraction or assay failure. Repeat sampling was performed in 40 cases and a result obtained in 27 (67.5%) of these. In 11 cases the risk score for trisomy 21 and in five cases that for trisomy 18 was > 99%, in one the risk for trisomy 13 was 34% and in 968 the risk for each of the three trisomies was < 0.01%. The suspected trisomies were confirmed by karyotyping after chorionic villus sampling (CVS), except in one case of trisomy 18 in which the karyotype was normal. On the basis of the maternal age distribution of the study population, the expected and observed numbers for each of the three trisomies were similar. Both cfDNA and combined testing detected all trisomies, but the estimated false-positive rates (FPR) were 0.1% and 3.4%, respectively.

Conclusion Routine screening for trisomies 21, 18 and 13 by cfDNA testing at 10 weeks is feasible and has a lower FPR than does combined testing, but abnormal results require confirmation by CVS. Copyright © 2013 ISUOG. Published by John Wiley & Sons Ltd.

INTRODUCTION

Several externally blinded validation studies in the last 2 years have shown that it is now possible, through analysis of cell-free (cf) DNA in maternal blood, to detect more than 99% of trisomy 21, 98% of trisomy 18 and 89% of trisomy 13 cases, with false-positive rates (FPR) of about 0.1%, 0.1% and 0.4%, respectively^{1–15}. Although most studies were in high-risk pregnancies, we have recently demonstrated that cfDNA testing is applicable not only to pregnancies at high risk for aneuploidies but also to the general population, in which the prevalence of fetal trisomy 21 is much lower¹⁰. Consequently, cfDNA testing is far superior to screening methods that are currently in use for these trisomies, and this will lead to widespread uptake of the test in routine clinical practice.

The best of the currently available methods of screening for trisomies 21, 18 and 13 is the first-trimester combined test, with a detection rate of about 90% for a FPR of 5%¹⁶. For the majority of parents first-trimester screening leads to early reassurance that their fetus is unlikely to be trisomic and for the few with an affected fetus the test provides the option of earlier and safer termination of pregnancy. In addition to effective screening for trisomies, first-trimester testing by ultrasound and biochemistry can identify many major fetal defects and can also lead to the prediction and potential prevention of a wide range of pregnancy complications¹⁷.

There are essentially two options for the introduction of cfDNA testing in screening for trisomies that would allow the advantages of the combined test to be retained in first, diagnosis of aneuploidies within the first trimester, and second, early detection of major defects and prediction of pregnancy complications. The first option is to carry out cfDNA testing together with serum biochemistry at 10 weeks' gestation, with an ultrasound scan at 12 weeks in all women; the second option is to perform cfDNA

testing contingent on the results of the combined test at 12 weeks.

The aim of this study was to explore the feasibility of introducing cfDNA testing in prenatal screening for trisomies according to the first option of maternal blood sampling at 10 weeks and ultrasound scanning at 12 weeks.

SUBJECTS AND METHODS

The data for this study were derived from clinical implementation of cfDNA testing in screening for trisomies 21, 18 and 13 during the 10th gestational week in women with singleton pregnancies attending The Fetal Medicine Centre in London, UK, between October 2012 and April 2013. A two-stage approach to screening was used, with two visits, one at 10 weeks and another at 12 weeks.

Clinical visit at 10 weeks

At 10 weeks' gestation, we recorded maternal characteristics and medical history, provided pretest counseling and obtained written consent for cfDNA testing. An ultrasound scan was carried out to determine if the pregnancy was singleton with a live fetus and to estimate gestational age by measurement of the fetal crown–rump length (CRL). Maternal blood was obtained by venipuncture for measurement of serum pregnancy-associated plasma protein-A (PAPP-A) and free β -human chorionic gonadotropin (β -hCG) (5 mL) using the Kryptor analyzer (Thermo Scientific, Berlin, Germany) and for cfDNA testing (20 mL, in Streck cfDNA BCTTM tubes) using the HarmonyTM Prenatal Test (Ariosa Diagnostics, Inc., San Jose, CA, USA).

Clinical visit at 12 weeks

At 12 weeks' gestation we performed an ultrasound scan to: first, diagnose any major fetal abnormalities; second, measure fetal CRL and nuchal translucency (NT) thickness; and third, assess the nasal bone as being present or absent, the flow across the tricuspid valve as being normal or regurgitant and the a-wave in the ductus venosus as being normal or reversed¹⁶. The maternal serum concentrations of PAPP-A and free β -hCG, measured at 10 weeks, were combined with maternal age, previous history of trisomic pregnancy and the ultrasound findings at 12 weeks to estimate the patient-specific risk for trisomies 21, 18 and 13¹⁸.

Pretest counseling

During pretest counseling it was explained that the cfDNA test is a high-performance screening test rather than a diagnostic test. If the results indicate a high risk for trisomies 21, 18 or 13 this does not mean that the fetus is definitely affected, but the parents should consider fetal karyotyping by chorionic villus sampling (CVS). In contrast, if the results indicate a low risk it is unlikely that the

fetus has one of these trisomies. It was also explained that the analysis of blood would be carried out in the USA and the results would be available at the time of the 12-week assessment, but that in about 5% of cases the test does not give a result and in these cases the risk for trisomies would be determined from the results of the combined test. The parents were informed that the cfDNA test does not provide information on other rare chromosomal abnormalities and if the ultrasound scan at 12 weeks shows a high NT thickness (> 3.5 mm) or major defects it may still be advisable to consider having CVS. Additionally, the cfDNA test does not provide information on physical defects, such as heart or brain abnormalities and spina bifida, and it is therefore advisable that detailed ultrasound scans at 12 weeks and at 20–22 weeks are carried out to examine the fetal anatomy.

Pregnancy management

The protocol for management of the pregnancies is summarized in Figure 1. In cases in which the Harmony Prenatal Test does not provide results, the risks from the combined test are used for counseling. If the cfDNA test indicates a high risk for trisomies the results of the combined test are ignored. If the cfDNA test indicates a low risk for trisomies 21 or 18, irrespective of the estimated risk from the combined test, the parents are reassured that the fetus is unlikely to be affected by these trisomies. In the case of trisomy 13, if the risk from the cfDNA test is low but the risk from the combined test is very high (primarily because of the presence of such defects as holoprosencephaly, megacystis or exomphalos) the parents are advised to consider CVS. Additional actions based on the results of the 12-week assessment include advice on the value of: first, CVS, if fetal NT > 3.5 mm or there are major fetal defects; second, follow-up scans for fetal anatomy, including fetal echocardiography, if there is increased NT > 3.5 mm or abnormal flow across the tricuspid valve or in the ductus venosus; and third, follow-up scans to monitor fetal growth if serum PAPP-A < 0.3 multiples of the median (MoM).

Laboratory analysis

Without any further processing, maternal blood samples were sent via courier to the USA for analysis using a chromosome-selective assay (Harmony Prenatal Test)^{6,7,11}. Risk scores for trisomies 21, 18 and 13 were provided in the test report and these were presented to each patient at the time of the 12-week assessment. The risk scores were represented as a percentage, with ranges capped at $> 99\%$ and $< 0.01\%$.

RESULTS

Study population

During the 7-month study period, 1111 women with presumed singleton pregnancies in the 10th gestational

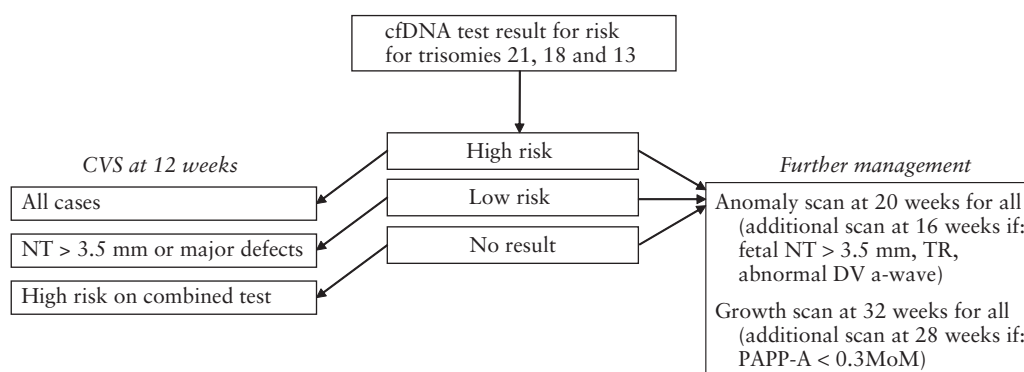


Figure 1 Protocol for pregnancy management according to results of maternal blood cell-free (cf) DNA testing and the combined test. CVS, chorionic villus sampling; DV, ductus venosus; MoM, multiples of the median; NT, nuchal translucency; PAPP-A, pregnancy-associated plasma protein-A; TR, tricuspid regurgitation.

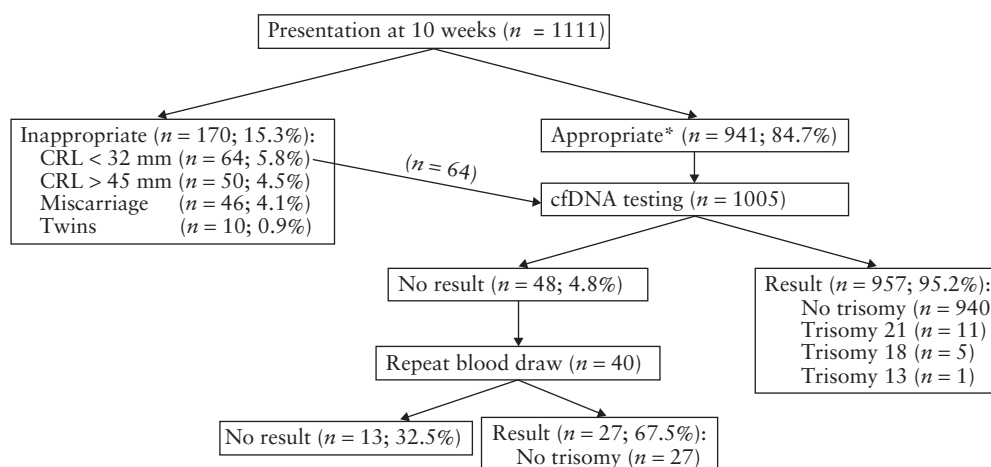


Figure 2 Flow-chart of ultrasound findings at 10 weeks' gestation and results of maternal blood cell-free (cf) DNA testing in women with presumed singleton pregnancies at 10 gestational weeks who requested cfDNA testing. *Appropriate pregnancies were singleton, with a live fetus with crown–rump length 32–45 mm.

week requested cfDNA testing (Figure 2). Ultrasound examination demonstrated a singleton pregnancy in 1101 cases and a twin pregnancy in 10 cases, including five with two live fetuses and five with one live fetus and one empty gestational sac. Of the singleton pregnancies, 46 had either a dead fetus or an anembryonic gestational sac and 1055 had a live fetus, including 941 with fetal CRL 32–45 mm, 64 with CRL < 32 mm and 50 with CRL > 45 mm. The women included in this study ($n = 1005$) were those with a singleton pregnancy and live fetus with CRL 32–45 mm ($n = 941$) and those with CRL < 32 mm who were given a new appointment a few days later, at which the CRL was 32–45 mm ($n = 64$).

The median maternal age of the study population was 36.7 (range, 20.4–48.8) years (Figure 3), the median maternal weight was 62.4 (42.5–133.0) kg and the median CRL at sampling was 38.0 (range, 32.0–45.0) mm. The racial origin of the women was Caucasian in 900 (89.5%), South Asian in 57 (5.7%), East Asian in 22 (2.2%), Afro-Caribbean in eight (0.8%) and mixed in 18 (1.8%). Conception was spontaneous in 861 (85.7%), by *in-vitro* fertilization (IVF) in 117 (11.6%) and after use of ovulation drugs in 27 (2.7%).

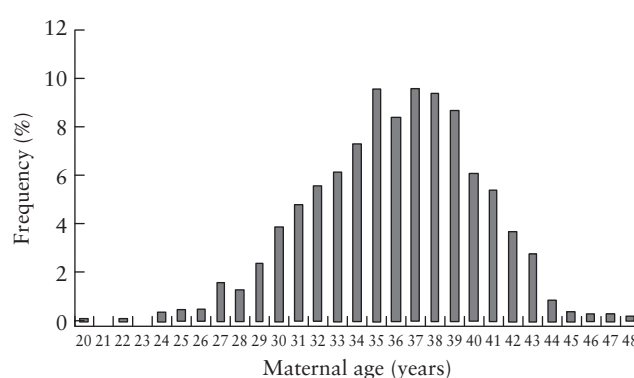


Figure 3 Age distribution of the study population of 1005 women with a singleton pregnancy and live fetus with crown–rump length 32–45 mm who underwent cell-free DNA testing.

Results of cfDNA testing

The median time interval between blood sampling and arrival of the samples at the laboratory was 1 (range, 1–6) days and the interval between blood sampling and receiving results was 9 (range, 6–20) days, with

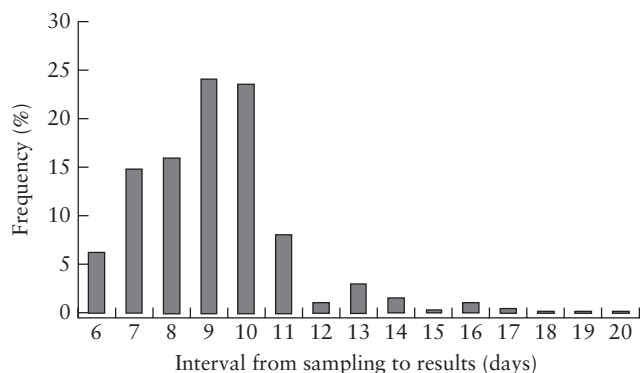


Figure 4 Distribution of time interval in days between blood sampling and obtaining results from maternal blood cell-free DNA testing ($n = 1005$).

985 (98.0%) results being available within 14 days from sampling (Figure 4).

Risk scores from cfDNA testing of the first blood sample were provided for 957 (95.2%) of the 1005 cases. In 48 (4.8%) of the 1005 cases, no result was provided because of, first, problems of blood collection and delivery to the laboratory ($n = 8$; one case of hemolyzed sample, one case in which the sample was not received by the laboratory and six cases in which the interval between sampling and delivery of the sample to the laboratory was 6 days), second, fetal fraction below the minimal requirement of 4% ($n = 23$) and third, assay failure ($n = 17$). In 40 of the 48 cases with no result, a further blood sample was obtained and a risk score was provided in 27 (67.5%), including all seven cases in which on first sampling there

was failure to obtain a result because of problems of blood collection and delivery to the laboratory, in 9 (50%) of the 18 in which on first sampling there was low fetal fraction and in 11 (73.3%) of the 15 in which on first sampling there was assay failure.

Results from cfDNA testing were provided for a total of 984 cases, including 957 from the first draw and 27 from the second draw. In 967 of these the risk scores for each of trisomies 21, 18 and 13 were $< 0.01\%$, in 11 cases the risk score for trisomy 21 was $> 99\%$ and the risk scores for trisomies 18 and 13 were $< 0.01\%$, in five cases the risk score for trisomy 18 was $> 99\%$ and the risk scores for trisomies 21 and 13 were $< 0.01\%$ and in one case the risk score for trisomy 13 was 34% and the risk scores for trisomies 21 and 18 were $< 0.01\%$ (Figure 5).

CVS was carried out in 16 of the 17 cases with a high-risk score for aneuploidies from cfDNA testing; in 15 of these the suspected abnormality was confirmed by cytogenetic analysis and the parents chose to undergo pregnancy termination (Table 1). In one case with a high-risk score for trisomy 21 there was miscarriage after blood sampling for cfDNA testing and before planned CVS for confirmation of results. In one of the five cases with a positive cfDNA test for trisomy 18, both molecular and cytogenetic analysis of chorionic villi demonstrated a normal karyotype. In this case, which was 20 weeks' gestation at the time of writing, detailed ultrasound examination did not show any of the usual sonographic features of trisomy 18.

Trisomy 21 was also diagnosed in a case for which cfDNA testing did not provide a result and CVS was carried out because the risk from the combined test was 1:2.

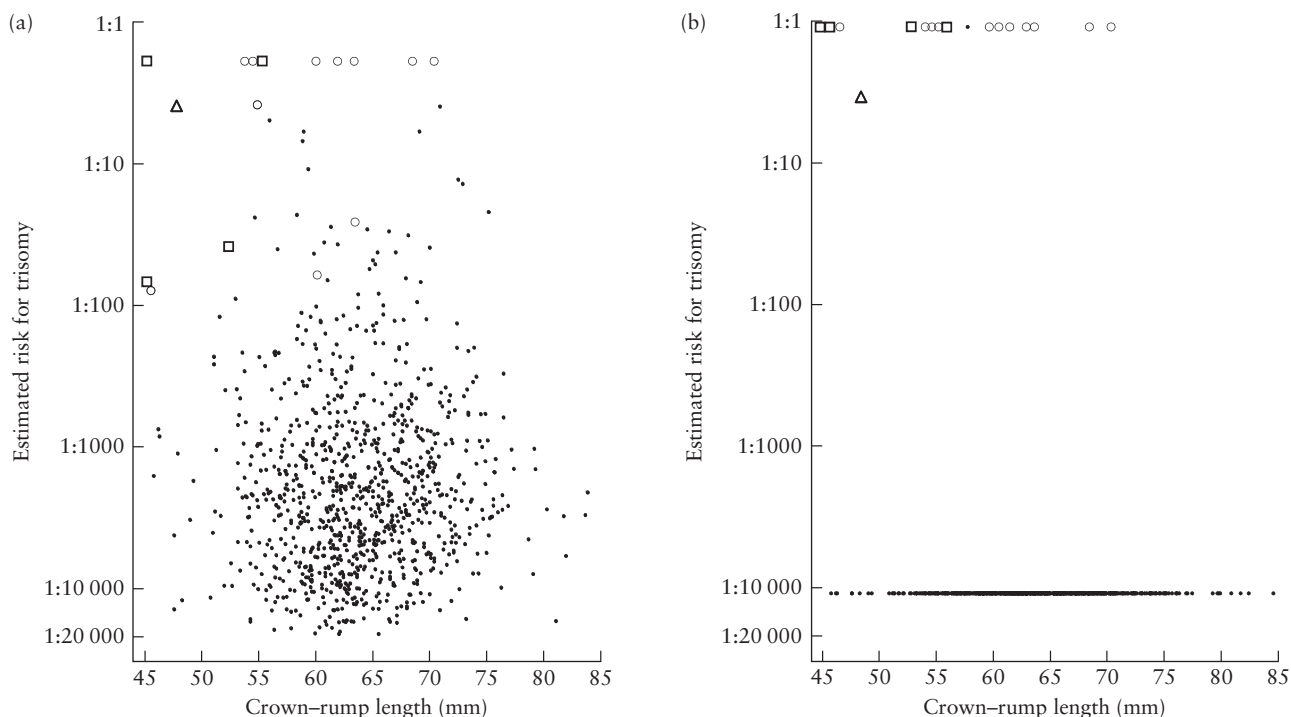


Figure 5 Estimated risk for trisomy in the pregnancies with trisomy 21 (\circ), trisomy 18 (\square) or trisomy 13 (Δ) and assumed euploid fetuses (\bullet), by combined test (a) and maternal blood cell-free DNA test provided at time of 12-week scan (b) ($n = 984$).

Table 1 Findings and outcome in the 17 pregnancies classified by cell-free DNA testing as being at high risk for aneuploidy

Cell-free DNA risk	Combined test risk	Karyotype	Pregnancy outcome
T21 risk > 99%	1:2	T21	TOP
T21 risk > 99%	1:2	T21	TOP
T21 risk > 99%	1:2	T21	TOP
T21 risk > 99%	1:2	T21	TOP
T21 risk > 99%	1:2	T21	TOP
T21 risk > 99%	1:2	T21	TOP
T21 risk > 99%	1:2	T21	TOP
T21 risk > 99%	1:4	T21	TOP
T21 risk > 99%	1:27	T21	TOP
T21 risk > 99%	1:81	Not done	Misc
T21 risk > 99%	1:65	T21	TOP
T18 risk > 99%	1:2	T18	TOP
T18 risk > 99%	1:2	T18	TOP
T18 risk > 99%	1:39	T18	TOP
T18 risk > 99%	1:71	T18	TOP
T18 risk > 99%	1:5861	Normal	Cont*
T13 risk 34%	1:4	T13	TOP

* Continuing at time of writing. Cont, continuing; Misc, miscarriage; T, trisomy; TOP, termination of pregnancy.

Results of combined screening

In 49 (5.0%) of the 984 cases with a cfDNA result, the estimated risk for trisomy 21 at the time of screening derived from the combined test (maternal age, fetal NT and serum free β -hCG and PAPP-A) was above the risk cut-off of 1:100, which is considered by the UK National Screening Committee as the cut-off for classifying pregnancies as high-risk (Figure 5).

In all 11 cases in which cfDNA testing gave a risk for trisomy 21 > 99%, in four of the five cases in which the test gave a risk for trisomy 18 of > 99% and in the one case in which the test gave a risk for trisomy 13 of 34%, the estimated risk from the combined test was more than 1:100. In the case with a positive cfDNA test for trisomy 18 but normal karyotype on CVS, the estimated risk from the combined test was less than 1:100.

Performance of screening

Invasive testing and fetal karyotyping was carried out in 37 of the 1005 pregnancies in the study population, including 16 with a high risk result from cfDNA testing, four with a high risk from combined testing and no cfDNA result, five with a high risk from combined testing and low risk from cfDNA testing and 12 with a low risk from both combined and cfDNA testing. The remaining 968 pregnancies included one case of suspected trisomy 21 ending in miscarriage, five other miscarriages diagnosed at the 12-week scan and 962 that had not yet delivered at the time of writing and it is therefore uncertain if there are any aneuploidies in this group.

The expected number of cases of trisomy 21, trisomy 18 and trisomy 13 in our study population, on the basis of the maternal age distribution and the age-related risk for these trisomies at 10 weeks' gestation, were 9.3, 4.4

and 1.4, respectively, similar to the observed numbers of 11, 4 and 1, respectively^{19,20}.

Assuming that all continuing pregnancies are normal, among the 984 cases with a cfDNA test result there were 16 trisomic and 968 unaffected pregnancies. The FPRs were 0.1% (1/968) for cfDNA testing and 3.4% (33/968) for the combined test.

DISCUSSION

The findings of this study demonstrate the feasibility of implementing cfDNA testing in screening for trisomies in singleton pregnancies at 10 weeks' gestation. In about 4% of women presenting for such screening there was a missed miscarriage and in another 1% there was an unexpected twin pregnancy. Additionally, in about 6% of cases the gestational age, assessed by sonographic measurement of fetal CRL, was lower than 10 weeks, requiring a further visit a few days later. In the group undergoing cfDNA testing, a result was obtained from a single blood draw in 95% of cases and this was available before the 12-week visit in 98% of cases.

Failure to obtain a result at the first blood draw, which occurred in 4.8% of our cases, was due to problems with blood collection and delivery to the laboratory in one fifth of the cases and due to low fetal fraction or assay failure in the other four fifths. At present, cfDNA testing is undertaken in a small number of laboratories around the world and problems arising from transcontinental transportation of samples may be unavoidable, but in such cases a repeat sample is very likely to provide a result. Repeat sampling can also provide a result in 60% of the cases in which the first sample does not give a result because of low fetal fraction or assay failure.

This study has shown that the approach of blood sampling at 10 weeks and ultrasound scanning at both 10 and 12 weeks retains the advantages of the combined test in achieving diagnosis of aneuploidies within the first trimester. In all cases of suspected aneuploidy in which the abnormality was confirmed by CVS, the parents elected to undergo pregnancy termination, which was carried out in the first trimester. It has been shown that the vast majority of pregnant women prefer screening to be performed in the first rather than the second trimester^{21,22}. The study has also demonstrated the necessity of confirming positive results of cfDNA testing by fetal karyotyping, because in one of the cases of suspected trisomy 18 the CVS result reported a normal karyotype. This is compatible with the results of previous studies which reported that maternal blood cfDNA testing is not a diagnostic but a screening test, with both false-positive and false-negative results^{1–15}.

In our population, with a median maternal age of 36.7 years, the screen-positive rate on cfDNA testing was 1.7%, compared with 5.0% for the combined test, at the recommended risk cut-off of 1:100. Since most pregnancies were continuing at the time of writing, it is not possible at present to assess the sensitivity of the screening tests in identifying trisomic pregnancies.

However, the number of affected pregnancies was similar to that estimated from the maternal age distribution of the study population. Although in this study both methods of screening may have identified all cases of trisomies 21, 18 and 13, there is evidence from previous studies that the sensitivity of screening for trisomies is considerably higher with cfDNA testing than with the combined test ($> 99\%$ vs 90%)^{1–16}. This study has shown that the main advantage of cfDNA testing, compared with the combined test, is the substantial reduction in FPR. Another major advantage of cfDNA testing is the reporting of results as very high or very low risk, which makes it easier for parents to decide in favor of or against invasive testing²³.

Invasive testing was carried out in the cases with a screen-positive result from cfDNA testing, but also in about 2.0% of the population with either a low or no risk from cfDNA testing and either a high- or a low-risk result from combined testing. Consequently, cfDNA testing has substantially reduced the rate of invasive testing, but some women still desire a diagnostic test to provide certainty of exclusion of not only the common trisomies but also other aneuploidies.

In this study we did not diagnose aneuploidies other than trisomies 21, 18 and 13. However, invasive testing should be recommended in cases with high fetal NT even if the cfDNA test gives low-risk results, because in such cases these trisomies account for only 75–80% of the associated clinically significant aneuploidies^{24,25}. Similarly, although we did not diagnose any major defects in the presumed euploid fetuses, previous studies have demonstrated the importance of the 12-week scan for early diagnosis of such defects²⁶.

In conclusion, this study has shown that routine screening for trisomies by cfDNA testing at 10 weeks is feasible, allowing diagnosis of aneuploidies and the option of pregnancy termination within the first trimester. The study has highlighted the advantages of cfDNA testing compared with the combined test, in terms of substantial reduction in FPR and clear separation of high- and low-risk results, but has also demonstrated the problem of the failure of cfDNA testing in providing results and the need to investigate abnormal results by invasive testing.

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