# Routine first-trimester screening for fetal trisomies in twin pregnancies: cell-free DNA test contingent on results from the combined test

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**KEYWORDS**: cell-free DNA; first-trimester screening; combined test; non-invasive prenatal testing; trisomy 21; trisomy 18; trisomy 13; twin pregnancy

**Running Head**: cfDNA testing in twins

**ABSTRACT**

**Objective:** To report on the routine clinical implementation of cell-free (cf)DNA analysis of maternal blood for trisomies 21, 18 and 13 contingent on the results of the first-trimester combined test in twin pregnancies.

**Methods:** Screening for trisomies 21, 18 and 13 was carried out by a combination of maternal age, fetal nuchal translucency (NT) thickness, and serum free ß-hCG and PAPP-A at 11-13 weeks' gestation in 959 twin pregnancies in two UK NHS hospitals. Women in the high-risk group (risk ≥1 in 100) were offered options of invasive testing, cfDNA testing or no further testing and those in the intermediate-risk group (risk 1 in 101 to 1 in 2500 in the first phase of the study and 1 in 101 to 1 in 500 in the second phase) were offered cfDNA or no further testing. The trisomic status of the pregnancies was determined by prenatal or postnatal karyotyping or examination of the neonates.

**Results:** In 42 (4.4%) of the 959 pregnancies there was termination, miscarriage or stillbirth with no known karyotype or there was loss to follow up. The 917 pregnancies with known trisomic status of both twins, included 6 that were discordant for trisomy 21, 4 discordant for trisomy 18 and 896 with no trisomies 21, 18 or 13. Following combined screening, 47 (5.1%), 203 (22.2%) and 667 (72.7%) of the pregnancies were classified as high-risk, intermediate-risk and low-risk, respectively. The high-risk group included 5 (83.3%) cases of trisomy 21 and 3 (75.0%) of trisomy 18. The cfDNA test was carried out in 224 pregnancies and results were provided in 214 (95.5%); this group included 6 with trisomy 21, 3 with trisomy 18 and 206 with no trisomies 21, 18 or 13. The cfDNA test correctly classified as screen positive all 6 cases of trisomy 21 and 2 of the 3 with trisomy 18 and as screen negative for each of the trisomies all 206 unaffected pregnancies. Contingent screening, led to prenatal detection of all cases of trisomy 21 and 3 of 4 with trisomy 18.

**Conclusions:** The study has demonstrated the feasibility of introducing cfDNA testing, contingent on the results of the first-trimester combined test for major trisomies, in a routine

population of twin pregnancies.

## INTRODUCTION

In singleton pregnancies screening for the major trisomies using a combination of fetal nuchal translucency (NT) thickness and serum β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein A (PAPP-A) can detect about 90% of cases of trisomies 21, 18 and 13, at false positive rate (FPR) of 5%.[1,2] In twin pregnancies use of the combined test can achieve a similarly high detection rate (DR) for trisomy 21 as in singletons, but with a higher FPR of about 6%.[3] A more effective method of screening for trisomy 21 is provided by analysis of cell-free (cf)DNA in maternal blood; a recent meta-analysis of clinical validation studies reported that in the combined total of 1963 cases of trisomy 21 and 223 932 non-trisomy 21 singleton pregnancies, the weighted pooled DR and FPR were 99.7% (95% CI, 99.1-99.9%) and 0.04% (95% CI, 0.02-0.07%), respectively.[4] In twin pregnancies, the performance of screening for trisomy 21 by cfDNA is encouraging but the number of cases reported is small; in a total of 24 cases of trisomy 21 and 1111 non-trisomy 21 cases, the DR was 100% (95% CI, 95.2-100%) and FPR was 0.0% (95% CI, 0.0-0.003%), respectively.[4]

In screening for the major trisomies in the general population, cfDNA testing can be used either as a first-line method of screening or contingent on the results of the combined test at 11-13 weeks' gestation. Contingent screening could potentially lead to a very high DR and very low invasive testing rate at a considerably lower cost than would be possible using cfDNA testing as a first-line method of screening based on current cfDNA testing costs.[5,6] We have previously reported on the clinical implementation of such policy in singleton pregnancies.[7,8]

The objective of this study is to examine the clinical implementation of cfDNA testing, contingent on the results of the combined test, in routine first-trimester screening for fetal trisomies in twin pregnancies.

## METHODS

### Study design and participants

This was a prospective study in women with twin pregnancies attending two national health service (NHS) hospitals in England (King's College Hospital, London, and Medway Maritime Hospital, Kent) for routine care between October 2013 and January 2018. Implementation of contingent screening was approved by the National Research Ethics Committee (REC reference 13/LO/0885).

During a routine visit at 11-13 weeks' gestation, we recorded maternal demographic characteristics and medical history, measured maternal serum free ß-hCG and PAPP-A (DELFIA Xpress system, PerkinElmer Life and Analytical Sciences, Waltham, USA) and carried out an ultrasound scan to determine gestational age from the measurement of the fetal crown-rump length (CRL)[9] of the larger fetus, and chorionicity by examining the junction of the intertwin membrane with the placenta,[10] to diagnose any major fetal abnormalities and measure fetal NT thickness. The measured NT was expressed as a difference from the expected normal mean for gestation (delta value).[11] Similarly, the measured free ß-hCG and PAPP-A were converted into multiple of the median (MoM) values adjusted for maternal characteristics, gestational age and chorionicity.[12,13]

The estimated risk for trisomy 21 and trisomies 18 and 13 was calculated and the highest of the two was considered in the stratification of the population. In the case of monochorionic twins a risk is given for the whole pregnancy; in dichorionic twins a risk is given for each fetus and the highest of the two was used for stratification. Women in the high-risk group (risk $\geq$1 in 100) were offered options of chorionic villous sampling (CVS), cfDNA testing or no further testing; this cut-off was selected because it is used by the NHS for offering invasive testing. Women in the intermediate-risk group (risk 1 in 101 to 1 in 2500 in the first phase of the study and 1 in 101 to 1 in 500 in the second phase) were offered cfDNA or no further testing. Women in the low-risk

group (risk <1 in 2500 in the first phase of the study and <1 in 500 in the second phase) were reassured that fetal trisomies were unlikely and no further testing was necessary.

Women provided written informed consent and maternal blood (20 mL) was sent via courier to the USA for cfDNA testing (Harmony™ Prenatal Test, Ariosa Diagnostics, Inc., San Jose, CA).[14,15] Digital analysis of selected regions (DANSR) by chromosome-selective sequencing or microarray was used to quantify chromosomes 21, 18 and 13. Risk scores for trisomy 21, 18, and 13 were provided as a percentage with ranges capped at >99% and <0.01%. In cases where the cfDNA test did not provide results the parents were offered repeat testing or to rely on the results of the combined test in deciding whether to have an invasive test or not. In cases with a high-risk result from the cfDNA test, the parents were advised to consider having invasive fetal karyotyping before deciding on the further management of their pregnancy.

Patient characteristics, results of the investigations and pregnancy outcome were recorded in a database. The outcomes were divided into first, trisomy 21, 18 or 13 if the karyotype of chorionic villi, amniotic fluid or neonatal blood demonstrated the relevant trisomy in one or both fetuses, second, no trisomy 21, 18 or 13 if the karyotype of chorionic villi, amniotic fluid or neonatal blood was normal or both neonates were phenotypically normal, third, no known karyotype in both fetuses because the pregnancies resulted in termination or embryo reduction, miscarriage or stillbirth and no karyotyping of fetal tissue was carried out, and fourth, outcome unknown because the pregnancies were lost to follow up.

**Statistical analysis**

Descriptive data were presented in median and interquartile range (IQR) for continuous variables and in numbers and percentages for categorical variables. Comparisons between outcome groups were by Mann-Whitney U-test for continuous variables and $\chi^2$-test or Fisher's exact test for categorical variables.

The statistical software package R version 3.3.3 (https://www.R-project.org/) was used for data analyses.

**Role of the funding source**

The study was supported by a grant from The Fetal Medicine Foundation (UK Charity No: 1037116). The cost of collection and analysis of the samples for the cell-free DNA test was covered by Ariosa Diagnostics, Inc. San Jose, CA, USA. These organizations had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

## RESULTS

### Study population

During the study period, 977 women with twin pregnancies and two live fetuses at 11-13 weeks' gestation were offered combined screening for trisomies; 959 (98.2%) accepted, but 42 (4.4%) of these were excluded from further analysis either because the pregnancies ended in termination, miscarriage or stillbirth with no known karyotype (n=29) or they were lost to follow up (n=13).

Maternal and pregnancy characteristics in the 917 pregnancies with known trisomic status of both twins are summarized in Table 1; these included 740 (80.7%) dichorionic and 177 (19.3%) monochorionic twins. In the monochorionic twin pregnancies there were no trisomic fetuses. In the dichorionic twin pregnancies there were 10 where one fetus was normal and the co-twin was trisomic (6 cases of trisomy 21 and 4 of trisomy 18).

On the basis of the maternal age distribution and the age-related risk for these trisomies at 12 weeks' gestation, the expected number of cases of trisomy 21 and trisomies 18 and 13 in our monochorionic twin pregnancies was 0.6 (95% CI: 0.07 – 4.87) and 0.3 (95% CI: 0.02 – 4.32) respectively.[16,17] In the dichorionic twin pregnancies, on the assumption that the trisomic risk for each of the 1,480 fetuses was the same as in singleton pregnancies, the expected number of cases of trisomy 21 and trisomies 18 and 13 in our study population, was 6.4 (95% CI: 3.03 – 13.54) and 3.4 (95% CI: 1.26 – 9.32) respectively, which were similar to the observed numbers of 6 and 4 respectively.[16,17]

### Stratification of risks and parental choices

Following combined screening, 47 (5.1%), 203 (22.2%) and 667 (72.7%) of the pregnancies were classified as high-risk, intermediate-risk and low-risk, respectively. The high-risk group can be subdivided into a group with estimated risk of ≥ 1 in 30, which contained 27 (2.9%) cases,

and another with a risk of 1 in 31 to 1 in 100, which contained 20 (2.2%) cases.

In the high-risk group, 36.2% (17/47) opted for CVS (including 4 cases of trisomy 21 and 2 of trisomy 18), 57.4% (27/47) for cfDNA testing (including 1 case of trisomy 21 and 1 of trisomy 18) and 6.4% (3/47) did not want any further investigations. In the subgroup with risk of ≥ 1 in 30, 55.6% (15/27) opted for CVS (including 4 cases of trisomy 21 and 2 of trisomy 18), 37.0% (10/27) for cfDNA testing (including 1 case of trisomy 21) and 7.4% (2/27) did not want any further investigations. In the intermediate-risk group, 91.6% (186/203) opted for cfDNA testing (including 1 case of trisomy 21) and 8.4% (17/203) did not want any further investigations.

**Results of the combined test**

Combined screening with estimated risk cut-off of 1 in 100 detected 83.3% (5/6) cases of trisomy 21 and 75% (3/4) with trisomy 18. One case of trisomy 21 had a risk of 1 in 939 and this was identified by cfDNA testing. In three of the six cases of trisomy 21, the parents chose to continue with the pregnancy and in the other three they had embryo reduction. One case of trisomy 18 had a risk of 1 in 3,450 and this case was identified by amniocentesis because at the routine 20 weeks scan the affected fetus had tetralogy of Fallot, clenched hands, strawberry shaped head and growth restriction.

**Implementation and performance of the cfDNA test**

In total, the cfDNA test was carried out in 224 pregnancies. These included 213 from the high- or intermediate-risk group that opted for cfDNA testing and 11 in the high-risk group that opted for CVS, but also had cfDNA testing for research; in the latter group the blood test was collected before invasive testing. Results from testing were provided after first sampling for 95.5% (214/224) of cases and the median fetal fraction was 8.5% (range 4 to 30%). The reasons for no result were insufficient fetal cfDNA for accurate evaluation in 7 cases and the sample did not meet thresholds for quality control in 3. In 7 of the 10 cases with no result, a further blood sample was obtained and a cfDNA result was provided in 1. In 8 of the 9 cases with no result from the cfDNA test the parents decided to avoid further testing and 1 chose to have amniocentesis.

The group of 215 pregnancies with a cfDNA result (214 from first sampling and 1 from second sampling) included 6 with trisomy 21, 3 with trisomy 18 and 206 with no trisomies 21, 18 or 13. The cfDNA test correctly classified as screen positive all 6 cases of trisomy 21 and 2 of the 3 with trisomy 18 and as screen negative for each of the trisomies all 206 unaffected pregnancies.

The distribution of estimated risk for trisomies by the combined test and the cfDNA test is given in Figure 1.

**Performance of contingent screening**

The study population of 917 pregnancies with known trisomic status of both twins included 6 with trisomy 21, 4 with trisomy 18 and 907 with no trisomies 21, 18 or 13. Contingent screening, led to prenatal detection of all cases of trisomy 21 and 3 of 4 with trisomy 18 (1 case was classified as low risk by the combined test).

Invasive tests were carried out in 33 (3.6%) of the study population. These included 18 (54.5%) for high-risk result from the combined test, 2 (6.1%) for positive result from the cfDNA test, 1 (3.0%) for failed cfDNA testing, 5 (15.2%) for fetal defects detected by ultrasound examination in the second trimester of pregnancy, 7 (21.2%) for endoscopic laser separation of communicating placental vessels in association with severe twin-to-twin transfusion syndrome or selective fetal growth restriction and 1 (3.0%) for prenatal diagnosis of sickle cell disease.

## DISCUSSION

### Main findings of the study

The study has demonstrated the feasibility of introducing cfDNA testing, contingent on the results of the first-trimester combined test for major trisomies, in a routine population of twin pregnancies. The observed number of trisomies was as expected on the basis of the maternal age distribution of the study population.

In our participating hospitals, about 98% of women attending for a routine ultrasound examination at 11-13 weeks' gestation accepted the offer of screening for fetal trisomies by the combined test and this was successfully carried out in all cases. In the high-risk group, 36% of women opted for invasive testing, 57% for cfDNA testing and 6% for no further tests; in the subgroup with risk of ≥ 1 in 30, 56% opted for invasive testing. In the intermediate-risk group, 92% opted for cfDNA testing and 8% for no further tests. These results on patient choices are very similar to those reported in our previous study for singleton pregnancies.[8] In the high-risk group the choice between CVS and cfDNA testing was influenced by objective evidence derived from the patient-specific risk obtained from the combined test. In 3 of the 6 cases of trisomy 21 the parents chose to continue with the pregnancy and in the other 3 they had embryo reduction; there were no obvious differences between the two groups in terms of maternal age, race, parity or method of conception.

The combined test, at risk cut-off of 1 in 100, could have potentially identified 5 of 6 cases of trisomy 21 and 3 of 4 of trisomy 18, at FPR of 4.3%; the number of affected cases is too small for accurate assessment of the performance of screening, but the results are consisted with the modelled performance of about 90% detection of the major trisomies at FPR of 6%.[3]

In the group undergoing cfDNA testing, results were provided for 96% of pregnancies; the failure rate in twin pregnancies was twice as high as that in our previous study for singleton pregnancies.[8] The cfDNA test detected all cases of trisomy 21 and 2 of 3 with trisomy 18 in the

population having this test, at FPR of 0%. As in the case of the combined test, the number of affected cases is too small for accurate assessment of the performance of cfDNA screening, but the results are consisted with those of previous reports.[18-25]

**Limitations of the study**

The main limitation of the study relates to the small number of trisomic pregnancies and the small number of cases which actually had cfDNA testing, preventing definitive conclusions to be drawn in terms of performance of screening by these two methods.

The results on the uptake of various options of screening and management of affected pregnancies depending on risk categories defined by the combined test highlight some general principles concerning the factors that influence patient decisions. However, the exact rates of uptake of a specific option may not be generalizable to all populations from different racial and socioeconomic backgrounds in different countries and healthcare systems.

**Previous studies of cfDNA testing in twin pregnancies**

There are only seven prospective studies with complete follow-up reporting on the performance of cfDNA testing in twin pregnancies.[19-25] Two studies examined a routine population,[24,25] three examined pregnancies at high-risk of aneuploidies,[21-23] and two were in a mixed population of high and low-risk pregnancies[19,20]. In the combined total of 31 cases of trisomy 21 and 2,008 non-trisomic pregnancies the detection rate was 100% and false positive rate was 0.05%. Although the number of twin pregnancies examined by cfDNA testing is considerably lower than singleton pregnancies,[4] the results suggest the test is equally effective in identifying trisomy 21.

**Conclusions**

Clinical implementation of cfDNA testing contingent on the results from a previously performed first-trimester combined test is feasible and it could potentially lead to the prenatal detection of a

higher proportion of affected pregnancies and a lower invasive-testing rate than in screening by the combined test alone. However, in clinical practice, prenatal detection of trisomies and pregnancy outcome depend not only on performance of screening tests but also on parental choices. Consequently, clinical implementation of cfDNA testing contingent on the results of the combined test may only have a modest impact in reducing the rate of invasive testing and a small effect on the rate of live births with trisomy 21.

**Acknowledgement:** This study was supported by a grant from the Fetal Medicine Foundation (Charity No: 1037116). The cost of collection and analysis of some of the samples was covered by Ariosa Diagnostics, Inc. San Jose, CA, USA.

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**Figure legend:**

**Figure 1** The distribution of estimated risk for trisomies by the combined test and the cfDNA test.

**Table 1.** Characteristics of the study population.

| Characteristics | High-risk (n=47) | Intermediate-risk (n=203) | Low-risk (n=667) |
|---|---|---|---|
| Maternal age in years, median (IQR) | 36.2 (32.5-40.0)* | 36.6 (33.3-38.9)* | 32.0 (28.3-35.5) |
| Maternal body mass index in kg/m$^2$, median (IQR) | 26.9 (22.1-30.5) | 25.0 (22.6-28.1) | 25.4 (22.3-30.0) |
| Racial origin | | | |
| White, n (%) | 33 (70.2) | 153 (75.4) | 512 (76.8) |
| Black, n (%) | 12 (25.5) | 32 (15.7) | 105 (15.7) |
| South Asian, n (%) | 2 (4.3) | 12 (5.9) | 25 (3.7) |
| East Asian, n (%) | - | 3 (1.5) | 5 (0.7) |
| Mixed, n (%) | - | 3 (1.5) | 21 (3.1) |
| Cigarette smoker, n (%)ew | 1 (2.1) | 3 (1.5) | 34 (5.1) |
| Parity | | | |
| Nulliparous, n (%) | 25 (53.2) | 87 (42.9) | 272 (40.8) |
| Parous, n (%) | 22 (46.8) | 116 (57.1) | 395 (59.2) |
| Method of conception | | | |
| Spontaneous, n (%) | 31 (66.0) | 129 (63.5) | 479 (71.8) |
| Assisted conception, n (%) | 16 (34.0) | 74 (36.5) | 188 (28.2) |
| Estimated risk for trisomies 21 or 18/13 (1 in ), median (IQR) | 24 (57-5)* | 463 (1,182 -276)* | 3,833 (7,321-1,952) |
| Patient choice for further testing | | | |
| Cell-free DNA test, n (%) | 27 (57.4) | 186 (91.6) | - |
| Chorionic villous sampling, n (%) | 17 (36.2) | - | - |
| Nothing, n (%) | 3 (6.4)* | 17 (8.4)* | 667 (100%) |

Comparisons between the high- and intermediate-risk groups was made to the low-risk group by Mann Whitney-U test for continuous variables and by Chi-square or Fisher exact test for categorical variables, with post-hoc Bonferroni correction with adjusted P-value of <0.025 (*). IQR = interquartile range.

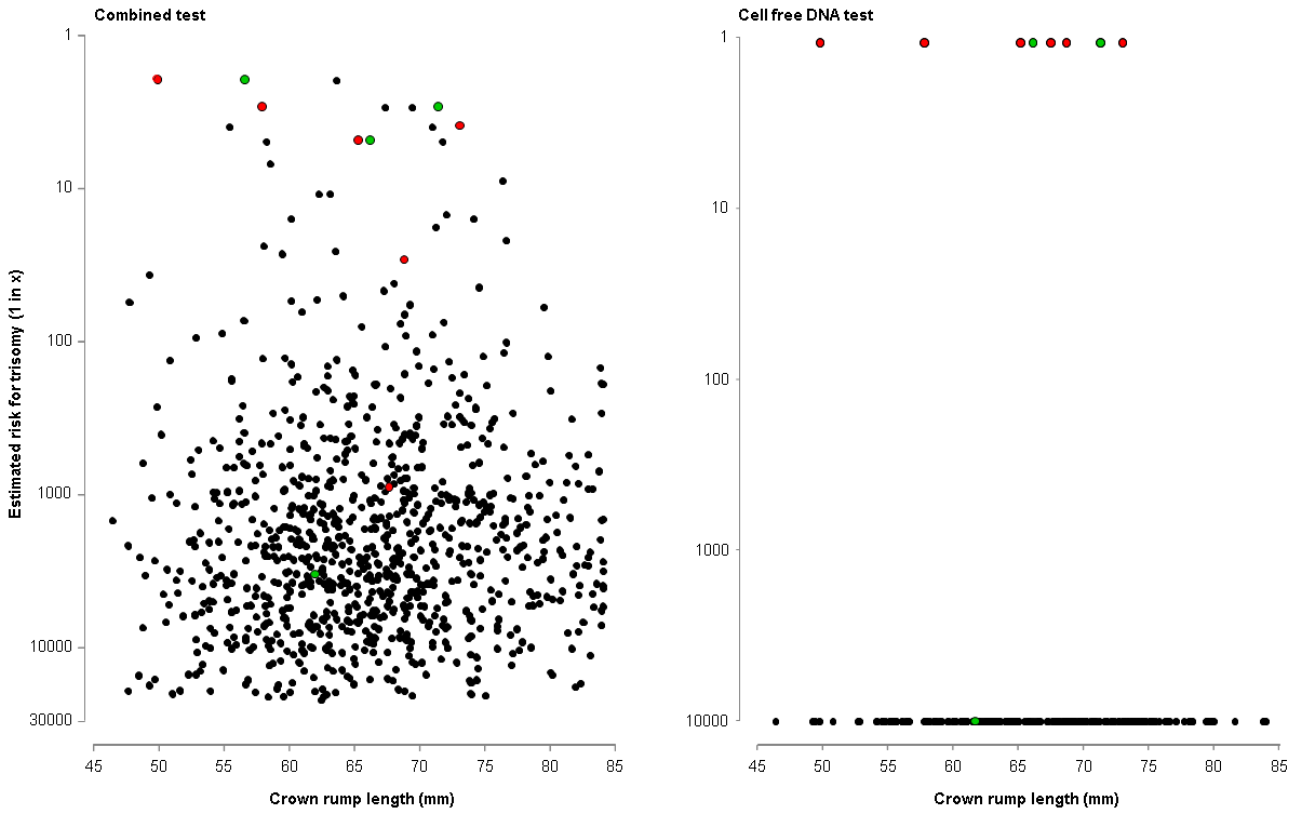


Figure 1