

**Cell free DNA testing of maternal blood in screening for trisomies in twin pregnancy:
Cohort study at 10-14 weeks and meta-analysis.**

Hannah JUDAH,¹ María del Mar GIL,^{1,2} Argyro SYNGELAKI,¹ Slavyana GALEVA,¹ Jacques JANI,³ Ranjit AKOLEKAR,^{4,5} Kypros H. NICOLAIDES.¹

1. Harris Birthright Research Centre of Fetal Medicine, King's College Hospital, London, UK.
2. Department of Obstetrics and Gynecology, Hospital Universitario de Torrejón, Torrejón de Ardoz, Madrid and School of Medicine, Universidad Francisco de Vitoria, UFV, Pozuelo de Alarcón, Madrid, Spain.
3. Department of Obstetrics and Gynecology, University Hospital Brugmann, Brussels, Belgium.
4. Department of Fetal Medicine, Medway Maritime Hospital, Kent.
5. Institute of Medical Sciences, Canterbury Christ Church University, Chatham, UK.

Short title: cfDNA testing in twin pregnancies

Acknowledgement: The study was supported by a grant from The Fetal Medicine Foundation (UK Charity No: 1037116). The cost of collection and analysis of some of the samples for the cell-free DNA test was covered by Roche/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.

Corresponding author

Professor K Nicolaidis
Fetal Medicine Research Institute,
King's College Hospital,
16-20 Windsor Walk,
Denmark Hill, London SE58BB
Telephone: +442032998256
Fax: +442077339534

What are the novel findings of this work

We investigated the performance of cfDNA testing of maternal blood in screening for trisomies 21, 18 and 13 in twin pregnancies. In the case of trisomy 21 the detection rate was 99% and the false positive rate was less than 0.1%. The reported number of twin pregnancies with trisomies 18 and 13 is too small for accurate assessment of the predictive performance of the cfDNA test.

What are the clinical implications of this work

In twin pregnancies cfDNA testing of maternal blood provides effective screening for trisomy 21. This can be used either as a primary method of screening or contingent on the results from first line screening by the first trimester combined test and / or second trimester biochemistry test. If cfDNA testing is adopted as a first line method of screening for trisomies in twin pregnancies it is still imperative that women should be offered an 11-14 weeks scan to determine gestational age, chorionicity and whether the fetuses are alive and if there is high nuchal translucency thickness or major defects that would merit invasive testing.

ABSTRACT

Objective: To expand the limited knowledge on cell-free (cf)DNA analysis of maternal blood for trisomies 21, 18 and 13 in twin pregnancies by updating the data from the Fetal Medicine Foundation (FMF) on prospective first trimester screening and those arising from systematic review of the literature.

Methods: The FMF data were derived from prospective screening for trisomies 21, 18 and 13 in twin pregnancies at 10⁺⁰-14⁺¹ weeks' gestation using the Harmony® prenatal test of Roche/Ariosa Diagnostics, Inc. Search of Medline, Embase, CENTRAL (The Cochrane Library), ClinicalTrials.gov and ICTRP (World Health Organization) was carried out to identify all peer-reviewed publications on clinical validation or implementation of maternal cfDNA testing for trisomies 21, 18 and 13 in twin pregnancies, irrespective of gestational age at testing, in which data on pregnancy outcome were provided for more than 85% of the study population. Meta-analysis was then performed using the FMF data and data from the studies identified by the literature search. This review was registered in PROSPERO international database for systematic reviews

Results: In the FMF study, cfDNA testing was carried out in 1442 twin pregnancies and a result was obtained, after first or second sampling, in 1367 (94.8%) cases. In 93.1% (1272/1367) cases there was prenatal or postnatal karyotyping or the birth of phenotypically normal babies; 95 cases were excluded from further analysis either because the pregnancies ended in termination, miscarriage or stillbirth with no known karyotype (n=56) or there was loss to follow up (n=39). In the 1272 pregnancies included in the study there were 20 cases with trisomy 21, 10 with trisomy 18, 2 with trisomy 13 and 1240 without trisomy 21, 18 or 13. The cfDNA test classified correctly 19 (95.0%) of the 20 cases of trisomy 21, 9 (90.0%) of 10 of trisomy 18, 1 (50.0%) of 2 of trisomy 13 and 1235 (99.6%) of 1240 cases without any of the three trisomies. The literature search identified 12 relevant studies, excluding our papers because their data are included in the current study. In the combined total of our study and the 12 studies identified by the literature search there were 137 trisomy 21 and 7507 non-trisomy 21 twin pregnancies; the pooled weighted detection rate (DR) and false positive rate (FPR) were 99.0% (95% CI 92.0, 100%) and 0.02% (95% CI 0.001, 0.43%), respectively. In the combined total of 50 cases of trisomy 18 and 6840 non-trisomy 18 pregnancies the pooled weighted DR and FPR were 92.8% (95% CI 77.6, 98.0%) and 0.01% (95% CI 0.00, 0.44%), respectively. In the combined total of 11 cases of trisomy 13 and 6290 non-trisomy 13 pregnancies the pooled weighted DR and FPR were 94.7% (95% CI 9.14, 99.97%) and 0.10% (95% CI 0.03, 0.39%), respectively.

Conclusions: In twin pregnancies the reported DR of trisomy 21 by cfDNA testing is high, but lower than in singleton pregnancies, whereas the FPR appears to be equally low. The number of cases of trisomy 18 and more so trisomy 13 is too small for accurate assessment of the predictive performance of the cfDNA test.

KEYWORDS: cell-free DNA; first-trimester screening; non-invasive prenatal testing; trisomy 21; trisomy 18; trisomy 13; twin pregnancy; meta-analysis.

INTRODUCTION

In singleton pregnancies, cell-free (cf) DNA analysis of maternal blood provides effective screening for trisomies 21, 18 and 13. A meta-analysis of clinical validation studies reported that in the combined total of 1963 cases of trisomy 21, 560 cases of trisomy 18 and 119 cases of trisomy 13 the weighted pooled detection rate (DR) was 99.7% (95% CI, 99.1-99.9%), 98.2% (95% CI 95.5-99.2%) and 99.0% (95% CI 65.8-100%), respectively, at an overall false positive rate (FPR) among the more than 200,000 cases of pregnancies unaffected by these trisomies was about 0.1%.¹ In contrast to singleton pregnancies, data of cfDNA testing in twins are very limited. In a study reporting the results of cfDNA testing in 928 twin pregnancies from the Fetal Medicine Foundation (FMF) and about 2,500 pregnancies from seven other studies identified by systematic review of the literature, there was a combined total of only 56 cases of trisomy 21, 18 cases of trisomy 18 and 3 cases of trisomy 13; in the meta-analysis of these eight studies the pooled weighted DR and FPR for trisomy 21 were 98.2% (95% CI 83.2, 99.8%) and 0.05% (95% CI 0.01, 0.26%), respectively, and the respective values for trisomy 18 were 88.9% (95% CI 64.8, 97.2%).²

The objectives of this study are first, to report our updated experience from the FMF on prospective first trimester screening for trisomies 21, 18 and 13 by cfDNA analysis of maternal blood in twin pregnancies and second, to carry out a meta-analysis of all studies of cfDNA testing in twin pregnancies published up to 20th March 2021.

METHODS

Update of the Fetal Medicine Foundation results

Study design and participants

The data for the study were derived from prospective screening for trisomies 21, 18 and 13 in twin pregnancies at 10⁺⁰-14⁺¹ weeks' gestation. Two populations were included; first self-referred women to the Fetal Medicine Centre in London, which is a private clinic, or Brugmann University Hospital, which is public hospital in Brussels and second, women selected for the cfDNA test after routine first-trimester combined testing in one of two National Health Service hospitals in England. The patients were examined between October 2012 and September 2020. The study was approved by the appropriate Ethics Committees (NREC reference 13/LO/0885, NREC reference 19/HRA/0576, CE 2014/5).

We recorded maternal demographic characteristics and medical history, and carried out an ultrasound scan to determine gestational age from the measurement of the crown-rump length³ of the larger fetus and chorionicity by examining the junction of the intertwin membrane with the placenta.⁴ Women provided written informed consent and maternal blood, collected into either Cell-Free DNA BCT tubes (Streck, Omaha, NE) or Roche Cell-Free DNA Collection Tubes (Roche, Pleasanton, CA), was shipped via courier to Roche/Ariosa Diagnostics, Inc. (San Jose, CA, USA) for analysis. Targeted cfDNA testing for fetal trisomy was performed using the Harmony® prenatal test as described previously.⁵⁻⁸ Harmony® uses DANSR assays targeting sequences on chromosomes 13, 18, and 21 for chromosome quantitation and single nucleotide polymorphisms on chromosomes 1 to 12 for fetal fraction measurement. Products of the DANSR assays can be quantified using either next-generation sequencing or a custom microarray; both were used during the course of this study. The data are analysed with the fetal fraction optimized FORTE algorithm, which calculates probability scores for fetal trisomy, with greater than 1% considered to be high probability. 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 babies, second, no trisomy 21, 18 or 13 if the karyotype 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 analyses

Descriptive data were presented in median and interquartile range (IQR) for continuous variables and in numbers and percentages for categorical variables.

Systematic review and meta-analysis

Literature search and study selection

Searches of Medline via PubMed, Embase and CENTRAL (The Cochrane Library) were performed to identify clinical validation or implementation studies of maternal cfDNA testing in screening for aneuploidies in twin pregnancies; additionally, ClinicalTrials.gov and WHO International Clinical Trials Registry Platform (ICTRP) were also searched for ongoing or recently completed trials. The study period was from January 2011, when the first such study was published, 20th March 2021; the initial search was 26th February 2021 and this was updated with autoalerts in Medline. A list of relevant citations was generated from these databases using the search strategies, which we used for our previous meta-analysis in twin pregnancies (Appendix 1). This review was registered in PROSPERO international database for systematic reviews (reference: CRD42021242564).

The abstracts of citations were examined by two reviewers (H.J and M.M.G.) to identify all potentially relevant articles, which were then examined in full-text form. Reference lists of relevant original and review articles were hand-searched for additional reports. Agreement about potential relevance was reached by consensus and by consultation with a third reviewer (K.H.N.). The inclusion criteria were peer-reviewed studies reporting on clinical validation or implementation of maternal cfDNA testing in screening for aneuploidies in twin pregnancies, in which data on pregnancy outcome were provided for more than 85% of the study population. Proof-of-principle articles and studies in which the laboratory scientists carrying out the tests were aware of fetal karyotype or pregnancy outcome were excluded. We also excluded case-control studies because they tend to introduce an optimistic bias to the estimates of diagnostic performance.

Data extraction and meta-analysis of data from all studies

Data regarding sample size, gestational age at analysis, method used for cfDNA testing and DR or sensitivity and FPR or specificity for non-mosaic trisomies 21,18 and 13 were obtained from each study included in the systematic review and documented in contingency tables. In the construction of these tables, we used the results from the cfDNA test and excluded those cases in which the test failed to give a result. In the calculation of FPR, we included all euploid and aneuploid cases other than the aneuploidy under investigation. Authors were contacted when clarifications were required in the interpretation of the data.

We extracted data from the primary studies to obtain the four cell values of a diagnostic 2×2 table to calculate test accuracy measures of DR and FPR. The analyses were stratified according to the three aneuploidies under investigation. We calculated DR and FPR with corresponding 95% CIs for individual studies and displayed them in forest plots to investigate heterogeneity. We pooled the DR and FPR estimates using bivariate random-effects regression models. The bivariate model assumes that logit transformations of DR and FPR are negatively correlated and follow a bivariate normal distribution⁹. We computed the positive and negative likelihood ratios from the pooled estimates of DR and FPR. Heterogeneity among studies was quantified with the variance of the logit of accuracy indices as estimated by the bivariate model.

For those analyses where more than 10 studies were included, we assessed publication bias by representing the diagnostic odds ratio (a single measure of diagnostic accuracy) against the effective sample size. With no bias, the plot shows an inverted symmetrical funnel shape (a vertical pattern indicates no publication bias whereas slope indicates the degree of bias). The degree of asymmetry was assessed statistically by regression of the logarithm of diagnostic odds ratio on the inverse of the square root of the effective sample size, weighted by this sample size. A value of $P < 0.1$ indicates significant publication bias.¹⁰

We conducted statistical analyses using the Metandi and Midas programs for the STATA software.¹¹

RESULTS

Update of the Fetal Medicine Foundation results

Study population

In the FMF study, cfDNA testing was carried in 1442 twin pregnancies and a result was obtained, after first or second sampling, in 1367 (94.8%) cases (Figure 1). In 93.1% (1272/1367) cases there was prenatal or postnatal karyotyping or the birth of phenotypically normal babies; 95 cases were excluded from further analysis either because the pregnancies ended in termination, miscarriage or stillbirth with no known karyotype (n=56) or there was loss to follow up (n=39). In the 75 cases with no result from cfDNA testing after first or second sampling, there were 62 (82.7%) without trisomy 21, 18 or 13, 11 (14.7%) in which the pregnancies ended in termination, miscarriage or stillbirth with no known karyotype and 2 with loss to follow up.

In the 1272 pregnancies included in the study, 1085 (85.3%) were dichorionic and 187 (14.7%) were monochorionic. The median maternal age was 37.0 (IQR 33.9-39.6) years, the median maternal weight was 65.0 (IQR 58.0-74.0) kg and the median gestational age at sampling was 11.7 (IQR 10.7-12.9, range 10.0-14.1) weeks. Conception was natural in 609 (47.8%) cases and after use of assisted reproductive techniques in 663 (52.2%). cfDNA testing was done by means of sequencing in 350 of the twin pregnancies and by microarray in 922.

In the 1272 pregnancies there were 20 cases with trisomy 21, 10 with trisomy 18, 2 with trisomy 13 and 1239 without trisomy 21, 18 or 13. One of the pregnancies with trisomy 21 and another with trisomy 18 were monochorionic where both fetuses were affected; all the other trisomic cases were in dichorionic pregnancies where only one fetus was affected and the cotwin was non-trisomic.

There were four cases with a false positive result for trisomy 13 and one with a false positive result for trisomy 21. In these five cases the risk from the combined test was low and there were no ultrasound features suggestive of these trisomies.

Performance of screening

The cfDNA test classified correctly 19 (95.0%) of the 20 cases of trisomy 21, 9 (90.0%) of 10 of trisomy 18, 1 (50.0%) of 2 of trisomy 13 and 1235 (99.7%) of 1240 cases without any of the three trisomies. One case each of trisomy 21, trisomy 18 and trisomy 13 was classified as normal. In the non-trisomic group, 4 cases were classified as trisomy 13 and 1 as trisomy 21 and therefore, the combined FPR was 0.4% (5/1240).

Systematic review and meta-analysis

Data sources

The search identified 381 potentially relevant citations, but 369 were excluded because they were non-relevant articles, conference abstracts rather than peer-reviewed papers, review articles or opinions, studies not confined to twins, case-control studies, or studies on clinical implementation of cfDNA testing in screening for aneuploidies in which pregnancy outcome data were provided for fewer than 85% of the study population, proof-of-principle studies reporting laboratory techniques rather than clinical validation of a predefined method of maternal blood cfDNA analysis (Figure 1). In total, 15 relevant studies were identified^{1,12-25} but three of these^{1,12,13} were excluded from the meta-analysis because their data are included in the updated Fetal Medicine Foundation results presented above. The characteristics of our current study and the 12 ones identified by the literature search are summarized in Table 1.

Methodological quality of the selected studies

The methodological quality of the selected studies, assessed by the Quality Assessment tool for Diagnostic Accuracy Studies (QUADAS-2)²⁶, is illustrated in Figure 3. This tool comprises four domains; each one is assessed in terms of risk of bias and the first three are also assessed in terms of concerns regarding applicability as previously explained². In brief, a study was considered at low risk of bias for each domain if first, it was explicitly stated that the cfDNA test had been carried out in a consecutive or random sample of patients; second, if the laboratory results from cfDNA testing were provided without prior knowledge of the fetal karyotype or pregnancy outcome; third, if the method of diagnosing the chromosomal abnormality under investigation was genetic testing or neonatal examination; and fourth, if all patients in the study population had a result from both the cfDNA test and pregnancy karyotype or outcome and if the method of classifying the outcome result (invasive testing or clinical examination) was the same in all cases in the study population. A study was classified as of not being of concern regarding applicability if it was a general population study, without any prior screening performed, and if the index test and reference standards reported dichotomous results, either normal or abnormal, in a previously specified manner.

Method of analyzing samples

The studies included in the meta-analysis used one of two methods for analysis of cfDNA in maternal blood: massively parallel sequencing or targeted analysis (either by next-generation sequencing or by a custom microarray) (Table 1).

Nature of the studies

All studies included in this meta-analysis were prospective; four studies were in high-risk pregnancies and nine examined a mixture of high-risk and routine populations (Table 1). The proportion of monozygotic twin pregnancies ranged from 3.2% to 42.4%.

Meta-analysis and performance of screening for aneuploidies

The DR and FPR for each study, weighted pooled data and heterogeneity between studies (variance of the logit sensitivity and specificity) are provided in Tables 2-4; sensitivity and specificity are illustrated in Figures 4-6. Heterogeneity²⁷ between studies was very low for sensitivity but substantial for specificity, the latter entirely due to the results from the study of Chibuk *et al.*²³ (Tables 2-4). Publication bias was only assessed for trisomy 21 (Figure 7). There was a low likelihood of publication bias for all conditions ($P > 0.10$).

Trisomy 21

A total of 13 studies reported on the performance of screening by cfDNA analysis for trisomy 21 in a combined total of 137 cases of trisomy 21 and 7507 non-trisomy 21 twin pregnancies (Table 2, Figure 4). There was a high heterogeneity between studies,²⁷ mainly because of the high FPR in the study by Chibuk *et al.*²³ Among individual studies, the DR varied between 95.0% and 100% and the FPR varied between 0% and 3.9%. The weighted pooled DR and FPR were 99.0% (95% CI 92.0, 100%) and 0.02% (95% CI 0.001, 0.43%), respectively. The positive and negative likelihood ratios were 4224 (95% CI 230 - 77525) and 0.010 (95% CI 0.001 - 0.085), respectively.

Trisomy 18

A total of nine studies reported on the performance of screening by cfDNA analysis for trisomy 18 in a combined total of 50 cases of trisomy 18 and 6840 non-trisomy 18 twin pregnancies (Table 3, Figure 5). There was a high heterogeneity between studies,²⁷ mainly because of the high FPR in the study by Chibuk *et al.*²³ Among individual studies, the DR varied between 50.0% and 100% and the FPR varied between 0% and 1.1%. The weighted pooled DR and FPR were 92.8% (95% CI 77.6, 98.0%) and 0.01% (95% CI 0.00, 0.44%), respectively. The positive and negative likelihood ratios were 7486 (95% CI 211 - 265331) and 0.072 (95% CI 0.021 - 0.240), respectively.

Trisomy 13

A total of seven studies reported on the performance of screening by cfDNA analysis for trisomy 13 in a combined total of 11 cases of trisomy 13 and 6290 non-trisomy 13 twin pregnancies (Table 4, Figure 6). There was a high heterogeneity between studies, mainly because of the high FPR in the study by Chibuk *et al.*²³ Among individual studies, the DR varied between 50.0% and 100% and the FPR varied between 0% and 0.8%. The weighted pooled DR and FPR were 94.7% (95% CI 9.14, 99.97%) and 0.10% (95% CI 0.03, 0.39%), respectively. The positive and negative likelihood ratios were 916 (95% CI 226 - 3714) and 0.053 (95% CI 0.000 - 7.173), respectively.

DISCUSSION

Principal findings of the study

The FMF study is the only one examining the performance of cfDNA testing of maternal blood in first trimester screening for trisomies in twin pregnancies. The study used targeted analysis of cfDNA, either by next-generation sequencing or by a custom microarray, and demonstrated that first, a result is

obtained, after first or second sampling, in about 95% of cases; second, in about 7% of cases undergoing first trimester screening it may not be possible to confirm the results with prenatal or postnatal karyotyping because such testing may not be undertaken if the pregnancies end in termination, miscarriage or stillbirth or there is loss to follow up; and third, even if our study population of 1442 cases is relatively large for twin pregnancies, the number of trisomic pregnancies (20 cases of trisomy 21, and 12 of trisomy 18 or 13) is too small for any valid conclusions to be drawn concerning the performance of screening of the cfDNA test. This study has not addressed the issue of cfDNA test failure because we have recently reported our experience in a larger cohort including both singleton and twin pregnancies.²⁸ In that study we found that important contributors to cfDNA test failure are increased maternal weight, conception by *in vitro* fertilization, Black or South Asian racial origin, dichorionicity, nulliparity, low gestational age and low serum PAPP-A and free β -hCG.

The literature search identified 12 studies reporting on screening for trisomies in twin pregnancies by cfDNA testing of maternal blood, in addition to our previous studies whose data are included in the current FMF study. The main features of these 12 studies are first, the method of cfDNA testing was by massively parallel sequencing in 11 studies and by targeted testing in only one; second, none of the studies was confined to the first trimester and in some cases testing was carried out as late as 36 weeks' gestation;^{15,25} and third, in nine of the studies the cfDNA test provided a result in >99% of cases^{14-17,19,21,22,24,25} and in six of the studies pregnancy outcome was apparently obtained in >99% of cases.^{14,15,17,20,22,23}

In the combined total of the FMF study and the 12 studies identified by the literature search there were 137 cases of trisomy 21, 50 of trisomy 18, 11 of trisomy 13 and more than 6000 pregnancies unaffected by trisomies 21, 18 or 13. The pooled weighted DRs were 99.0% (95% CI 92.0, 99.9%) for trisomy 21, 92.8% (95% CI 77.6, 98.0%) for trisomy 18 and 94.7% (95% CI 9.13, 99.97%) for trisomy 13, at a combined FPR of about 0.13%. Although there was no obvious difference in performance of screening between the FMF study and that of the other studies, the small number of cases prevented meaningful subgroup analyses, including chorionicity, cfDNA method for analysis, patient's background risk or gestational age at testing. For example, in the case of trisomy 21, 11 of the 13 studies had a very small number of affected pregnancies and they all reported a DR of 100%; however there was a false negative result in each of the two biggest studies, the one by the FMF using targeted testing in 20 cases of trisomy 21 and the one by Chibuk *et al.*,²³ using massively parallel sequencing in 52 cases of trisomy 21.

Comparison with results of previous meta-analyses in twin pregnancies

We performed three prospective studies on screening for fetal trisomies by cfDNA testing in twin pregnancies and used meta-analyses to combine our data with those of previous clinical validation or implementation studies; the inclusion criteria were peer-reviewed studies in twin pregnancies, in which data on pregnancy outcome were provided for more than 85% of the study population. The inclusion of only prospective cohort studies with follow-up in at least 85% of cases was aimed at minimizing reporting bias and the exclusion of case-control studies was aimed at avoiding optimistic bias to the estimates of diagnostic performance. We pooled the sensitivity and specificity estimates using bivariate random-effects regression models in order to account for the potential trade-off between sensitivity and specificity (the sensitivity may improve with worsening in specificity and vice versa) and incorporate this negative correlation into the analysis.

In our first meta-analysis there were five studies and in a total of 24 cases of trisomy 21 and 1111 unaffected cases the DR was 100% and FPR was 0%.¹ In the second meta-analysis there were eight studies and in a total of 56 cases of trisomy 21 and 3718 non-trisomy 21 twin pregnancies the pooled weighted DR and FPR were 98.2% and 0.05%, respectively; the study also reported 18 cases of trisomy

18 with pooled weighted DR of 88.9% and three cases of trisomy 13 with DR of 66.7%.² In our current meta-analysis, the number of included studies increased to 13 and the number of trisomies 21, 18 and 13 increased to 137, 50 and 11, respectively.

There are a further four meta-analyses reporting on the performance of cfDNA testing for trisomies in twin pregnancies. The first examined four studies that included both singletons and twins and three studies that included only twins; it was not possible to extract the number of twin pregnancies that were evaluated but the authors reported that the DR of trisomy 21 in twins was 89.4% (95% CI 75-96%) and the FPR was 0.4%.²⁹ The second meta-analysis examined 10 studies in twin pregnancies, including prospective or retrospective studies, cohort and case-control studies with no criteria on degree of follow up; in a combined total of 69 cases of trisomy 21, 13 cases of trisomy 18 and 3 cases of trisomy 13 the respective DRs were 99%, 85% and 100% and the combined FPR was 0.05%.³⁰ The third meta-analysis examined 25 studies in twin pregnancies, including prospective or retrospective studies, cohort and case-control studies with no criteria on degree of follow up; in a combined total of 199 cases of trisomy 21, 58 cases of trisomy 18 and 14 cases of trisomy 13 the respective DRs were 99%, 88% and the combined FPR was 0%.²⁴ The fourth meta-analysis,²⁵ examined 12 studies in twin pregnancies and although their stated inclusion criteria were identical to those of Gil et al.,² in reality they included one study that was primarily in singleton pregnancies which presumably by accident had a few twin pregnancies³¹ and another in which data on pregnancy outcome were not provided for a minimum of 85% of the study population;³² in a combined total of 74 cases of trisomy 21, 22 cases of trisomy 18 and 5 cases of trisomy 13 the respective DRs were 95%, 82% and 80% and the combined FPR was 0.3%.²⁵

Implication for clinical practice

The finding of high DR and low FPR for trisomy 21 demonstrates the superiority of the cfDNA test over previous methods of screening, including maternal age, second trimester biochemical testing or first trimester combined test.³³ However, the performance of the cfDNA test for trisomy 21 in twin pregnancies may not be as high as that in singleton pregnancies; in our meta-analysis of studies in singleton pregnancies the weighted pooled DR was 99.7% (95% CI, 99.1-99.9%).¹ Similarly, in trisomy 18 and 13 the weighted pooled DR in twin pregnancies was lower than in singleton pregnancies (97.9%, 95% CI, 94.9-99.1% and 99.0%, 95% CI, 65.8-100%, respectively)¹ and the 95% CI was much wider and ranged from 77.9% to 97.9% for trisomy 18 and from 9.1% to 99.97% for trisomy 13, because of the relatively small number of cases. Consequently, parents can be counselled that cfDNA testing in twin pregnancies is mainly useful in screening for trisomy 21 and that there is considerable uncertainty concerning trisomies 18 and 13.

Irrespective of a decision to offer cfDNA testing as first or second line screening for trisomies, a positive or high-risk result should be confirmed by invasive testing. In the case of first-trimester screening and positive cfDNA result for trisomy 21 the diagnostic test can be chorionic villous sampling. In the case of trisomies 18 and 13 a positive result should be followed by a detailed ultrasound examination and if the characteristic defects associated with these trisomies are detected then chorionic villous sampling can be carried out; if no defects are detected in the scan the preferred diagnostic test is amniocentesis to avoid an erroneous result due to placenta confined mosaicism. On the other hand, a negative or low-risk cfDNA result is reassuring that the fetus is unlikely to be affected by the trisomy under investigation. The *posterior* risk for a given patient can be obtained by multiplying the *prior* risk with the negative likelihood ratios calculated in this meta-analysis; the risk for trisomies 21 and 18 is reduced by a factor of 100 and 14, respectively. For example, if prior screening by the combined test had shown that the risk for trisomy 21 was 1 in 10 and cfDNA testing gives a low-risk result the chance that the fetus is affected is 1 in 1000; in contrast, if the risk for trisomy 18 from the combined test was 1 in 2 and cfDNA testing gives a low-risk result the chance that the fetus is affected is 1 in 28.

If cfDNA testing is adopted as a first line method of screening for trisomies in twin pregnancies it is still imperative that women should be offered an 11-14 weeks scan for first, accurate dating of pregnancy from the crown-rump length of the biggest twin, second, diagnosis of chorionicity which is the main determinant of a wide range of adverse outcomes,³⁴ third, measurement of fetal nuchal translucency thickness and assessment of intertwin discordance in crown-rump length which have a strong influence on adverse pregnancy outcomes,^{35,36} fourth, diagnosis of a vanishing twin because this would preclude the use of cfDNA testing for at least 16 weeks from the estimated time of fetal demise,^{37,38} fifth, early diagnosis of major fetal abnormalities,³⁹ sixth, in the presence of nuchal translucency thickness ≥ 3.5 mm and in cases with major defects, such as holoprosencephaly or exomphalos, an invasive test with microarray rather than cfDNA testing would be the appropriate pregnancy management,⁴⁰ seventh, early screening for preterm preeclampsia which is 8-9 times more common than in singleton pregnancies,^{41,42} and eighth, measurement of cervical length because if this is short prophylactic use of vaginal progesterone could substantially reduce the risk of preterm birth.⁴³

In the studies included in this meta-analysis there was a wide range of failure rates to obtain a result from cfDNA testing and there was no obvious relationship between such failure rate and the method, MPSS or targeted, used for the analysis of samples (Table 1). The main cause of cfDNA test failure is reduced fetal fraction; we have previously reported that the median fetal fraction in pregnancies with fetal trisomy 21 is not significantly different from unaffected pregnancies, but in trisomies 18 and 13 the fetal fraction is significantly reduced.⁴⁴ Consequently, in pregnancies with a failed result the risk for trisomies 18 and 13 is increased, but this is not the case for trisomy 21. In pregnancies with cfDNA test failure repeat sampling will provide a result in half to two-thirds of cases.⁴⁴ The options for the management of pregnancies with second failed cfDNA test are invasive testing and no further investigations; an important determinant for selecting the appropriate management option is the estimated risk for trisomies from prior screening and the results of detailed ultrasound examination looking for specific features of trisomies 18 and 13.^{44,45}

Limitations

The main limitation of the FMF study and the meta-analysis relates to the relatively small number of trisomic twin pregnancies investigated by cfDNA testing of maternal blood by comparison with singleton pregnancies;¹ one of the consequences of small numbers is the wide 95% CI in DR.

In the assessment of the quality of the included studies, all of them were considered to be at high-risk of selection bias and at high-risk of concerns regarding applicability in relation to patient selection because they were not performed as part of routine primary screening but they were carried out in preselected populations, where a previous method of screening had already been performed, including maternal age, first trimester combined test or second trimester serum biochemistry. However, unlike predictive values, the ability to detect aneuploidy with cfDNA analysis is dependent upon assay precision and fetal DNA percentage in the sample rather than the prevalence of the disease in the study population; therefore, detection rate and false positive rate would remain the same if only routine populations were included. Most studies were also classified as being at high-risk of bias in relation to flow and timing; this is essentially because cfDNA testing did not provide results in all cases, there was no complete follow up, or the method of determining outcome was not the same in all cases. However, such criticisms could be applied to any clinical study; all methods of traditional screening occasionally fail to give a result and no screening study in pregnancy can have complete follow up, especially because some women miscarry and karyotyping is not carried out.

Conclusions

In twin pregnancies the reported DR of trisomy 21 by cfDNA testing is high, but lower than in singleton pregnancies, whereas the FPR appears to be equally low. The number of cases of trisomies 18 and more so trisomy 13 is too small for accurate assessment of the predictive performance of the cfDNA test.

REFERENCES

1. Gil MM, Accurti V, Santacruz B, Plana MN, Nicolaides KH. Analysis of cell-free DNA in maternal blood in screening for aneuploidies: updated meta-analysis. *Ultrasound Obstet Gynecol* 2017; **50**: 302-314.
2. Gil MM, Galeva S, Jani J, Konstantinidou L, Akolekar R, Plana MN, Nicolaides KH. Screening for trisomies by cfDNA testing of maternal blood in twin pregnancy: update of The Fetal Medicine Foundation results and meta-analysis. *Ultrasound Obstet Gynecol* 2019; **53**: 734-742.
3. Robinson HP, Fleming JE. A critical evaluation of sonar crown rump length measurements. *Br J Obstet Gynaecol* 1975; **82**: 702-710.
4. Sepulveda W, Sebire NJ, Hughes K, Odibo A, Nicolaides KH. The lambda sign at 10-14 weeks of gestation as a predictor of chorionicity in twin pregnancies. *Ultrasound Obstet Gynecol* 1996; **7**: 421-423.
5. Sparks AB, Struble CA, Wang ET, Song K, Oliphant A. Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18. *Am J Obstet Gynecol* 2012; **206**: 319.e1-9.
6. Ashoor G, Syngelaki A, Wang E, Struble C, Oliphant A, Song K, Nicolaides KH. Trisomy 13 detection in the first trimester of pregnancy using a chromosome-selective cell-free DNA analysis. *Ultrasound Obstet Gynecol* 2013; **41**: 21-25.
7. Struble CA, Syngelaki A, Oliphant A, Song K, Nicolaides KH. Fetal fraction estimate in twin pregnancies using directed cell-free DNA analysis. *Fetal Diagn Ther* 2014; **35**: 199-203.
8. Juneau K, Bogard PE, Huang S, Mohseni M, Wang ET, Ryvkin P, Kingsley C, Struble CA, Oliphant A, Zahn JM. Microarray-based cell-free DNA analysis improves noninvasive prenatal testing. *Fetal Diagn Ther* 2014; **36**: 282-286.
9. Reitsma JB, Glas AS, Rutjes AW, Scholten RJ, Bossuyt PM, Zwinderman AH. Bivariate analysis of sensitivity and specificity produces informative summary measures in diagnostic reviews. *J Clin Epidemiol* 2005; **58**: 982-990.
10. Deeks JJ, Macaskill P, Irwig L. The performance of tests of publication bias and other sample size effects in systematic reviews of diagnostic test accuracy was assessed. *J Clin Epidemiol* 2005; **58**: 882-893.
11. StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, USA.
12. Sarno L, Revello R, Hanson E, Akolekar R, Nicolaides KH. Prospective first-trimester screening for trisomies by cell-free DNA testing of maternal blood in twin pregnancy. *Ultrasound Obstet Gynecol* 2016; **47**: 705-711.
13. Galeva S, Konstantinidou L, Gil MM, Akolekar R, Nicolaides KH. Routine first-trimester screening for fetal trisomies in twin pregnancies: cell-free DNA test contingent on results from the combined test. *Ultrasound Obstet Gynecol* 2019; **53**: 208-213.

14. Lau TK, Jiang F, Chan MK, Zhang H, Lo PS, Wang W. Non-invasive prenatal screening of fetal Down syndrome by maternal plasma DNA sequencing in twin pregnancies. *J Matern Fetal Neonatal Med* 2013; **26**: 434-437.
15. Huang X, Zheng J, Chen M, Zhao Y, Zhang C, Liu L, Xie W, Shi S, Wei Y, Lei D, Xu C, Wu Q, Guo X, Shi X, Zhou Y, Liu Q, Gao Y, Jiang F, Zhang H, Su F, Ge H, Li X, Pan X, Chen S, Chen F, Fang Q, Jiang H, Lau TK, Wang W. Noninvasive prenatal testing of trisomies 21 and 18 by massively parallel sequencing of maternal plasma DNA in twin pregnancies. *Prenat Diagn* 2014; **34**: 335-340.
16. Tan Y, Gao Y, Lin G, Fu M, Li X, Yin X, Du J, Li J, Li W, Peng H, Yuan Y, Chen F, Jiang F, Zhang H, Lu G, Gong F, Wang W. Noninvasive prenatal testing (NIPT) in twin pregnancies with treatment of assisted reproductive techniques (ART) in a single center. *Prenat Diagn* 2016; **36**: 672-679.
17. Du E, Feng C, Cao Y, Yao Y, Lu J, Zhang Y. Massively Parallel Sequencing (MPS) of Cell-Free Fetal DNA (cffDNA) for Trisomies 21, 18, and 13 in twin pregnancies. *Twin Res Hum Genet* 2017; **20**: 242-249.
18. Le Conte G, Letourneau A, Jani J, Kleinfinger P, Lohmann L, Costa JM, Benachi A. Cell-free fetal DNA analysis in maternal plasma as screening test for trisomies 21, 18 and 13 in twin pregnancy. *Ultrasound Obstet Gynecol* 2018; **52**: 318-324.
19. Yang J, Qi Y, Hou Y, Guo F, Peng H, Wang D, Haoxin OY, Wang Y, Huang H, Yin A. Performance of non-invasive prenatal testing for trisomies 21 and 18 in twin pregnancies. *Mol Cytogenet* 2018; **11**: 4-9.
20. Norwitz ER, McNeill G, Kalyan A, Rivers E, Ahmed E, Meng L, Vu P, Egbert M, Shapira M, Kobara K, Parmar S, Goel S, Prins SA, Aruh I, Persico N, Robins JC, Kirshon B, Demko ZP, Ryan A, Billings PR, Rabinowitz M, Benn P, Martin KA, Hedriana HL. Validation of a single-nucleotide polymorphism-based non-invasive prenatal test in twin gestations: determination of zygosity, individual fetal sex, and fetal aneuploidy. *J Clin Med* 2019; **8**: 937.
21. Yin Y, Zhu H, Qian Y, Jin J, Mei J, Dong M. Noninvasive prenatal screening for twin pregnancy: an analysis of 2057 cases. *Journal of Zhejiang University (Medicine Edition)* 2019; **48**: 403-408.
22. Yu W, Lv Y, Yin S, Liu H, Li X, Liang B, Kong L, Liu C. Screening of fetal chromosomal aneuploidy diseases using noninvasive prenatal testing in twin pregnancies. *Expert Rev Mol Diagn* 2019; **19**: 189-196.
23. Chibuk J, Rafalko J, Boomer T, McCullough R, McLennan G, Wyatt P, Almasri E. Cell-free DNA screening in twin pregnancies: A more accurate and reliable screening tool. *Prenat Diagn* 2020; **40**: 1321-1329.
24. He Y, Wang Y, Li Z, Chen H, Deng J, Huang H, He X, Zeng W, Liu M, Huang B, Chen P. Clinical performance of non-invasive prenatal testing for trisomies 21, 18 and 13 in twin pregnancies: A cohort study and a systematic meta-analysis. *Acta Obstet Gynecol Scand* 2020; **99**: 731-743.
25. Khalil A, Archer R, Hutchinson V, Mousa HA, Johnstone ED, Cameron MJ, Cohen KE, Ioannou C, Kelly B, Reed K, Hulme R, Papageorgiou AT. Noninvasive prenatal screening in twin pregnancies with cell-free DNA using the IONA test: a prospective multicenter study. *Am J Obstet Gynecol* 2021 Jan 15;S0002-9378(21)00028-4.

26. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, Leeflang MM, Sterne JA, Bossuyt PM; QUADAS-2 Group. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Ann Intern Med* 2011; **155**: 529-536.
27. Plana MN, Pérez T, Zamora J. New measures improved the reporting of heterogeneity in diagnostic test accuracy reviews: a metaepidemiological study. *J Clin Epidemiol* 2021; **131**:101-112.
28. Galeva S, Gil MM, Konstantinidou L, Akolekar R, Nicolaides KH. First-trimester screening for trisomies by cfDNA testing of maternal blood in singleton and twin pregnancies: factors affecting test failure. *Ultrasound Obstet Gynecol* 2019; **53**: 804-809.
29. Taylor-Phillips S, Freeman K, Geppert J, Agbebiyi A, Uthman OA, Madan J, Clarke A, Quenby S, Clarke A. Accuracy of non-invasive prenatal testing using cell-free DNA for detection of Down, Edwards and Patau syndromes: a systematic review and meta-analysis. *BMJ Open* 2016; **6**: e010002.
30. Liao H, Liu S, Wang H. Performance of non-invasive prenatal screening for fetal aneuploidy in twin pregnancies: a meta-analysis. *Prenat Diagn* 2017; **37**: 874–882.
31. Garshasbi M, Wang Y, Zadeh S, Giti S, Piri S, Hekmat M. Clinical application of cell-free DNA sequencing-based non-invasive prenatal testing for trisomies 21, 18, 13 and sex chromosome aneuploidy in a mixed-risk population in Iran. *Fetal Diagn Ther* 2020; **47**: 220-227.
32. Motevasselian, M, Saleh Gargari, S, Younesi, S, Pooransari P, Saadati P, Mirzamoradi M, Savad S, Mahdi Taheri Amin M, Modarresi M-H, Afrakhteh M, Ghafouri-Fard S. Non-invasive prenatal test to screen common trisomies in twin pregnancies. *Mol Cytogenet* 2020; **13**: 5.
33. Audibert F, Gagnon, A. Prenatal screening for and diagnosis of aneuploidy in twin pregnancies. *J Obstet Gynaecol Canada* 2017;**39**:e329-46.
34. Litwinska E, Syngelaki A, Cimpoca B, Frei L, Nicolaides KH. Outcome of twin pregnancy with two live fetuses at 11-13 weeks' gestation. *Ultrasound Obstet Gynecol* 2020; **55**: 32-38.
35. Cimpoca B, Syngelaki A, Litwinska E, Muzaferovic A, Nicolaides KH. Increased nuchal translucency at 11-13 weeks' gestation and outcome in twin pregnancy. *Ultrasound Obstet Gynecol* 2020; **55**: 318-325.
36. Litwinska E, Syngelaki A, Cimpoca B, Sapantzoglu I, Nicolaides KH. Intertwin discordance in fetal size at 11-13 weeks' gestation and pregnancy outcome. *Ultrasound Obstet Gynecol* 2020; **55**: 189-197.
37. Chaveeva P, Wright A, Syngelaki A, Konstantinidou L, Wright D, Nicolaides KH. First-trimester screening for trisomies in pregnancies with vanishing twin. *Ultrasound Obstet Gynecol* 2020; **55**: 326-331.
38. Chen M, Su F, Wang J, Zhou L, Liu Q, Chai X, Yuan Y, Cen M, Wu Y, Wang Y, Chen F, Zhang Y, Chen D, Gao Y. Temporal persistence of residual fetal cell-free DNA from a deceased cotwin after

selective fetal reduction in dichorionic diamniotic twin pregnancies. *Prenat Diagn* 2021 Jan 13. doi: 10.1002 /pd.5898.

39. Syngelaki A, Cimpoa B, Litwinska E, Akolekar R, Nicolaides KH. Diagnosis of fetal defects in twin pregnancies at routine 11-13-week ultrasound examination. *Ultrasound Obstet Gynecol* 2020; **55**: 474-481.
40. Syngelaki A, Guerra L, Ceccacci I, Efeturk T, Nicolaides KH. Impact of holoprosencephaly, exomphalos, megacystis and increased nuchal translucency on first-trimester screening for chromosomal abnormalities. *Ultrasound Obstet Gynecol* 2017; **50**: 45-48.
41. Francisco C, Wright D, Benkő Z, Syngelaki A, Nicolaides KH. Hidden high rate of pre-eclampsia in twin compared with singleton pregnancy. *Ultrasound Obstet Gynecol* 2017; **50**: 88-92.
42. Benkő Z, Wright A, Rehal A, Cimpoa B, Syngelaki A, Delgado JL, Tsokaki T, De Alvarado M, Vojtassakova D, Malligiannis Ntalianis K, Chaveeva P, Del Campo A, De Ganzo T, Resta C, Atanasova V, Accurti V, Villalain C, Aguilera J, Dojcinovska D, O'Gorman N, Plasencia W, Zingler E, Dutemeyer V, Alvar B, Casanova MC, Nicolaides KH. Prediction of pre-eclampsia in twin pregnancy by maternal factors and biomarkers at 11-13 weeks' gestation: data from EVENTS trial. *Ultrasound Obstet Gynecol* 2021; **57**: 257-265.
43. Rehal A, Benkő Z, De Paco Matallana C, Syngelaki A, Janga D, Cicero S, Akolekar R, Singh M, Chaveeva P, Burgos J, Molina FS, Savvidou M, De La Calle M, Persico N, Quezada Rojas MS, Sau A, Greco E, O'Gorman N, Plasencia W, Pereira S, Jani JC, Valino N, Del Mar Gil M, Maclagan K, Wright A, Wright D, Nicolaides KH. Early vaginal progesterone versus placebo in twin pregnancies for the prevention of spontaneous preterm birth: a randomized, double-blind trial. *Am J Obstet Gynecol* 2021; **224**: 86.e1-86.e19.
44. Revello R, Sarno L, Ispas A, Akolekar R, Nicolaides KH. Screening for trisomies by cell-free DNA testing of maternal blood: consequences of a failed result. *Ultrasound Obstet Gynecol* 2016; **47**: 698-704.
45. Sarno L, Revello R, Hanson E, Akolekar R, Nicolaides KH. Prospective first-trimester screening for trisomies by cell-free DNA testing of maternal blood in twin pregnancy. *Ultrasound Obstet Gynecol* 2016; **47**: 705-711.

FIGURE LEGENDS

Figure 1. Flow chart for the Fetal Medicine Foundation study.

Figure 2. Flow chart for the systematic review.

Figure 3. Summary of the quality of included studies using the Quality Assessment tool for Diagnostic Accuracy Studies (QUADAS-2) checklist.

Figure 4. Forest plots of sensitivity and specificity with 95% confidence intervals (CI) and weighted pooled summary statistics using bivariate random-effects model in assessing cell-free DNA analysis in screening for trisomy 21.

Figure 5. Forest plots of sensitivity and specificity with 95% confidence intervals (CI) and weighted pooled summary statistics using bivariate random-effects model in assessing cell-free DNA analysis in screening for trisomy 18.

Figure 6. Forest plots of sensitivity and specificity with 95% confidence intervals (CI) and weighted pooled summary statistics using bivariate random-effects model in assessing cell-free DNA analysis in screening for trisomy 13.

Figure 7. Funnel plots demonstrating assessment of publication bias of studies reporting on screening for trisomy 21. A vertical pattern indicates no publication bias, whereas a slope indicates a degree of bias.

Table 1. Summary of characteristics of studies reporting on cell-free (cf) DNA analysis of maternal blood in screening for trisomies (T) 21, 18 and 13 in twin pregnancies.

Study	cf DNA testing				Pregnancies	cfDNA result	Outcome	Monochorionic	Karyotype		
	Method	GA (weeks)	Population	Aneuploidy					T21	T18	T13
Lau (2013) ¹⁴	MPSS	13 (11-20)	High-risk	T21	12	12 (100%)	12 (100%)	2 (16.7%)	1		
Huang (2014) ¹⁵	MPSS	19 (11-36)	High-risk	T21, T18	189	189 (100%)	189 (100%)	33 (17.5%)	9	2	
Tan (2016) ¹⁶	MPSS	12 (11-28)	Mixture	T21, T18, T13	565	560 (99.1%)	510 (91.1%)	(3.2%)*	4		
Du (2017) ¹⁷	MPSS	18 (14-23)	High-risk	T21, T18, T13	91	91 (100%)	91 (100%)	39 (42.9%)	2		
Le Conte (2018) ¹⁸	MPSS	16 (10-35)	Mixture	T21, T18, T13	492	-	420 (85.4%)	86 (20.6%)	3	1	
Yang (2018) ¹⁹	MPSS	≥ 9	Mixture	T21, T18, T13	432	432 (100%)	373 (86.3%)	(22.0%)*	4	1	
Norwitz (2019) ²⁰	Targeted	>9	Mixture	T21, T18, T13	117	107 (91.5%)	107 (100%)	-	5	5	1
Yin (2019) ²¹	MPSS	(12-27)	Mixture	T21, T18, T13	2057	2057 (100%)	2004 (97.4%)	-	7	2	0
Yu (2019) ²²	MPSS	18 (8-31)	Mixture	T21, T18, T13	1160	1160 (100%)	1157 (99.7%)	308 (26.6%)	16	4	1
Chibuk (2020) ²³	MPSS	12 (9-34)	High-risk	T21, T18, T13	422	387 (91.7%)	385 (99.5%)	-	52	24	6
He (2020) ²⁴	MPSS	16 (10-23)	Mixture	T21, T18, T13	146	146 (100%)	141 (96.6%)	39 (27.7%)	1		
Khalil (2021) ²⁵	MPSS	16 (10-36)	Mixture	T21, T18, T13	998	995 (99.7%)	958 (96.3%)	276 (28.8%)	13	1	1
Current study	Targeted	11 (10-14)	Mixture	T21, T18, T13	1441	1366 (94.8%)	1271 (93.0%)	187 (14.7%)	20	10	2

Only first author is given for each study. MPSS, massively parallel shotgun sequencing. Gestational age (GA) is given as median or mean (range).

* this is the value in the original sample before exclusions for failed results and no follow up.

Table 2. Studies reporting on the application of cell-free DNA analysis of maternal blood in screening for trisomy 21.

Study	Trisomy 21		Non trisomy 21	
	Total	Detection rate n (% , 95% CI)	Total	False positive rate n (% , 95% CI)
Lau (2013) ¹⁴	1	1 (100, 2.5 - 100)	11	0 (0, 0 - 28.49)
Huang (2014) ¹⁵	9	9 (100, 66.4 - 100)	180	0 (0, 0 - 2.03)
Tan (2016) ¹⁶	4	4 (100, 39.8 - 100)	506	0 (0, 0 - 0.73)
Du (2017) ¹⁷	2	2 (100, 15.8 - 100)	89	0 (0, 0 - 4.06)
Le Conte (2018) ¹⁸	3	3 (100, 29.2 - 100)	415	1 (0.24, 0.01 - 1.34)
Yang (2018) ¹⁹	4	4 (100, 39.8 - 100)	396	0 (0, 0 - 0.93)
Norwitz (2019) ²⁰	5	5 (100, 47.8 -100)	102	0 (0, 0 - 3.55)
Yin (2019) ²¹	7	7 (100, 59.0 - 100)	1997	1 (0.05, 0 - 0.28)
Yu (2019) ²²	16	16 (100, 79.4 - 100)	1141	0 (0, 0 - 0.32)
Chibuk (2020) ²³	52	51 (98.1, 89.7 - 100)	333	13 (3.9, 2.09 - 6.58)
He (2020) ²⁴	1	1 (100, 2.5 - 100)	140	0 (0, 0 - 2.60)
Khalil (2021) ²⁵	13	13 (100, 75.3 - 100)	945	0 (0, 0 - 0.39)
Current study	20	19 (95.0, 75.1 - 100)	1252	1 (0.08, 0 - 0.44)
Pooled analysis, %, (95% CI)*	137	99.0 (92.0 - 100)	7507	0.02 (0.001 - 0.43)
Heterogeneity assessment	0.115		5.264	
Likelihood ratio positive, (95% CI)	4224 (230 - 77525)			
Likelihood ratio negative, (95% CI)	0.010 (0.001 - 0.085)			

Only first author is given for each study. Cases with mosaicism were excluded from calculations.

*Bivariate random-effects model; CI, confidence interval.

Table 3. Studies reporting on the application of cell-free DNA analysis of maternal blood in screening for trisomy 18.

Study	Trisomy 18		Non trisomy 18	
	Total	Detection rate n (% , 95% CI)	Total	False positive rate n (% , 95% CI)
Huang (2014) ¹⁵	2	1 (50.0, 1.3 - 98.7)	187	0 (0, 0 - 1.95)
Le Conte (2018) ¹⁸	1	1 (100, 2.5 - 100)	417	0 (0, 0 - 0.88)
Yang (2018) ¹⁹	1	1 (100, 2.5 - 100)	399	0 (0, 0 - 0.92)
Norwitz (2019) ²⁰	5	5 (100, 47.8 - 100)	102	0 (0, 0 - 3.55)
Yin (2019) ²¹	2	2 (100, 15.8 - 100)	2002	0 (0, 0 - 0.18)
Yu (2019) ²²	4	4 (100, 39.8 - 100)	1153	0 (0, 0 - 0.32)
Chibuk (2020) ²³	24	24 (100, 85.8 - 100)	361	4 (1.11, 0.30 - 2.81)
Khalil (2021) ²⁵	1	0 (0, 0 - 97.5)	957	1 (0.10, 0 - 0.58)
Current study	10	9 (90.0, 55.5 - 99.7)	1262	0 (0, 0 - 0.29)
Pooled analysis, %, (95% CI)*	50	92.8 (77.6 – 98.0)	6840	0.01 (0.00 - 0.44)
Heterogeneity assessment	0.458		5.079	
Likelihood ratio positive, (95% CI)	6198 (253 - 151590)			
Likelihood ratio negative, (95% CI)	0.072 (0.021 - 0.240)			

Only first author is given for each study. Cases with mosaicism were excluded from calculations.

*Bivariate random-effects model; CI, confidence interval.

Table 4. Studies reporting on the application of cell-free DNA analysis of maternal blood in screening for trisomy 13.

Study	Trisomy 13		Non trisomy 13	
	Total	Detection rate n (%; 95% CI)	Total	False positive rate n (%; 95% CI)
Le Conte (2018) ¹⁸	0	-	418	1 (0.24, 0.01 - 1.33)
Norwitz (2019) ²⁰	1	1 (100, 2.5 - 100)	106	0 (0, 0 - 3.42)
Yin 2019 ²¹	0	-	2004	1 (0.05, 0 - 0.28)
Yu (2019) ²²	1	1 (100, 2.5 - 100)	1156	0 (0, 0 - 0.32)
Chibuk (2020) ²³	6	6 (100, 54.1 - 100)	379	3 (0.79, 0.16 - 2.30)
Khalil (2021) ²⁵	1	1 (100, 2.5 - 100)	957	0 (0, 0 - 0.38)
Current study	2	1 (50, 1.3 - 98.7)	1270	4 (0.31, 0.09 - 0.80)
Pooled analysis, %, (95% CI)*	11	94.7 (9.14 - 99.97)	6290	0.10 (0.03 - 0.39)
Heterogeneity assessment		1.555		1.236
Likelihood ratio positive, (95% CI)		916 (226 - 3714)		
Likelihood ratio negative, (95% CI)		0.053 (0.000 - 7.173)		

Only first author is given for each study. Cases with mosaicism were excluded from calculations.

*Bivariate random-effects model; CI, confidence interval.