

## OBSTETRICS

# Noninvasive prenatal screening in twin pregnancies with cell-free DNA using the IONA test: a prospective multicenter study



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**BACKGROUND:** In singleton pregnancies, studies investigating cell-free DNA in maternal blood have consistently reported high detection rate and low false-positive rate for the 3 common fetal trisomies (trisomies 21, 18, and 13). The potential advantages of noninvasive prenatal testing in twin pregnancies are even greater than in singletons, in particular lower need for invasive testing and consequent fetal loss rate. However, several organizations do not recommend cell-free DNA in twin pregnancies and call for larger prospective studies.

**OBJECTIVE:** In response to this, we undertook a large prospective multicenter study to establish the screening performance of cell-free DNA for the 3 common trisomies in twin pregnancies. Moreover, we combined our data with that reported in published studies to obtain the best estimate of screening performance.

**STUDY DESIGN:** This was a prospective multicenter blinded study evaluating the screening performance of cell-free DNA in maternal plasma for the detection of fetal trisomies in twin pregnancies. The study took place in 6 fetal medicine centers in England, United Kingdom. The primary outcome was the screening performance and test failure rate of cell-free DNA using next generation sequencing (the IONA test). Maternal blood was taken at the time of (or after) a conventional screening test. Data were collected at enrolment, at any relevant invasive testing throughout pregnancy, and after delivery until the time of hospital discharge. Prospective detailed outcome ascertainment was undertaken on all newborns. The study was undertaken and reported according to the Standards for Reporting of Diagnostic Accuracy Studies. A pooled analysis was also undertaken using our data and those in the studies identified by a literature search (MEDLINE, Embase, CENTRAL, Cochrane Library, and [ClinicalTrials.gov](https://www.clinicaltrials.gov)) on June 6, 2020.

**RESULTS:** A total of 1003 women with twin pregnancies were recruited, and complete data with follow-up and reference data were available for 961 (95.8%); 276 were monochorionic and 685 were dichorionic. The failure

rate was 0.31%. The mean fetal fraction was 12.2% (range, 3%–36%); all 9 samples with a 3% fetal fraction provided a valid result. There were no false-positive or false-negative results for trisomy 21 or trisomy 13, whereas there was 1 false-negative and 1 false-positive result for trisomy 18. The IONA test had a detection rate of 100% for trisomy 21 ( $n=13$ ; 95% confidence interval, 75–100), 0% for trisomy 18 ( $n=1$ ; 95% confidence interval, 0–98), and 100% for trisomy 13 ( $n=1$ ; 95% confidence interval, 3–100). The corresponding false-positive rates were 0% (95% confidence interval, 0–0.39), 0.10% (95% confidence interval, 0–0.58), and 0% (95% confidence interval, 0–0.39), respectively. By combining data from our study with the 11 studies identified by literature search, the detection rate for trisomy 21 was 95% ( $n=74$ ; 95% confidence interval, 90–99) and the false-positive rate was 0.09% ( $n=5598$ ; 95% confidence interval, 0.03–0.19). The corresponding values for trisomy 18 were 82% ( $n=22$ ; 95% confidence interval, 66–93) and 0.08% ( $n=4869$ ; 95% confidence interval, 0.02–0.18), respectively. There were 5 cases of trisomy 13 and 3881 non-trisomy 13 pregnancies, resulting in a computed average detection rate of 80% and a false-positive rate of 0.13%.

**CONCLUSION:** This large multicenter study confirms that cell-free DNA testing is the most accurate screening test for trisomy 21 in twin pregnancies, with screening performance similar to that in singletons and very low failure rates (0.31%). The predictive accuracy for trisomies 18 and 13 may be less. However, given the low false-positive rate, offering first-line screening with cell-free DNA to women with twin pregnancy is appropriate in our view and should be considered a primary screening test for trisomy 21 in twins.

**Key words:** aneuploidy, cell-free fetal DNA, detection rate, diagnostic accuracy, Down syndrome, false-positive rate, NIPT, noninvasive, screening, sensitivity, specificity, trisomy, twin

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## Introduction

Many health systems offer routine antenatal screening for trisomy 21 (Down syndrome), which is the most common chromosomal abnormality at birth. In the first trimester of pregnancy, screening tests available include ultrasound measurement of the fetal nuchal translucency and other sonographic markers, to which measurement of maternal serum free beta human chorionic gonadotropin and pregnancy-associated plasma protein A can be

added (the “combined test”). More recently, cell-free DNA (cfDNA) in maternal blood has been either utilized as a first-line screening test or offered to women who after combined screening demonstrate an intermediate or high chance (in contingent screening)<sup>1</sup> or a high chance (in secondary screening).<sup>2</sup>

In singleton pregnancies, the combined test has a detection rate (DR) for trisomy 21 of 90% and a false-positive rate (FPR) of 5%.<sup>3</sup> The chief advantages of cfDNA screening are a higher

## AJOG at a Glance

**Why was this study conducted?**

National and international guidelines highlighted that the total number of reported affected cases in studies reporting on the predictive accuracy of cell-free DNA for common trisomies in twins is small and called for more studies.

**Key findings**

The screening performance of noninvasive prenatal testing for trisomy 21 in twin pregnancies is similar to what is reported in singletons. However, the predictive accuracy for trisomies 18 and 13 may be less.

**What does this add to what is known?**

This prospective multicenter study included nearly 1000 twin pregnancies and reported very low failure rate (<0.5%) and excellent predictive accuracy for trisomy 21. The detection rate for trisomy 21 according to pooled data from 5598 twin pregnancies and 74 cases of trisomy 21 is 95% for a false-positive rate of 0.1%.

associated DR of 99.7% (95% confidence interval [CI], 99.1–99.9) and much lower FPR of 0.04% (95% CI, 0.02–0.07), which translates into a much lower need for invasive testing and consequent fetal loss rate.<sup>4</sup>

In principle, these advantages of cfDNA screening should be greater still in twins: this is because the incidence of trisomy 21 is higher in twin than singleton pregnancies<sup>5</sup> and because combined screening in twin pregnancies has a lower DR (75%) and higher FPR (9% of pregnancies and 7% of fetuses).<sup>6</sup> This high FPR is of particular concern because pregnancy loss rates after invasive prenatal diagnosis are higher in twin pregnancies<sup>7–9</sup> and because of the additional complexity of selective termination of pregnancy, an option that is given when there is discordance between fetuses in any abnormality, which carries additional risks to the ongoing pregnancy.<sup>10</sup>

However, there is greater uncertainty regarding the predictive accuracy of cfDNA in twin pregnancies than that of singleton pregnancies.<sup>10</sup> Studies in singletons are very large, with a recent meta-analysis including 1963 cases of trisomy 21 and 223,932 of non-trisomy 21, 563 cases of trisomy 18 and 222,013 unaffected pregnancies, and 119 cases of trisomy 13 and 212,883 unaffected pregnancies.<sup>4</sup> In contrast, data in twin pregnancies are much scarcer. A recent meta-analysis included only 7 studies, 56

cases with trisomy 21 and 3718 non-trisomy 21 twin pregnancies, 18 cases of trisomy 18 and 3143 non-trisomy 18 pregnancies, and 3 affected cases with trisomy 13. The respective pooled weighted DR and FPR were 98.2% (95% CI, 83.2–99.8) and 0.05% (95% CI, 0.01–0.26) for trisomy 21 and 88.9% (95% CI, 64.8–97.2) and 0.03% (95% CI, 0.00–0.33) for trisomy 18, whereas for trisomy 13, based on just 3 cases, DR was 66.7% and FPR was 0.19%. It is of note that screening characteristics in the individual studies included varied: for trisomy 21, the DR ranged from 94.1% and 100% and FPR between 0% and 0.24%, whereas for trisomy 18, the DR was from 50.0% and 100% and FPR between 0% and 0.10% (the numbers for trisomy 13 are too small to report).

A lower screening performance of cfDNA testing in twin pregnancies may be expected biologically because of a number of factors. Aneuploidy in dizygotic twins is likely to affect only 1 fetus; however, the cfDNA contribution of the 2 fetuses can vary up to 2-fold.<sup>11,12</sup> In addition, the fetal fraction of cfDNA in the maternal circulation is lower in singleton pregnancies when the fetus is affected by trisomy 21,<sup>13</sup> and this is likely also true in dizygotic twin pregnancies discordant for aneuploidy, so that a high contribution from a normal cotwin could “mask” the low fetal fraction of the

affected fetus, leading to a false-negative result. This challenge in the use of cfDNA in dizygotic twin pregnancies could explain the higher failure rate (almost double) in dichorionic than monochorionic twins.<sup>14</sup> Moreover, other factors known to be associated with a lower fetal fraction in singleton pregnancies include in vitro fertilization (IVF) conception and higher maternal weight, 2 factors that are more common in twin than singleton pregnancies.<sup>13</sup>

In part owing to these concerns on the small number of twin pregnancies affected by common trisomies that are reported in the published literature, national guidelines call for more data on the performance of screening by cfDNA testing in twin pregnancies.<sup>15</sup> In response to this, we undertook a large, prospective, multicenter study to establish the screening performance of cfDNA testing for the 3 common fetal trisomies (trisomies 21, 18, and 13) in twin pregnancies and to assess the performance of the test in different types of twin pregnancies including dizygotic dichorionic, monozygotic dichorionic, and monozygotic monochorionic. Finally, we combined our data from those available in the literature to obtain the best estimate of screening performance based on the totality of the evidence.

**Methods**

This is a prospective, multicenter study evaluating the screening characteristics of measurement of maternal cfDNA in maternal plasma samples, obtained from women with a twin pregnancy, for the detection of fetal trisomies. The study was undertaken in 6 fetal medicine centers in England: St George's University Hospitals NHS Foundation Trust, Leicester Royal Infirmary, the Central Manchester University Hospitals NHS Foundation Trust, Norfolk and Norwich University Hospitals NHS Foundation Trust, Leeds General Infirmary, and John Radcliffe Hospital in Oxford, United Kingdom. Participants were recruited between February 2015 and June 2018. The study was undertaken and reported according to the 2015 Standards for Reporting of Diagnostic Accuracy Studies.<sup>16</sup>

## Participants

Eligible women were at least 16 years old with fetal ultrasound demonstrating a viable twin pregnancy of at least 10 weeks' gestation and were considered able to give their informed consent and not from a vulnerable group. The definition of vulnerable group was in accordance with ISO14155:2020 and excluded the following subjects: participants who have Down syndrome or other chromosomal abnormality themselves, children under 16 years old, adults with learning disabilities or mental illness or who are unable to give informed consent for themselves, adults who are unconscious or very severely ill, adults who have a terminal illness or current malignancy, adults in emergency situations, prisoners and young offenders, or any person considered to have a particularly dependent relationship with investigators. The exclusion criteria included higher order multiple pregnancies, fetal demise or vanishing twin, known mosaicism, partial trisomy or translocations, or known aneuploidy or malignancy in the pregnant woman.

After providing a written informed consent, participating women provided a blood sample at the time of (or after) a conventional screening test, such as combined test, quadruple serum screening, screening ultrasound scan, or other clinically available cfDNA test. We included 2 groups of pregnant women with twin pregnancies: the first group consisted of those with a low chance of carrying a fetus with a chromosomal abnormality, on the basis of the conventional prenatal screening tests; the second group included women with a high chance, on the basis of conventional prenatal screening tests (in the United Kingdom, this is defined as greater than 1:150 at term), and who attended the fetal medicine clinics at the study sites for prenatal counseling and possible diagnostic testing (chorionic villus sampling [CVS] or amniocentesis).

Information was collected at subject recruitment and included demographic characteristics (maternal age, parity, height, weight) and current pregnancy history (method of conception,

screening test results, relevant ultrasound findings, chorionicity, estimated due date, and gestational age at blood sampling). Data collection forms were completed at enrolment, at any relevant invasive testing throughout pregnancy, and at follow-up (delivery and until the time of hospital discharge). Prospective detailed outcome ascertainment was undertaken on all newborns at each center, including birth outcome, the presence of any congenital abnormalities, phenotypic appearance at birth, and any chromosomal testing undertaken in the antenatal or immediate postnatal period (such as CVS, amniocentesis, or postnatal cord blood testing). For birth outcome, where newborn examination results were not available directly, data were obtained from referring hospitals or through direct contact with the participant. There were 103 cases where amniocentesis was performed and 12 who had CVS, whereas the birth outcomes were obtained by newborn examination, from data from referring hospitals, or directly from mother or birth outcome in 846 participants.

The test laboratory staff were blinded to any ultrasound or clinical test results, including the karyotype. Test results of cfDNA testing were made available to the clinicians managing the pregnant women participants.

## Test characteristics

All tests were undertaken using the IONA test, using next generation sequencing, and using a proprietary algorithm. The IONA test was Conformité Européenne (CE) marked in February 2015 for the screening of trisomies 21, 18, and 13 by using a sample of maternal blood, which contains fragments of cfDNA from both the placenta and the mother. A CE mark (CE marking, which represents regulatory approval of a medical device in the United Kingdom and Europe) is placed on medical devices to show they conform to the requirements in the directives. It shows that the device is fit for its intended purpose stated and meets legislation relating to safety. Although considered

validated for use in twin pregnancies in the Instructions for Use, only 11 twin pregnancies were included in the validation study for CE marking, all of which were correctly assayed.<sup>17</sup>

## Cell-free DNA preparation and sequencing

At each site, peripheral maternal blood (10 mL) was collected into standard Streck blood sample tubes from each participant. The samples were then sent to a central laboratory (Yourgene Health Clinical Service Laboratory, Manchester, United Kingdom) to be tested for trisomies 21, 18, and 13 using the IONA test. Samples were initially processed to isolate cfDNA and prepared for sequencing following methods detailed by Crea et al.<sup>18</sup> On arrival at the central laboratory, the maternal blood samples were centrifuged at 1600 grams for 10 minutes and the plasma fraction removed; this was then centrifuged for a further 10 minutes at 16,000 grams to remove any cellular material. Unless analyzed on the same day as being received, the plasma samples were held at  $-80^{\circ}\text{C}$ . Samples were either analyzed on the day of arrival or held frozen, for up to a week if insufficient samples were available to make up an analysis run. Before analysis, the plasma sample was defrosted and centrifuged for 1 minute at 3000 grams. The resultant cfDNA was isolated, extracted, and sequenced from 1.0 to 2.5 mL of maternal plasma, which contained both maternal and fetal cfDNA. The cfDNA was extracted using an automated DNA extraction platform (QIA Symphony, QIAGEN, Hilden, Germany) and used for DNA library preparation using the IONA Library Preparation Kit in combination with an automated liquid handling platform (Sciclone, PerkinElmer Inc, Waltham, MA). The IONA run control DNA library was included to ascertain the validity of the sequencing run performed. Semiconductor whole-genome sequencing was performed using Ion Chef and Ion Proton systems (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions, as a multiplex of 8 samples in

addition to the IONA run control (4 sequencing runs per day were performed to achieve the 32-sample optimal throughput). The assessment of fetal fraction was made using a proprietary method developed by Premaitha Health.

All sample processing and analysis were performed by appropriately trained and experienced operators who were blinded to karyotype results throughout the study. The IONA test results were compared with the results from invasive testing or birth outcome.

### Primary outcomes and clinical reference standard

The primary outcome measures of the study were the screening performance of the IONA test in terms of DR and specificity in twin pregnancies for each of the 3 trisomies and the test failure rate. This was considered important to overcome the challenge encountered in the use of cfDNA in twin pregnancies. The reference test (gold standard) was chromosomal analysis undertaken prenatally (CVS or amniocentesis) or postnatally (umbilical cord or newborn blood) or the absence of phenotypic features for chromosomal abnormalities after birth.

### Ethical approval

This multicenter study was approved by the ethics committee of South Berkshire B. All women gave a written informed consent to participate. Subjects were free to withdraw from participation in this study at any time for any reason without affecting their standard of care. The study was registered on [ClinicalTrials.gov](https://www.clinicaltrials.gov) with the registration number NCT03200041.

### Systematic review

To determine the predictive accuracy of cfDNA analysis for trisomies 21, 18, and 13 in twin pregnancy based on the totality of evidence, the study results were combined with those results identified in a review of the literature ([Supplemental Table](#)).<sup>19–29</sup> We searched MEDLINE, Embase, CENTRAL, Cochrane Library, and [ClinicalTrials.gov](https://www.clinicaltrials.gov) on June 6, 2020, to identify published cohort studies on the performance of maternal cfDNA testing for trisomies 21, 18, and 13 in twin

pregnancy. We included studies in which data on pregnancy outcome were provided for at least 85% of the study population. Data on the number of pregnancies, chorionicity, gestational age at sampling, method of analyzing samples, DR, and FPR were extracted from these studies.

### Statistical analysis

The resulting fragment count data from each sample were used as input to a set of mixture models that incorporate distributions of expected values under both trisomy-affected and trisomy-unaffected hypotheses for trisomies 21, 18, and 13. Each model generated a test likelihood ratio that was then used, together with maternal age—derived previous probability of trisomy, to quantify the probability of each trisomy, taking into account both maternal age and the corresponding DNA test result. In this way, the IONA software interprets the results, so the pipeline generates a final result without the need for further local bioinformatics analysis. The software used for analysis in the IONA test includes entry of multiple pregnancy status (singleton or twin) and chorionicity (monochorionic, dichorionic, or unknown) for every sample. In the event of a twin pregnancy which is not known to be monochorionic, an inbuilt validity check algorithm is modified. A twin correction factor is applied which effectively increases the minimum fetal fraction which must be reached for the check to pass. This accounts for the possibility that 1 of the 2 fetuses in a dichorionic twin pregnancy could be trisomy affected, with the other fetus being unaffected, a situation which could lead to a reduced trisomy chromosome level measurement for the pregnancy as a whole. The correction also accounts for the fact that in trisomy 18 and 13 affected pregnancies, lower levels of trisomy chromosome measurements may occur. This extra correction further increases the required minimum fetal fraction for the check to pass.

To investigate the performance of the IONA test, we calculated DR, specificity, failure rates, and CIs for aneuploidy and euploid twin pregnancies.

For the pooled analysis, we calculated the DR and FPR with corresponding 95% CI for individual studies and the pooled DR and FPR using random-effect meta-analyses. Between-study heterogeneity was assessed using the I<sup>2</sup> statistic. The analyses were stratified according to the type of aneuploidy (trisomy 21 or trisomy 18). Because there were a very small number of cases of trisomy 13 for a meaningful pooled analysis, we computed average DR and FPR values. Statistical analyses were performed using the StatsDirect statistical software (version 2.7.8, StatsDirect Ltd, Cheshire, United Kingdom).

### Sample size considerations

The sample size estimate in singleton pregnancies calculated that samples from 92 pregnancies complicated by trisomy 21 and 738 euploid fetuses were required.<sup>17</sup> However, this took into account the numbers needed for assay robustness studies, in-use optimization studies, and sample stability studies, which have since been established and therefore did not require repetition. Furthermore, at the time of planning, the largest study reporting data on the use of cfDNA in twin pregnancies included 515 twin pregnancies (351 with known outcomes, 12 cases of trisomy 21). Owing to the rarity of aneuploidy and on the basis of sample sizes utilized to support existing noninvasive prenatal testing (NIPT) testing claims, the study design presented was to target 15 cases affected by trisomy; this was more than the existing literature claimed at the time of study design and included unaffected samples in excess of the population prevalence to maximize chances of obtaining sufficient trisomy cases and accounting for “loss to follow-up” at a rate of 15%. The initial aim for this study was to include 350 euploid twin pregnancies and 15 pregnancies with 1 or both fetuses affected by trisomy 21. To achieve this, the plan was to recruit 500 subjects pregnant with twins, approximately 350 twin pregnancies at a low chance of aneuploidy (based on conventional screening methods), and at least 150 high-chance twin pregnancies confirmed by prenatal invasive



diagnostic procedures or birth outcome. However, at interim analysis, a smaller than expected number of women with a high chance of trisomy 21 were identified; to reach our target number of 15 trisomy 21 affected pregnancies, the number of subjects to be recruited was increased to 1000 twin pregnancies (2000 fetuses).

## Results

During the study, we enrolled 1003 women and samples were collected from all study participants meeting the entry criteria. Complete data with follow-up and reference data (chromosomal analysis or birth outcome) were available for 961 of women (95.8%) because 37 women were lost to follow-up and a further 5 patients were excluded (4 did not meet the inclusion criteria and 1 patient withdrew from the study). The results from all remaining 961 patient samples were available for analysis and assessment of clinical accuracy. In the population, there were 16 chromosomal abnormalities, comprising 14 with trisomy 21 and 1 each with trisomy 18 and trisomy 13 (Figure).

The mean gestational age at enrolment was 15 weeks and 6 days (ranging from 10 to 36 weeks), and the mean maternal age was 32.4 years (range, 17–60 years). In 260 patients, the pregnancy was the result of IVF treatment (Table 1). Cases in which cfDNA screening was performed before 20 weeks' gestation and at or greater than 20 weeks' gestation are presented in Supplemental Figures 1 and 2, respectively.

Of the 961 samples available for analysis, 276 of the twin pregnancies were monochorionic and 685 were dichorionic. A breakdown of the initial screening results, per fetus, found 1129 of the fetuses had a low chance of trisomies 21, 18, and 13, whereas 793 had a high chance; the breakdown is presented in Table 2.

Of the 961 women included in the final patient sample, 958 samples generated a result from the IONA test, whereas in 3 cases the samples failed to generate a result, a failure rate of 0.31%. All 3 were followed up and subsequently found to have normal chromosomes in 2

### FIGURE Study flowchart

The study took place in 6 United Kingdom centres and enrolled 1003 twin pregnancies; 961 (685 dichorionic and 276 monochorionic) had results available for analysis and 958 were included in the final analysis.

T13, trisomy 13; T18, trisomy 18; T21, trisomy 21.

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cases and trisomy 21 (as determined on CVS) in 1 case. The fetal fraction in the analyzed samples ranged from 3% to 36% (median, 13.7%) in dichorionic twins and 4% to 29% (median, 11.6%) in monochorionic twins; the 9 samples with 3% fetal fraction provided a valid result. Samples that failed to generate a result were excluded from the DR and screening characteristics, to better distinguish between “no calls” (caused through events such as machine breakdown or insufficient fetal fraction within a sample) and the alternative of a false-negative or false-positive result, where a result is obtained using the test but is in disagreement with the birth outcome or amniocentesis result. “No calls” are reported as a failed test percentage; in practice, these are reported back to the healthcare provider to ensure that counseling is given regarding the options (such as a blood sample redraw, other form of screening, or invasive testing).

The DR and specificity of the test, based on the 958 women with a result, and the results are presented in Table 3. Of note is that, compared with the reference test, there were no false-positive or false-negative results for trisomy 21 or trisomy 13. For trisomy 18, there was 1 false-negative and 1 false-positive result. The false-negative result occurred with a likelihood ratio of 5.3 and a background chance of 1:2301 resulting in an adjusted chance of 1:435, which was considered screen negative. The algorithm of the test has since been amended to include an additional sample validity check for cases where maternal age risk is low ( $<1:150$ ) and the likelihood ratio is small and positive; this sample would no longer produce a false-negative result. The false-positive result was associated with a likelihood ratio of 74 and a background chance of 1:174, which resulted in adjusted chance of 1:33.

TABLE 1

**Baseline characteristics and pregnancy history in the study population (n=961) and pregnancy outcomes of the 1922 fetuses**

Characteristic	Median (IQR), mean ( $\pm$ SD), or n (%)
Maternal age, y	33 (31–38)
Gestational age at blood draw, wk	14.1 (12.7–18.7)
Maternal weight, kg	72 (39–168)
Maternal height, cm	163.5 ( $\pm$ 6.6)
Maternal body mass index, kg/m <sup>2</sup>	25.1 (22.2–29.0)
Maternal blood transfusion within 6 mo	6 (0.6)
Previous pregnancy with aneuploidy	16 (1.7)
Conception by IVF	245 (25.5)
Monochorionic twin pregnancy	276 (28.8)
Dichorionic twin pregnancy	685 (72.2)
Fetal fraction, %	11 (8–15)
Live birth	1804 (95.9)
Intrauterine fetal death	41 (2.1)
Termination of pregnancy	31 (1.6)
Miscarriage	46 (2.4)
Number recruited in the first trimester <sup>a</sup>	223 (23.2)
Number recruited in the second trimester <sup>a</sup>	685 (71.3)
Number recruited in the third trimester <sup>a</sup>	53 (5.5)

IQR, interquartile range; IVF, in vitro fertilization; SD, standard deviation.

<sup>a</sup> Of the 16 aneuploid cases, 13, 2, and 1 were diagnosed in the first, second, and third trimester, respectively.

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TABLE 2

**Previous screening results and indications leading to cell-free DNA testing**

Indications	Number of patients
Maternal age of $>35$ y as the sole indication	560
No specific indication (“routine” screening)	168
Ultrasound findings	106
High-chance combined test result	59
Previous chromosomal abnormality	26
High-chance quadruple test result	14
Nuchal translucency of $\geq 3.5$ mm	14
Nuchal translucency of $\geq 3.5$ and ultrasound findings	6
Ultrasound findings and previous chromosomal abnormality	2
Ultrasound findings and high-chance NIPT result	2
Nuchal translucency of $\geq 3.5$ and previous chromosomal abnormality	1
High-chance previous NIPT test result	1
Other	2

NIPT, noninvasive prenatal testing.

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## Systematic review

The literature search identified 11 relevant studies, excluding potentially overlapping studies (Supplemental Table). In the combined populations of our study and the 11 studies identified by the literature search, there were 74 cases of trisomy 21 and 5598 non-trisomy 21 twin pregnancies, 22 cases of trisomy 18 and 4869 non-trisomy 18 pregnancies, and 5 cases of trisomy 13 and 3881 non-trisomy 13 pregnancies. Overall, 73 of 74 pregnancies with trisomy 21 were detected using cfDNA, giving a pooled DR of 95% (95% CI, 90–99) and FPR of 0.09 (95% CI, 0.02–0.19) (Table 4). The DR of cfDNA for trisomy 18 was 82% (95% CI, 66–93) and FPR was 0.08 (95% CI, 0.02–0.18) (Table 5). Because there were a very small number of cases of trisomy 13 for meaningful pooled analysis, we computed average DR and FPR values; these were 80% and 0.13%, respectively. The main limitations of previous studies were the small sample size, retrospective design, and use of stored samples (Videos 1 and 2).

## Discussion

### Summary of the study findings

The performance of cfDNA analysis of maternal blood for trisomies 21, 18, and 13 in twin pregnancy was investigated in a large, prospective multicenter study in the United Kingdom which recruited more than 1000 pregnancies. There was a very low failure rate for cfDNA testing, with only 3 cases where results could not be obtained. All cases of trisomy 21 and the 1 case of trisomy 13 were detected with an FPR of 0%. For trisomy 18, there was 1 false-positive and 1 false-negative result giving a DR of 0% (95% CI, 0%–97%) in which the 95% CI is much wider for twins owing to the small number of cases available.

### Interpretation of study findings and comparison with existing literature

Our findings of high predictive accuracy of cfDNA for trisomy 21 in twins similar to that reported in singleton pregnancies are consistent with the published studies and pooled analysis.<sup>26</sup> DRs obtained in this study are equivalent to those shown

**TABLE 3**  
Screening characteristics for trisomies 21, 18, and 13 in the study

Trisomy	Affected	True positive	Unaffected	True negative	False positive	Sensitivity, % (95% CI)	Specificity, % (95% CI)
Trisomy 21	13	13 <sup>a</sup>	945 <sup>a</sup>	945	0	100 (75–100)	100 (99.6–100)
Trisomy 18	1	0	957 <sup>a</sup>	956	1	0 (0–97)	99.9 (99.4–100)
Trisomy 13	1	1	957 <sup>a</sup>	957	0	100 (3–100)	100 (99.6–100)

CI, confidence interval.

Data are expressed as number unless noted otherwise.

<sup>a</sup> Three samples, 2 unaffected and 1 trisomy 21, were excluded from the screening characteristics because they failed to provide a test result that could be compared for concordance with the reference method. These samples form the basis of the test failure rate that is reported for this study as 0.31%.

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in the pooled literature review which suggests consistently high performance. This is likely because the minimal requirement for fetal fraction in twins is increased compared with singleton pregnancies to prevent the reporting of wrong results and to ensure sufficient fetal material is present within the sample. This test has been designed to maintain the performance level as seen with singleton pregnancies but expects to return a higher failure rate corresponding to the need for higher fetal fraction. Although this study was not powered to investigate this and achieved

very low failure rates, we believe the findings to be consistent with this approach. Because the study cohort included only 1 pregnancy affected by trisomy 13, no firm conclusions could be drawn from this study on the screening performance for trisomy 13. The FPR for trisomy 18 is consistent with previous studies,<sup>26</sup> and it is suggested that such false-positive result could be caused by cotwin demise,<sup>30</sup> confined placental mosaicism,<sup>31</sup> maternal mosaicism,<sup>32</sup> malignancy,<sup>33</sup> and uniparental disomy.<sup>24</sup> None of these factors were identified in this twin pregnancy.

Moreover, sex chromosome analysis was not included in the screening algorithm owing to limited clinical utility in singletons or twins and owing to ethical constraints related to the risk of selective sex-specific termination. Screening for sex aneuploidy is also not recommended by the National Screening Committee in the United Kingdom.

### Clinical and research implications

In this study, the results could not be obtained in only 3 cases. Previous studies have identified dichorionicity as a risk factor for NIPT failure.<sup>34</sup> Galeva et al<sup>34</sup>

**TABLE 4**  
Studies reporting on screening for trisomy 21 using cell-free DNA in twin pregnancy

Study	Trisomy 21, n	Detected, n	Sensitivity, % (95% CI)	Non-trisomy 21, n	False positive, n	False-positive rate, % (95% CI)
Lau et al, <sup>19</sup> 2013	1	1	100 (3–100)	11	0	0 (0–28.49)
Huang et al, <sup>20</sup> 2014	9	9	100 (66–100)	180	0	0 (0–2.03)
Tan et al, <sup>21</sup> 2016	4	4	100 (40–100)	506	0	0 (0–0.73)
Du et al, <sup>22</sup> 2017	2	2	100 (16–100)	89	0	0 (0–4.06)
Le Conte et al, <sup>23</sup> 2018	3	3	100 (29–100)	415	1	0.2 (0–1.34)
Yang et al, <sup>24</sup> 2018	4	4	100 (40–100)	396	0	0 (0–0.93)
Yu et al, <sup>25</sup> 2019	16	16	100 (79–100)	1141	0	0 (0–0.32)
Gil et al, <sup>26</sup> 2019	17	16	94 (71–100)	980	1	0.1 (0–0.57)
Garshasbi et al, <sup>27</sup> 2020	3	3	100 (29–100)	440	0	0 (0–0.83)
Motevasselian et al, <sup>28</sup> 2020	1	1	100 (3–100)	355	1	0.3 (0–1.56)
He et al, <sup>29</sup> 2020	1	1	100 (3–100)	140	0	0 (0–2.60)
Current study	13	13	100 (75–100)	945	0	0 (0–0.39)
Pooled analysis	74	73	95 (90–99)	5598	3	0.09 (0.03–0.19)
Heterogeneity (I <sup>2</sup> )			0			0

CI, confidence interval.

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TABLE 5

## Studies reporting on screening for trisomy 18 using cell-free DNA in twin pregnancy

Study	Trisomy 18, n	Detected, n	Sensitivity, % (95% CI)	Non-trisomy 21, n	False positive, n	False-positive rate, % (95% CI)
Huang et al, <sup>20</sup> 2014	2	1	50 (1–99)	187	0	0 (0–1.95)
Le Conte et al, <sup>23</sup> 2018	1	1	100 (3–100)	417	0	0 (0–0.88)
Yang et al, <sup>24</sup> 2018	1	1	100 (3–100)	399	0	0 (0–0.92)
Yu et al, <sup>25</sup> 2019	4	4	100 (40–100)	1153	0	0 (0–0.32)
Gil et al, <sup>26</sup> 2019	10	9	90 (55–100)	987	1	0.10 (0–0.56)
Garshasbi et al, <sup>27</sup> 2020	1	1	100 (3–100)	442	0	0 (0–0.83)
Motevasselian et al, <sup>28</sup> 2020	2	2	100 (16–100)	354	0	0 (0–1.04)
Current study	1	0	0 (0–98)	957	1	0.10 (0–0.58)
Pooled analysis	22	19	82 (66–93)	4869	2	0.08 (0.02–0.18)
Heterogeneity (I <sup>2</sup> )			1.6			0

CI, confidence interval.

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have estimated that cfDNA test failure after first sampling in dichorionic twins to be 3.3 times higher than in singletons. The authors reported that this is likely to be attributed to the higher incidence of IVF conception and nulliparity among twin pregnancies than singletons. Given the very low failure rate in this study, it was not possible to examine risk factors of failed cfDNA.

Initial reports on the performance of screening for trisomy 21 by cfDNA in twin pregnancies are encouraging, and therefore, the American College of Obstetricians and Gynecologists has recommended its use.<sup>15</sup> Similarly, other guidelines focusing on twin pregnancies, such as those by the International Society of Ultrasound in Obstetrics and Gynecology and the Royal College of Obstetricians and Gynaecologists,<sup>35,36</sup> have acknowledged the potential role of cfDNA as a screening tool for trisomy 21 in twin pregnancies and highlighted the need for more studies to validate its performance.

Although the focus of some guidelines is on the possible lower DR in twins than singletons, we believe that is an irrelevant comparison for pregnant women. Rather, the data are of importance when it comes to the choice of screening test. Our data, combined with those in the existing literature, show that there is now

strong evidence that cfDNA is more accurate than the combined test or second trimester serum biochemistry in twin pregnancy.<sup>35,36</sup> In our opinion, perhaps the most important advantage is the much lower FPR of cfDNA in twin pregnancies than other tests: the FPR in combined screening is high (9% of pregnancies and 7% of fetuses),<sup>6</sup> and this means that consideration for invasive testing is relevant in 1 in 11 twin pregnancies. A meta-analysis published in 2012 suggests that overall pregnancy loss rates after invasive prenatal diagnosis are considerable<sup>7–9</sup> (3.8% and 3.1% after CVS and amniocentesis)<sup>7</sup>; this is certainly higher than in singleton pregnancies, where the loss rates have recently been shown to be lower than previously estimated.<sup>37</sup> Of note, the most recent meta-analysis that included 16 studies and performed a head-to-head comparison between twin pregnancies undergoing and those not undergoing invasive testing demonstrated that there was no difference in the risk of fetal loss. The pooled incidence of fetal loss in twin pregnancies undergoing CVS was 2.0% (compared with 1.8%) and in those undergoing amniocentesis was 2.4% (compared with 2.4%).<sup>38</sup> Finally, combined screening for aneuploidy in twin pregnancy is more complex than that in singletons: additional

factors to be considered include chorionicity-related predictive accuracy and the option of selective termination of pregnancy, which carries additional risk to the ongoing pregnancy.<sup>10,35</sup>

### Strengths and weaknesses

Our study is one of the largest twin studies investigating the performance of cfDNA analysis of maternal blood for trisomies 21, 18, and 13 in twin pregnancy. Despite this, 1 limitation was the relatively small number of pregnancies affected with trisomies. However, this reflects the nature of the low prevalence of trisomies 21, 18, and 13 and the fact that the study included a mainly low-chance population for aneuploidy.

The strengths of this study include the prospective multicenter design with input from the patient and public representatives in the content of the study documents, study publicity, interpretation of study findings, and dissemination of study findings.

The cfDNA test was performed and the results were given by the laboratory without previous knowledge of the fetal karyotype or pregnancy outcome. We report detailed methodology and laboratory techniques used. The algorithm for assessment takes into account maternal age and fetal fraction while calculating the likelihood ratio.



Moreover, the study included pregnancies with both low and high chances on previous screening, which reflects clinical practice, ensuring its generalizability. Furthermore, the loss to follow-up rate was low, further reducing the risk of bias.

Finally, the study results were combined with those identified in a review of the literature, and therefore, it is possible to present the most up-to-date summary of the performance of cfDNA analysis of maternal blood for trisomies 21, 18, and 13 in twin pregnancy (Supplemental Table, Table 4, and Table 5).

It is important to highlight the fact that the DRs only apply to the twin cases that received results. Routine implementation of NIPT needs to take into account the test failure rate (which at the time of the first draw was 3.4% in singletons, 4.9% in monochorionic twins, and 11.3% in dichorionic twins),<sup>35</sup> although it was much lower in our study (0.31%). Furthermore, studies report on the screening performance at a wide gestational age window, beyond 16 weeks' gestation in a number of the studies with some testing performed as late as 36 weeks' gestation, making this less clinically relevant. Moreover, cfDNA is unable to predict which twin is affected. Therefore, nuchal translucency and detailed ultrasound scan have a potential role in suggesting which twin might have aneuploidy, facilitating the choice of which fetus to be tested genetically.

## Conclusions

The results of an algorithm for cfDNA testing in twin pregnancies taking into account maternal age and fetal fraction is presented. This large multicenter study confirms that cfDNA testing is the most accurate screening test for trisomy 21 in twin pregnancies, and the failure rate was reassuringly very low (0.31%); this screening performance of NIPT in twin pregnancies seems similar to that in singletons. The predictive accuracy for trisomies 18 and 13 may be less. The greater likelihood of trisomy 21 in multiple pregnancy, higher FPR of other screening approaches, higher likelihood of being offered invasive testing, and the greater likelihood of complications of

invasive testing<sup>10,35</sup> all mean that cfDNA has an important role to play in screening for trisomies in twins. In our view, such testing has the potential to overcome many of these complex issues and, given the much lower FPR (and high DR) in combination with the relatively low incidence of twin pregnancy, should be considered as a primary screening test in twins. ■

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## References

- Nicolaides KH, Wright D, Poon LC, Syngelaki A, Gil MM. First-trimester contingent screening for trisomy 21 by biomarkers and maternal blood cell-free DNA testing. *Ultrasound Obstet Gynecol* 2013;42:41–50.
- Guy GP, Hargrave J, Dunn R, et al. Secondary non-invasive prenatal screening for fetal trisomy: an effectiveness study in a public health setting. *BJOG* 2020 [Epub ahead of print].
- Nicolaides KH. Screening for fetal aneuploidies at 11 to 13 weeks. *Prenat Diagn* 2011;31:7–15.
- 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;50:302–14.
- Mutton D, Alberman E, Hook EB. Cytogenetic and epidemiological findings in Down syndrome, England and Wales 1989 to 1993. National Down syndrome Cytogenetic Register and the Association of Clinical Cytogeneticists. *J Med Genet* 1996;33:387–94.
- Spencer K, Nicolaides KH. Screening for trisomy 21 in twins using first trimester ultrasound and maternal serum biochemistry in a one-stop clinic: a review of three years experience. *BJOG* 2003;110:276–80.
- Agarwal K, Alfirevic Z. Pregnancy loss after chorionic villus sampling and genetic amniocentesis in twin pregnancies: a systematic review. *Ultrasound Obstet Gynecol* 2012;40:128–34.
- Hansen M, Kurinczuk JJ, Milne E, de Klerk N, Bower C. Assisted reproductive technology and birth defects: a systematic review and meta-analysis. *Hum Reprod Update* 2013;19:330–53.
- Royal College of Obstetricians and Gynaecologists. Amniocentesis and chorionic villus sampling (Green-top Guideline No. 8). London, England: Royal College of Obstetricians and Gynaecologists Press; 2010.
- National Collaborating Centre for Women's and Children's Health (UK). Multiple pregnancy:

the management of twin and triplet pregnancies in the antenatal period. London, England: Royal College of Obstetricians and Gynaecologists Press; 2011.

- Qu JZ, Leung TY, Jiang P, et al. Noninvasive prenatal determination of twin zygosity by maternal plasma DNA analysis. *Clin Chem* 2013;59:427–35.
- Leung TY, Qu JZ, Liao GJ, et al. Noninvasive twin zygosity assessment and aneuploidy detection by maternal plasma DNA sequencing. *Prenat Diagn* 2013;33:675–81.
- Ashoor G, Syngelaki A, Poon LC, Rezende JC, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: relation to maternal and fetal characteristics. *Ultrasound Obstet Gynecol* 2013;41:26–32.
- Bevilacqua E, Gil MM, Nicolaides KH, et al. Performance of screening for aneuploidies by cell-free DNA analysis of maternal blood in twin pregnancies. *Ultrasound Obstet Gynecol* 2015;45:61–6.
- Bossuyt PM, Reitsma JB, Bruns DE, et al. for the STARD Group. Screening for fetal chromosomal abnormalities. ACOG Practice Bulletin No. 226. American College of Obstetricians and Gynecologists. *Obstet Gynecol* 2020;136(16). Epub Aug 14 2020.
- Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ* 2015;351:h5527.
- Papageorgiou AT, Khalil A, Forman M, et al. Clinical evaluation of the IONA test: a non-invasive prenatal screening test for trisomies 21, 18, 13. *Ultrasound Obstet Gynecol* 2016;47:188–93.
- Crea F, Forman M, Hulme R, et al. The IONA test: development of an automated cell-free DNA-based screening test for fetal trisomies 13, 18, and 21 that employs the ion proton semiconductor sequencing platform. *Fetal Diagn Ther* 2017;42:218–24.
- 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;26:434–7.
- Huang X, Zheng J, Chen M, et al. Noninvasive prenatal testing of trisomies 21 and 18 by massively parallel sequencing of maternal plasma DNA in twin pregnancies. *Prenat Diagn* 2014;34:335–40.
- Tan Y, Gao Y, Lin G, et al. Noninvasive prenatal testing (NIPT) in twin pregnancies with treatment of assisted reproductive techniques (ART) in a single center. *Prenat Diagn* 2016;36:672–9.
- 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;20:242–9.
- Le Conte G, Letourneau A, Jani J, et al. Cell-free fetal DNA analysis in maternal plasma as screening test for trisomies 21, 18 and 13 in twin

pregnancy. *Ultrasound Obstet Gynecol* 2018;52:318–24.

24. Yang J, Qi Y, Hou Y, et al. Performance of non-invasive prenatal testing for trisomies 21 and 18 in twin pregnancies. *Mol Cytogenet* 2018;11:47.

25. Yu W, Lv Y, Yin S, et al. Screening of fetal chromosomal aneuploidy diseases using noninvasive prenatal testing in twin pregnancies. *Expert Rev Mol Diagn* 2019;19:189–96.

26. Gil MM, Galeva S, Jani J, et al. 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;53:734–42.

27. Garshasbi M, Wang Y, Hantoosh Zadeh S, Giti S, Piri S, Reza Hekmat M. Clinical application of cell-free DNA sequencing-based noninvasive prenatal testing for trisomies 21, 18, 13 and sex chromosome aneuploidy in a mixed-risk population in Iran. *Fetal Diagn Ther* 2020;47:220–7.

28. Motevasselian M, Saleh Gargari S, Younesi S, et al. Non-invasive prenatal test to screen common trisomies in twin pregnancies. *Mol Cytogenet* 2020;13:5.

29. He Y, Wang Y, Li Z, et al. 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;99:731–43.

30. Futch T, Spinosa J, Bhatt S, de Feo E, Rava RP, Sehner AJ. Initial clinical laboratory experience in noninvasive prenatal testing for fetal aneuploidy from maternal plasma DNA samples. *Prenat Diagn* 2013;33:569–74.

31. Choi H, Lau TK, Jiang FM, et al. Fetal aneuploidy screening by maternal plasma DNA

sequencing: 'false positive' due to confined placental mosaicism. *Prenat Diagn* 2013;33:198–200.

32. Yao H, Zhang L, Zhang H, et al. Noninvasive prenatal genetic testing for fetal aneuploidy detects maternal trisomy X. *Prenat Diagn* 2012;32:1114–6.

33. Bianchi DW, Chudova D, Sehner AJ, et al. Noninvasive prenatal testing and incidental detection of occult maternal malignancies. *JAMA* 2015;314:162–9.

34. 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;53:804–9.

35. Khalil A, Rodgers M, Baschat A, et al. ISUOG Practice Guidelines: role of ultrasound in twin pregnancy. *Ultrasound Obstet Gynecol* 2016;47:247–63.

36. Management of monochorionic twin pregnancy: Green-top Guideline No. 51. *BJOG* 2017;124:e1–45.

37. Salomon LJ, Sotiriadis A, Wulff CB, Odibo A, Akolekar R. Risk of miscarriage following amniocentesis or chorionic villus sampling: systematic review of literature and updated meta-analysis. *Ultrasound Obstet Gynecol* 2019;54:442–51.

38. Di Mascio D, Khalil A, Rizzo G, et al. Risk of fetal loss following amniocentesis or chorionic villus sampling in twin pregnancy: systematic review and meta-analysis. *Ultrasound Obstet Gynecol* 2020;56:647–55.

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**SUPPLEMENTAL FIGURE 1****Study flowchart of participants recruited at or after 20 weeks' gestation**

The study enrolled 194 twin pregnancies at 20 weeks' gestation or beyond; 189 (130 dichorionic and 59 monochorionic) had results available for analysis and 187 were included in the final analysis.

*T18*, trisomy 18; *T21*, trisomy 21.

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## SUPPLEMENTAL FIGURE 2

**Study flowchart of participants recruited prior to 20 weeks' gestation**

The study enrolled 809 twin pregnancies before 20 weeks' gestation; 772 (555 dichorionic and 217 monochorionic) had results available for analysis and 771 were included in the final analysis.

T13, trisomy 13.

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## SUPPLEMENTAL TABLE

## Existing screening studies using cfDNA in twins for the detection of trisomies 21, 18, and 13

Study	Aneuploidy studied	n	Monochorionic, n (%)	Trisomy 21 (n)	Trisomy 18 (n)	Trisomy 13 (n)	Outcome known (%)	cfDNA method	GA (wk)	Population
Lau et al, <sup>19</sup> 2013	Trisomy 21	12	2 (16.7)	1	—	—	100	MPSS	13 (11–20)	High risk
Huang et al, <sup>20</sup> 2014	Trisomies 21 and 18	189	33 (17.5)	9	2	—	100	MPSS	19 (11–36)	High risk
Tan et al, <sup>21</sup> 2016	Trisomy 21	560	18 (3.2) <sup>a</sup>	4	—	—	90	MPSS	12 (11–28)	Mixture
Du et al, <sup>22</sup> 2017	Trisomy 21	92	39 (42.4)	2	—	—	100	MPSS	18 (14–23)	High risk
Le Conte et al, <sup>23</sup> 2018	Trisomies 21, 18, and 13	418	86 (20.6)	3	1	0	85	MPSS	16 (10–35)	Mixture
Yang et al, <sup>24</sup> 2018	Trisomies 21 and 18	432	95 (22.0)	4	1	—	91	MPSS	>9	Mixture
Yu et al, <sup>25</sup> 2019	Trisomies 21, 18, and 13	1157	308 (26.6)	16	4	1	99	MPSS	18 (8–30)	Mixture
Gil et al, <sup>26</sup> 2019	Trisomies 21, 18, and 13	997	143 (14.3)	17	10	2	94	Targeted	11 (10–14)	Mixture
Garshasbi et al, <sup>27</sup> 2020	Trisomies 21 and 18	443	N/A	3	1	—	100	MPSS	>9	Mixture
Motevasselian et al, <sup>28</sup> 2020	Trisomies 21, 18, and 13	356	76 (21.3) <sup>a</sup>	2	2	1	100	MPSS	15+4 d (±5 d)	Mixture
He et al, <sup>29</sup> 2020	Trisomy 21	141	39 (27.7) <sup>a</sup>	1	—	—	100	MPSS	16.1 (10–23)	Mixture
Our study	Trisomies 21, 18, and 13	958	276 (28.8)	13	2	1	96	NGS	14.1 (12.7–18.7)	Mixture

Numbers reported are those after exclusion of cases without cfDNA test result or pregnancy outcome.

GA is given as mean (range), mean (SD) or actual value.

cfDNA, cell-free DNA; GA, gestational age; MPSS, massively parallel signature sequencing; NGS, next generation sequencing.

<sup>a</sup> Value in original sample before exclusion for failed results and no follow-up.

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