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Prospective first-trimester screening for trisomies by cell-free DNA testing of maternal blood in twin pregnancy

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KEYWORDS: cell-free DNA; fetal fraction; first-trimester screening; non-invasive prenatal testing; trisomy 21; twin pregnancy

ABSTRACT

Objectives *First, to examine in twin pregnancies the performance of first-trimester screening for fetal trisomies 21, 18 and 13 by cell-free (cf) DNA testing of maternal blood and, second, to compare twin and singleton pregnancies regarding the distribution of fetal fraction of cfDNA and rate of failure to obtain a result.*

Methods *This was a prospective study in 438 twin and 10 698 singleton pregnancies undergoing screening for fetal trisomies by cfDNA testing at 10 + 0 to 13 + 6 weeks' gestation. Chromosome-selective sequencing of cfDNA was used and, in twin pregnancies, an algorithm was applied that relies on the lower fetal fraction contributed by the two fetuses. Multivariate regression analysis was used to determine significant predictors of fetal fraction and a failed result.*

Results *In twin pregnancies, the median fetal fraction was lower (8.0% (interquartile range (IQR), 6.0–10.4%)* vs *11.0% (IQR, 8.3–14.4%); P < 0.0001) and failure rate after first sampling was higher (9.4%* vs *2.9%; P < 0.0001) compared to in singletons. Multivariate logistic regression analysis demonstrated that the risk of test failure increased with increasing maternal age and body mass index and decreased with fetal crown–rump length. The risk was increased in women of South Asian racial origin and in pregnancies conceived by* in-vitro *fertilization (IVF). The main contributor to the higher rate of failure in twins was conception by IVF which was observed in 9.5% of singletons and 56.2% of twins. In the 417 twin pregnancies with a cfDNA result after first or second sampling, the detection rate was 100% (8/8) for trisomy 21 and 60% (3/5) for trisomies 18 or 13, at a false-positive rate (FPR) of 0.25% (1/404). In the 10 530 singleton pregnancies with a cfDNA result after first or second sampling, the detection rate was 98.7% (156/158) for trisomy 21 and 80.3% (49/61) for trisomies 18 or 13, at a FPR of 0.22% (23/10 311).*

Conclusions *In twin pregnancies undergoing first-trimester screening for trisomies by cfDNA testing, the fetal fraction is lower and failure rate higher compared to in singletons. The number of trisomic twin pregnancies examined was too small for an accurate assessment of performance of screening, but it may be similar to that in singleton pregnancies. Copyright © 2016 ISUOG. Published by John Wiley & Sons Ltd.*

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 and implementation studies in large numbers of affected and unaffected pregnancies reported that the detection rate (DR) and false-positive rate (FPR) for trisomy 21 were 99.2% (95% CI, 98.5–99.6%) and 0.09% (95% CI, 0.05–0.14%), respectively, and the respective values for trisomy 18 were 96.3% (95% CI, 94.3–97.9%) and 0.13% (95% CI, 0.07–0.20%) and for trisomy 13 were 91.0% (95% CI, 85.0–95.6%) and 0.13% (95% CI, 0.05–0.26%)[1]. In contrast to singleton pregnancies, for which there are extensive data on the performance of screening for trisomies by cfDNA testing, very few studies report data on twins[2–7]; most studies were retrospective, using stored plasma samples, or prospective but with incomplete follow up[2–5]. Only two studies examined twin pregnancies prospectively and reported the outcome in all cases; in their combined data from 201 cases at 11–36 weeks' gestation, they classified correctly all 10 cases of trisomy 21 and one of the two cases of trisomy 18, and the FPR was 0% in the 189 cases with euploid fetuses[6,7]. Consequently, the results are promising but the

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number of examined cases is too small for an accurate assessment of screening performance.

In cfDNA testing, the ability to detect the small increase in the amount of a given chromosome present in maternal plasma in a trisomic, compared to a disomic, pregnancy is directly related to the relative proportion of the fetal-to-maternal origin of the cfDNA[8]. When the fetal fraction is low, it is more difficult to discriminate between trisomic and unaffected pregnancies and a minimum fraction of about 4% is usually necessary for accurate cfDNA analysis; companies that routinely measure fetal fraction report a failed result when the fraction is below 4%. In twin pregnancies, cfDNA testing is more complex than in singleton pregnancies, because if the two fetuses are dizygotic only one is likely to have aneuploidy when present. Although, in dizygotic twins each fetus can contribute different amounts of cfDNA into the maternal circulation[9,10], if the fetal fraction of a trisomic fetus is below the 4% threshold but there is a high contribution from the disomic cotwin such that there is a satisfactory total fetal fraction, a false-negative result could be obtained. To avoid this potential mistake it was proposed that, in cfDNA testing in twin pregnancies, the lower fetal fraction of the two fetuses, rather than the total, should be estimated in the assessment of risk for aneuploidies[11]. An inevitable consequence of such a policy is that the failure rate of the cfDNA test in twin pregnancies is higher than in singletons[3,5].

The objectives of this prospective study were first, to examine the performance of cfDNA screening in the first trimester for fetal trisomies 21, 18 and 13 in twin pregnancies and second, to compare twin and singleton pregnancies regarding the distribution of fetal fraction and rate of failure to obtain a result.

METHODS

The data for this study were derived first from cfDNA testing as an option following first-trimester combined testing in women with singleton or twin pregnancies attending routine care at 11 + 0 to 13 + 6 weeks' gestation in one of two National Health Service (NHS) hospitals in England[12] and second from cfDNA testing as part of routine screening in pregnancies at 10 + 0 to 13 + 6 weeks attending the Fetal Medicine Centre in London, which is a private clinic[13]. The patients were examined between October 2012 and August 2015.

We recorded maternal characteristics and medical history, including maternal age, racial origin (Caucasian, African, South Asian, East Asian and mixed), method of conception (spontaneous/assisted conception requiring the use of ovulation drugs/IVF), cigarette smoking during pregnancy (yes/no) and parity (parous/nulliparous if no previous pregnancy at or after 24 weeks' gestation). We also measured maternal weight and height. In all cases free beta-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) were measured within 10 min of blood collection at 10 + 0 to 13 + 6 weeks (DELFIA Xpress system, PerkinElmer Life and Analytical Sciences, Waltham, USA, or Kryptor, Thermo Scientific, Berlin, Germany). An ultrasound examination was carried out at 11 + 0 to 13 + 6 weeks to determine gestational age from measurement of fetal crown–rump length (CRL)[14], diagnose any major fetal abnormalities and measure fetal nuchal translucency (NT) thickness. In twin pregnancies, gestational age was determined from the CRL of the larger fetus and chorionicity was determined by examining the junction of the intertwin membrane with the placenta[15]. The measured NT was expressed as a difference from the expected normal mean for gestation (delta value)[16]. Similarly, the measured free β-hCG and PAPP-A were converted into multiples of the median (MoM) for gestational age, adjusted for maternal weight, racial origin, smoking status, method of conception, parity, chorionicity and machine used for the assays[17,18]. Biophysical and biochemical markers were combined to estimate the patient-specific risk for trisomies 21, 18 and 13.

Women provided written informed consent and maternal blood (20 mL) was sent via courier to the USA for cfDNA testing (Harmony™ Prenatal Test, Ariosa Diagnostics, Inc., San Jose, CA, USA)[19,20]. Chromosome-selective sequencing, referred to as digital analysis of selected regions (DANSR), and fetal-fraction optimized risk of trisomy evaluation (FORTE™) were used to assay non-polymorphic and polymorphic loci, where fetal alleles differ from maternal alleles, enabling simultaneous determination of chromosome proportion and fetal fraction. In twin pregnancies, the FORTE algorithm used for singletons was modified so that the smallest contribution of fetal fraction from the two fetuses was considered[11]. Risk scores for trisomies 21, 18 and 13 were provided as a percentage with ranges capped at > 99% and < 0.01%.

For cases in which 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 and results of the investigations were recorded in a fetal database. Results from invasive testing, obtained from laboratories, and pregnancy outcome, obtained from obstetricians, general practitioners or the patients, were recorded in the same database. The outcomes were divided first into trisomy 21, 18 or 13 if the karyotype of chorionic villi, amniotic fluid or neonatal blood demonstrated the relevant trisomy, second, into no trisomy 21, 18 or 13 if the karyotype of chorionic villi, amniotic fluid or neonatal blood was normal or the neonate was phenotypically normal, third, into no known karyotype because the pregnancies resulted in miscarriage or stillbirth and no karyotyping of fetal tissue was carried out, and fourth, into unknown outcome because the pregnancies were lost to follow up.

Statistical analysis

Descriptive data are presented as median and interquartile range (IQR) for continuous variables and as numbers and percentages for categorical variables. The measured fetal fraction was $\log_{10}$ transformed to make the distribution Gaussian, which was assessed using histograms and probability plots. In the combined data of singleton and twin pregnancies and separately in the twin pregnancies, univariate and multivariate regression analysis were used to determine which of the factors amongst maternal age, body mass index (BMI), racial origin, smoking status, method of conception, fetal CRL, serum PAPP-A and free β-hCG, fetal NT and fetal karyotype were significant predictors of $\log_{10}$ fetal fraction. Similarly, in the combined data of singleton and twin pregnancies and separately in the twin pregnancies, logistic regression analysis was undertaken to examine the maternal and pregnancy characteristics providing significant contribution to a prediction of failed cfDNA test result after first sampling.

The statistical software package SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses.

RESULTS

Characteristics of the study population

A total of 467 twin pregnancies had cfDNA testing and combined screening for trisomies, but 29 (6.2%) of these were excluded from further analysis either because the pregnancy ended in termination, miscarriage or stillbirth with no known karyotype ($n = 23$), were lost to follow up ($n = 4$) or had chromosomal abnormalities other than trisomies 21, 18 or 13 ($n = 2$). In the 438 cases included in the study, 373 (85.2%) were dichorionic and 65 (14.8%) were monochorionic. Maternal and pregnancy characteristics of the 438 twin pregnancies from this study and 10 698 singleton pregnancies from a previous study[21] are summarized in Table 1.

The 438 twin pregnancies included eight cases of trisomy 21, four of trisomy 18, one of trisomy 13 and 425 unaffected cases. The 10 698 singleton pregnancies included 160 cases of trisomy 21, 50 of trisomy 18, 16 of trisomy 13 and 10 472 unaffected by these trisomies[21].

Performance of screening

In the 417 twin pregnancies with a cfDNA result after first or second sampling, the detection rate was 100% (8/8) for trisomy 21, 75% (3/4) for trisomy 18 and 0% (0/1) for trisomy 13, at a FPR of 0.25% (1/404). All trisomic pregnancies were dichorionic with one affected and one normal twin.

In the 10 530 singleton pregnancies with a cfDNA result after first or second sampling, the detection rate was 98.7% (156/158) for trisomy 21, 89.1% (41/46) for trisomy 18 and 53.3% (8/15) for trisomy 13, at a FPR of 0.22% (23/10 311).

Table 1 Maternal and pregnancy characteristics of study population of singleton and twin pregnancies with cell-free DNA testing for aneuploidies

Characteristic	*Singleton* (n = *10 698*)	*Twin* (n = *438*)
Maternal age (years)	36.3 (33.2–39.3)	37.3 (34.6–40.0)*
Maternal BMI (kg/m^2)	23.3 (21.1–26.5)	23.5 (21.0–26.9)
Racial origin		
Caucasian	8751 (81.8)	358 (81.7)
African	698 (6.5)	26 (5.9)
South Asian	663 (6.2)	30 (6.8)
East Asian	386 (3.6)	19 (4.3)
Mixed	200 (1.9)	5 (1.1)
Nulliparous	4760 (44.5)	261 (59.6)*
Cigarette smoker	263 (2.5)	3 (0.7)†
Mode of conception		
Spontaneous/ovulation induction	9683 (90.5)	192 (43.8)
In-vitro fertilization	1015 (9.5)	246 (56.2)*
Origin of oocyte		
Self	904 (89.1)	216 (87.8)
Donor	111 (10.9)	30 (12.2)
GA at sampling (weeks)	11.9 (10.6–12.9)	11.7 (10.4–12.9)‡
Crown–rump length (mm)	53.7 (38.5–65.7)	54.2 (38.9–66.3)

Data are given as median (interquartile range) or *n* (%). Significant difference: *$P < 0.0001$; †$P < 0.01$; ‡$P < 0.05$. BMI, body mass index; GA, gestational age.

Factors affecting fetal fraction in singleton and twin pregnancies

The distribution of $\log_{10}$ fetal fraction from first sampling was Gaussian in both the singleton and twin pregnancies (Figure 1), but the median fetal fraction was lower in twins than in singletons (8.0% (IQR, 6.0–10.4%) *vs* 11.0% (IQR, 8.3–14.4%); $P < 0.0001$); in these calculations, it was assumed that, in cases with a failed result, the fetal fraction was 3%.

For the combined data from singleton and twin pregnancies, multivariate logistic regression analysis demonstrated that the fetal fraction increased with increasing fetal CRL, PAPP-A MoM and free β-hCG MoM, decreased with increasing maternal age and BMI, was lower in twin pregnancies than in singletons and decreased in women of South Asian racial origin and in pregnancies conceived by IVF (Table S1).

In twin pregnancies, multivariate logistic regression analysis demonstrated that the fetal fraction increased with increasing PAPP-A MoM and free β-hCG MoM and decreased with increasing maternal BMI. The median fetal fraction was higher in monochorionic than dichorionic twins (10.1% (IQR, 7.6–14.5%) *vs* 7.7% (IQR, 5.8–10.0%); $P < 0.0001$) and was decreased in pregnancies conceived by IVF (Table S2).

Factors affecting cfDNA test failure in singleton and twin pregnancies

There was no result from cfDNA testing after first sampling in 2.9% (316/10 698) of singleton pregnancies and in 9.4% (41/438) of twin pregnancies ($P < 0.0001$).

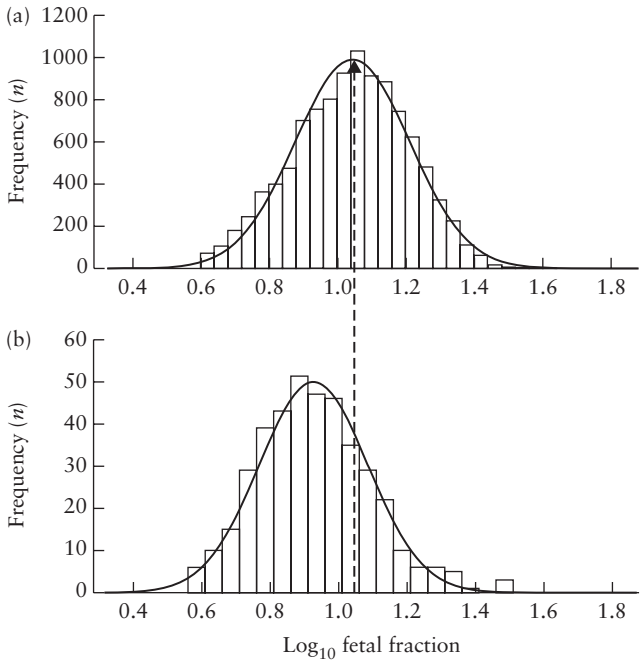

Figure 1 Frequency distribution of $\log_{10}$ fetal fraction of cell-free (cf) DNA in maternal blood in singleton (a) and twin (b) pregnancies undergoing cfDNA testing for aneuploidies. Dashed line indicates median fetal fraction in singleton pregnancies.

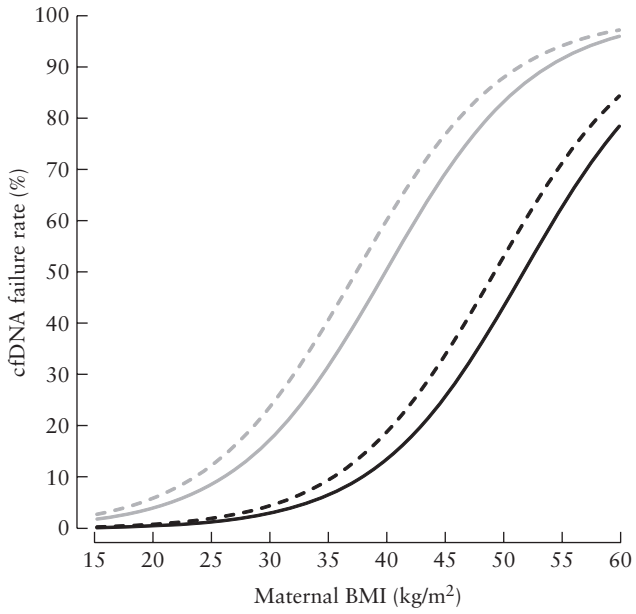

Figure 2 Failure rate of cell-free (cf) DNA testing plotted against maternal body mass index (BMI) in singleton (solid lines) and twin (dashed lines) pregnancies conceived by *in-vitro* fertilization (gray lines) or spontaneously or through ovulation induction (black lines). In these estimates it was assumed that maternal age was 35 years, racial origin was Caucasian and fetal crown–rump length was 55 mm.

In the 41 twin pregnancies with failed cfDNA testing after first sampling, 39 had repeat cfDNA testing and this provided results in 20 (51.3%) cases. In the 316 singleton pregnancies with failed cfDNA testing after first sampling, 235 had repeat cfDNA testing and this provided results in 148 (63.0%) cases[21].

For the combined data from singleton and twin pregnancies, multivariate logistic regression analysis demonstrated that the risk of test failure after first sampling increased with increasing maternal age and BMI and decreased with increasing fetal CRL. The risk was higher in twin pregnancies than in singletons and increased in women of South Asian racial origin and in pregnancies conceived by IVF (Table 2). The relationship of test failure with BMI and method of conception in singleton and twin pregnancies is illustrated in Figure 2 and reported in Table 3.

In twin pregnancies, multivariate logistic regression analysis demonstrated that the risk of test failure after first sampling increased with increasing maternal BMI and in pregnancies achieved by IVF, and decreased with increasing fetal CRL (Table S3).

Table 2 Univariate and multivariate logistic regression analyses demonstrating factors from maternal and pregnancy characteristics that contribute significantly to the prediction of a failed cell-free DNA test

	Univariate		*Multivariate*	
Independent variable	*Odds ratio (95% CI)*	P	*Odds ratio (95% CI)*	P
Constant			−8.470	< 0.0001
Age in years	1.051 (1.026–1.076)	< 0.0001	1.031 (1.007–1.055)	0.011
Body mass index in kg/m^2	1.138 (1.121–1.155)	< 0.0001	1.174 (1.154–1.194)	< 0.0001
Racial origin				
Caucasian	Reference	< 0.0001		
African	1.915 (1.356–2.705)	< 0.0001		
South Asian	2.006 (1.420–2.834)	< 0.0001	1.851 (1.284–2.669)	0.001
East Asian	0.592 (0.277–1.262)	0.174		
Mixed	1.561 (0.791–3.079)	0.199		
Cigarette smoker	0.692 (0.306–1.564)	0.376		
In-vitro fertilization	5.702 (4.572–7.110)	< 0.0001	6.487 (5.009–8.401)	< 0.0001
Fetal crown–rump length in mm	0.991 (0.983–0.998)	0.009	0.985 (0.977–0.993)	< 0.0001
Pregnancy type				
Singleton	Reference	< 0.0001		
Twin	3.393 (2.414–4.769)	< 0.0001	1.477 (1.006–2.170)	0.040

Table 3 Estimate of failure rate of cell-free (cf) DNA testing in singleton and twin pregnancies conceived spontaneously or by *in-vitro* fertilization (IVF), stratified according to maternal body mass index (BMI) for a fixed maternal age of 35 years, Caucasian racial origin and fetal crown–rump length of 55 mm

	Failure rate of cfDNA testing (95% CI) (%)			
	Singleton		*Twin*	
BMI (kg/m²)	*Spontaneous*	*IVF*	*Spontaneous*	*IVF*
15	0.3 (0.2–0.4)	1.9 (1.6–2.2)	0.4 (0.3–0.5)	2.7 (2.4–3.0)
16	0.3 (0.2–0.4)	2.2 (1.9–2.5)	0.5 (0.4–0.6)	3.2 (2.8–3.6)
17	0.4 (0.3–0.5)	2.5 (2.2–2.8)	0.6 (0.4–0.8)	3.7 (3.3–4.1)
18	0.5 (0.4–0.6)	3.0 (2.6–3.4)	0.7 (0.5–0.9)	4.3 (3.9–4.7)
19	0.6 (0.4–0.8)	3.5 (3.1–3.9)	0.8 (0.6–1.0)	5.0 (4.5–5.5)
20	0.6 (0.4–0.8)	4.0 (3.6–4.4)	1.0 (0.8–1.2)	5.9 (5.4–6.4)
21	0.8 (0.6–1.0)	4.7 (4.3–5.1)	1.1 (0.9–1.3)	6.8 (6.3–7.3)
22	0.9 (0.7–1.1)	5.5 (5.0–6.0)	1.3 (1.1–1.5)	7.9 (7.3–8.5)
23	1.0 (0.8–1.2)	6.4 (5.9–6.9)	1.5 (1.2–1.8)	9.1 (8.5–9.7)
24	1.2 (1.0–1.4)	7.4 (6.9–7.9)	1.8 (1.5–2.1)	10.6 (10.0–11.2)
25	1.4 (1.2–1.6)	8.6 (8.0–9.2)	2.1 (1.8–2.4)	12.2 (11.5–12.9)
26	1.7 (1.4–2.0)	9.9 (9.3–10.5)	2.5 (2.2–2.8)	14.0 (13.3–14.7)
27	2.0 (1.7–2.3)	11.5 (10.8–12.2)	2.9 (2.6–3.2)	16.0 (15.2–16.8)
28	2.3 (2.0–2.6)	13.2 (12.5–13.9)	3.3 (2.9–3.7)	18.3 (17.5–19.1)
29	2.7 (2.4–3.0)	15.1 (14.4–15.8)	3.9 (3.5–4.3)	20.8 (20.0–21.6)
30	3.1 (2.7–3.5)	17.3 (16.5–18.1)	4.6 (4.2–5.0)	23.6 (22.7–24.5)
31	3.7 (3.3–4.1)	19.7 (18.9–20.5)	5.3 (4.8–5.8)	26.6 (25.7–27.5)
32	4.3 (3.9–4.7)	22.4 (21.5–23.3)	6.2 (5.7–6.7)	29.9 (29.0–30.8)
33	5.0 (4.5–5.5)	25.3 (24.4–26.2)	7.2 (6.7–7.7)	33.3 (32.3–34.3)
34	5.8 (5.3–6.3)	28.4 (27.5–29.3)	8.3 (7.7–8.9)	37.0 (36.0–38.0)
35	6.7 (6.2–7.2)	31.8 (30.8–32.8)	9.6 (9.0–10.2)	40.8 (39.8–41.8)
36	7.8 (7.2–8.4)	35.4 (34.4–36.4)	11.1 (10.5–11.7)	44.7 (43.7–45.7)
37	9.0 (8.4–9.6)	39.1 (38.1–40.1)	12.8 (12.1–13.5)	48.7 (47.7–49.7)
38	10.4 (9.8–11.0)	43.0 (42.0–44.0)	14.7 (14.0–15.4)	52.7 (51.7–53.7)
39	12.0 (11.3–12.7)	47.0 (46.0–48.0)	16.8 (16.0–17.6)	56.7 (55.7–57.7)
40	13.8 (13.1–14.5)	51.0 (50.0–52.0)	19.1 (18.3–19.9)	60.6 (59.6–61.6)
41	15.8 (15.0–16.6)	55.0 (54.0–56.0)	21.7 (20.8–22.6)	64.3 (63.3–65.3)
42	18.1 (17.3–18.9)	58.9 (57.9–59.9)	24.6 (23.7–25.5)	67.9 (66.9–68.9)
43	20.6 (19.8–21.4)	62.7 (61.7–63.7)	27.7 (26.8–28.6)	71.3 (70.4–72.2)
44	23.3 (22.4–24.2)	66.4 (65.4–67.4)	31.0 (30.0–32.0)	74.5 (73.6–75.4)
45	26.3 (25.4–27.2)	69.9 (69.0–70.8)	34.5 (33.5–35.5)	77.4 (76.5–78.3)
46	29.5 (28.6–30.4)	73.1 (72.2–74.0)	38.3 (37.3–39.3)	80.1 (79.3–80.9)
47	33.0 (32.0–34.0)	76.2 (75.3–77.1)	42.1 (41.1–43.1)	82.5 (81.7–83.3)
48	36.6 (35.6–37.6)	78.9 (78.1–79.7)	46.1 (45.1–47.1)	84.7 (84.0–85.4)
49	40.4 (39.4–41.4)	81.5 (80.7–82.3)	50.1 (49.1–51.1)	86.7 (86.0–87.4)
50	44.3 (43.4–45.3)	83.8 (83.0–84.6)	54.0 (53.0–55.0)	88.4 (87.7–89.1)
51	48.3 (47.3–49.3)	85.8 (85.1–86.5)	58.0 (57.0–59.0)	90.0 (89.4–90.6)
52	52.3 (51.3–53.3)	87.7 (87.0–88.4)	61.8 (60.8–62.8)	91.3 (90.7–91.9)
53	56.3 (55.3–57.3)	89.3 (88.7–89.9)	65.5 (64.5–66.5)	92.5 (92.0–93.0)
54	60.2 (59.2–61.2)	90.7 (90.1–91.3)	69.1 (68.1–70.1)	93.5 (93.0–94.0)
55	64.0 (63.0–65.0)	92.0 (91.4–92.6)	72.4 (71.5–73.3)	94.5 (94.0–95.0)
56	67.6 (66.6–68.6)	93.1 (92.6–93.6)	75.5 (74.6–76.4)	95.2 (94.8–95.6)
57	71.0 (70.1–71.9)	94.1 (93.6–94.6)	78.3 (77.4–79.2)	95.9 (95.5–96.3)
58	74.2 (73.3–75.1)	94.9 (94.4–95.4)	80.9 (80.1–81.7)	96.5 (96.1–96.9)
59	77.1 (76.2–78.0)	95.6 (95.2–96.0)	83.3 (82.5–84.1)	97.0 (96.6–97.4)
60	79.8 (79.0–80.6)	96.3 (95.9–96.7)	85.4 (84.7–86.1)	97.4 (97.1–97.7)

DISCUSSION

Principal findings of the study

This prospective study demonstrates the feasibility of chromosome-selective sequencing of cfDNA in maternal blood for the assessment of risk for fetal trisomies 21, 18 and 13 in twin pregnancies at 10–13 weeks' gestation. In twin pregnancies, compared to singletons, the median fetal fraction was lower (8% *vs* 11%) and the failure rate after first sampling was three times higher (9.4% *vs* 2.9%). In those with a failed test, repeat testing provided a result in 51% of twins and in 63% of singletons.

There are two main factors contributing to the higher failure rate in twins compared to singletons. First, lower fetal fraction is an inevitable consequence of selecting the lower fetal fraction of the two fetuses rather than the total in estimating the risk for aneuploidies[11]; the rationale for this choice is to avoid a false-negative result in a dizygotic twin pregnancy, discordant for aneuploidy, for which the total fetal fraction is satisfactory but the contribution from the affected fetus could be less than 4%. Second, a considerably higher rate of conception by IVF in twin than singleton pregnancies, which was 56.2% *vs* 9.5% in our series.

In both singleton and twin pregnancies, the main contributors to low fetal fraction and high failure rate are high maternal BMI, conception by IVF, low fetal CRL and serum free β-hCG and PAPP-A. The source

of fetal cfDNA in maternal plasma is dying cells in the placenta, and the observed associations between fetal fraction and fetal CRL and serum free β-hCG and PAPP-A are likely to be the consequence of placental mass. The inverse association between fetal fraction and maternal weight could be attributed to a dilutional effect. Low fetal fraction in IVF pregnancies could be the consequence of the associated impaired placentation; in such pregnancies the serum concentration of PAPP-A is decreased by 10–25%[17,22,23] and the incidence of pre-eclampsia is increased[24,25].

The performance of screening for trisomies by cfDNA testing in twin pregnancies in our study was similar to that in singletons. However, the number of trisomic fetuses was too small for definitive conclusions to be drawn.

Comparison of findings to those of previous studies

Screening for trisomies in twin pregnancies by cfDNA testing has been carried out by massively parallel shotgun sequencing (MPSS) or by chromosome-selective sequencing. The MPSS studies examined stored plasma or prospectively collected blood from a combined total of 280 twin pregnancies[2,4,6,7]. In these studies no attempt was made to determine the fetal fraction for each twin and it was assumed that the contribution from each fetus to the maternal plasma cfDNA was adequate for accurate results. The cfDNA test provided results for all cases, the DR was 100% (23/23) for trisomy 21, 67% (2/3) for trisomies 18 or 13 and the FPR was 0% (0/254).

One previous study used chromosome-selective sequencing and an algorithm that relies on the lower fetal fraction of the twins, as in the present study, to assess risk for trisomies in either stored plasma samples or prospectively in blood obtained at 11–13 weeks' gestation[3]. In the retrospective study, the DR was 90% (9/10) for trisomy 21, 100% for trisomy 13 (1/1) and the FPR was 0% (0/181). In the prospective study, including 68 twin pregnancies that are included in the current study, there was test failure in 13.2% after first sampling which was reduced to 7.4% after second sampling. Both cases of trisomy 21 and the one case of trisomy 18 were detected, at a FPR of 0% (0/60); most euploid pregnancies were continuing at the time of publication and they have now delivered and the result of the cfDNA test was correct.

One multicenter study, which included 129 of the cases in the current study, used chromosome-selective sequencing for cfDNA testing in 515 twin pregnancies at 10–28 weeks' gestation[5]. The objective of the study was to compare the failure rate in twin pregnancies with that in 1847 singleton pregnancies; in twins the failure rate after first sampling was higher (5.6% *vs* 1.7%) and the main contributors to test failure were increased maternal weight and conception by IVF.

For the combined data from the MPSS studies and the current one, results from cfDNA testing have been reported in a total of 697 twin pregnancies with known outcome, the DR was 100% (31/31) for trisomy 21, 63% (5/8) for trisomies 18 or 13 and the FPR was 0.15% (1/658).

Implications for clinical practice

Monochorionic twins are monozygotic and do not pose any special problems in relation to cfDNA testing, invasive testing or subsequent management of possible trisomies; the fetal fraction is similar to that in singleton pregnancies, if the parents wish for invasive testing this can easily be carried out in the first trimester, allowing for the option of early pregnancy termination if this is the parental choice following diagnosis of trisomies in both fetuses. In contrast, dichorionic twins are usually dizygotic and they pose major challenges in screening and diagnosis of trisomies and subsequent management of pregnancies discordant for such aneuploidies. Over the last 20 years, the rate of twinning has increased, due mainly to the increasing maternal age of the population and the widespread use of IVF. The consequence of increased maternal age is that the proportion of twin pregnancies that are screen positive by traditional methods of screening is considerably higher than in singleton pregnancies. The consequence of increased conception by IVF is the high risk of cfDNA test failure. It could be argued that, in twin pregnancies conceived by IVF in women with high BMI and identified by traditional screening as being at high risk for trisomies, it would be preferable to select invasive testing rather than the cfDNA test. If the pregnancies are discordant for an aneuploidy and the parents choose to have selective feticide, the subsequent risks of miscarriage or early preterm birth increase with gestational age at feticide[26]. The high failure rate of cfDNA testing would shift the option of prenatal diagnosis and selective feticide from the first to the second trimester with a consequent increase in the rate of miscarriage. The counterargument is that the risk of miscarriage from invasive testing in twins is likely to be higher than in singletons and many older women conceiving by IVF would be reluctant to select invasive testing unless their risk from traditional screening is very high.

Conclusions

Screening by cfDNA testing of maternal blood in twin pregnancies, has a similarly high DR for trisomy 21 and a low FPR as in singleton pregnancies. The number of cases of trisomies 18 and 13 examined was too small for reliable conclusions to be drawn. Chromosome-selective sequencing with estimation of fetal fraction from each twin, which aims to minimize the risk of providing false-negative results by ensuring that the lower of the two is at least 4%, is associated with a higher failure rate than methods which do not measure fetal fraction or ignore assessment of the contribution of each fetus. In twin pregnancies, as in singletons, the main contributors to test failure of chromosome-selective sequencing are increased maternal weight and conception by IVF.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Table S1 Univariate and multivariate regression analyses demonstrating factors from maternal and pregnancy characteristics that provide significant contribution to the prediction of $\log_{10}$ fetal fraction in singleton and twin pregnancies combined

Table S2 Univariate and multivariate regression analyses demonstrating factors from maternal and pregnancy characteristics that provide significant contribution to prediction of $\log_{10}$ fetal fraction in twin pregnancies

Table S3 Univariate and multivariate logistic regression analyses demonstrating factors from maternal and pregnancy characteristics that provide significant contribution to prediction of failed cell-free DNA testing in twin pregnancies

