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

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Running head: Cell free DNA testing in twins

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ABSTRACT

Objectives: First, to examine the performance of screening for fetal trisomies 21, 18 and 13 in twin pregnancies by cell-free (cf) DNA testing of maternal blood and second, to compare twin and singleton pregnancies for the distribution of fetal fraction and failure rate 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}-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 contribution of the two fetuses. Multivariate regression analysis was used to determine significant predictors of fetal fraction and failed result.

Results: In twin pregnancies, compared to singletons, the median fetal fraction was lower (8.0, 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%; $p<0.0001$). 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. The main contributor to the higher rate of failure in twins was conception by *in vitro* fertilization 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% for trisomy 21 (8 of 8) and 60% for trisomy 18 or 13 (3 of 5), at false positive rate of 0.25% (1 of 404). In the 10,530 singleton pregnancies with a cfDNA result after first or second sampling, the detection rate was 98.7% for trisomy 21 (156 of 158) and 80.3% for trisomy 18 or 13 (49 of 61), at false positive rate of 0.22% (23 of 10,311).

Conclusions: In twin pregnancies, compared to singletons, undergoing first trimester screening for trisomies by cfDNA testing, the fetal fraction is lower and failure rate higher. The number of trisomic twin pregnancies examined is too small for accurate assessment of performance of screening, but this may be similar to that in singleton pregnancies.

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%), 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 where there is extensive data on the performance of screening for trisomies by cfDNA testing, very few studies reported data on twins [2-7]; most studies were retrospective on stored plasma samples or prospective but with incomplete follow up [2-5]. There are only two studies that examined twin pregnancies prospectively and reported outcome in all cases; in their combined data from 201 cases at 11-36 weeks' gestation, they correctly classified all 10 cases of trisomy 21, one of 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 number of examined cases is too small for accurate assessment of performance of screening.

In cfDNA testing the ability to detect the small increase in the amount of a given chromosome 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. Since 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 threshold of 4% but there is a high contribution from the disomic co-twin so 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 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 are first, is to examine the performance of cfDNA screening for fetal trisomies 21, 18 and 13 in twin pregnancies and second, to compare twin and singleton pregnancies for the distribution of fetal fraction and failure rate to obtain a result.

METHODS

The data for this study were derived from first, cfDNA testing as an option following first-trimester combined testing in women with singleton or twin pregnancies attending for routine care at 11^{+0}-13^{+6} weeks' gestation in one of two National Health Service (NHS) hospitals in England [12] and second, cfDNA testing as part of routine screening in pregnancies at 10^{+0}-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 (natural or assisted conception requiring the use of ovulation drugs or *in-vitro* fertilization), cigarette smoking during pregnancy (yes or no) and parity (parous or nulliparous if no previous pregnancy at or after 24 weeks' gestation). We also measured maternal weight and height. In all cases free ß-hCG and PAPP-A were measured within 10 minutes of blood

collection at 10^{+0}-13^{+6} weeks (DELFIA Xpress system, PerkinElmer Life and Analytical Sciences, Waltham, USA, or Kryptor, Thermo Scientific, Berlin, Germany). An ultrasound scan was carried out at 11^{+0}-13^{+6} weeks to determine gestational age from the measurement of the 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 ß-hCG and PAPP-A were converted into multiple of the median (MoM) for gestational age adjusted for maternal weight, racial origin, smoking status, method of conception, parity, chorionicity and machine 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) [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 fetal fraction contribution of the two fetuses was considered [11]. Risk scores for trisomy 21, 18, and 13 were provided as a percentage with ranges capped at >99% and <0.01%.

In cases where the cfDNA test did not provide results the parents were offered repeat testing or to rely on the results of the combined test in deciding whether to have an invasive test or not. In cases with a high-risk result from the cfDNA test, the parents were advised to consider having invasive fetal karyotyping before deciding on the further management of their pregnancy.

Patient characteristics 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 into firstly, trisomy 21, 18 or 13 if the karyotype of chorionic villi, amniotic fluid or neonatal blood demonstrated the relevant trisomy, secondly, 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, thirdly, no known karyotype because the pregnancies resulted in miscarriage or stillbirth and no karyotyping of fetal tissue was carried out, and fourthly, 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. 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, racial origin, smoking status, method of conception, fetal CRL, serum PAPP-A and free ß-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 prediction of failed cfDNA test result after first sampling.

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

RESULTS

Characteristics of 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 pregnancies ended in termination, miscarriage or stillbirth with no known karyotype (n=23), they were lost to follow up (n=4) or they 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 from a previous study in singleton pregnancies [21], are summarized in Table 1.

The 438 twin pregnancies included 8 cases of trisomy 21, 4 of trisomy 18, 1 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% for trisomy 21 (8 of 8), 75% for trisomy 18 (3 of 4) and 0% for trisomy 13 (0 of 1), at false positive rate of 0.25% (1 of 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% for trisomy 21 (156 of 158), 89.1% for trisomy 18 (41 of 46) and 53.3% for trisomy 13 (8 of 15), at false positive rate of 0.22% (23 of 10,311).

Factors affecting fetal fraction in singleton and twin pregnancies

The distribution of log10 fetal fraction from first sampling was Gaussian in both the singleton and twin pregnancies (Figure 1), but the median fetal fraction in twins was lower 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 the cases of failed result, the fetal fraction was 3%.

In the combined data from singleton and twin pregnancies, multivariate logistic regression analysis demonstrated that the fetal fraction increased with increasing fetal CRL, PAPP-A and free β-hCG MoM and decreased with increasing maternal age and body mass index, and it was lower in twin pregnancies than in singletons and decreased in women of South Asian racial origin and in pregnancies achieved by *in vitro* fertilization (Supplementary Table 1)

In twin pregnancies, multivariate logistic regression analysis demonstrated that the fetal fraction increased with increasing PAPP-A and free β-hCG MoM and decreased with increasing maternal body mass index. The fetal fraction was higher in monochorionic than dichorionic twins (median 10.1, IQR 7.6-14.5% vs median 7.7, IQR 5.8-10.0%; $p<0.0001$) and it was decreased in pregnancies achieved by *in vitro* fertilization (Supplementary Table 2).

Factors affecting cfDNA test failure in singleton and twin pregnancies

There was no result from cfDNA testing after first sampling in 2.9% (316 of 10,698) singleton pregnancies and in 9.4% (41 of 438) twin pregnancies ($p<0.0001$). In the 41 twin pregnancies with failed cfDNA testing after first sampling, 39 patients 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 patients had repeat cfDNA testing and this provided results in 148 (63.0%) cases [21].

In the combined data from singleton and twin pregnancies, multivariate logistic regression analysis demonstrated that risk of test failure after first sampling increased with increasing maternal age and body mass index and decreased with fetal CRL. The risk was higher in twin pregnancies than in singletons and increased in women of South Asian racial origin and in pregnancies achieved by *in vitro* fertilization (Table 2). The relation of test failure to body mass index and method of conception in singleton and twin pregnancies is illustrated in Figure 2 and reported in supplementary Table 3.

In twin pregnancies, multivariate logistic regression analysis demonstrated that risk of test failure after first sampling increased with increasing body mass index, decreased with fetal CRL and was increased in pregnancies achieved by *in vitro* fertilization (Table 3).

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. The reason for the lower fetal fraction and higher failure rate in twins, compared to singletons, is the 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 where the total fetal fraction is satisfactory but the contribution of the affected fetus could be less than 4%.

In both singleton and twin pregnancies, the main contributors to low fetal fraction and high failure rate are high maternal body mass index, conception by *in vitro* fertilization, low fetal CRL and low serum free β-hCG and PAPP-A. The main factor for the higher rate of failure in twins was conception by *in vitro* fertilization which was observed in 9.5% of our singleton and 56.2% of our twin pregnancies. 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 conceptions by *in vitro* fertilization could be the consequence of the associated inpaired placentation; in such pregnancies the serum concentration of PAPP-A is decreased by 10-25% [17,22,23] and the incidence of preeclampsia is increased [24,25].

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

Comparison of findings to those in 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 of 23) for trisomy 21, 67% (2 of 3) for trisomy 18 or 13 and the FPR was 0% (0 of 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 of 10) for trisomy 21, 100% for trisomy 13 (1 of 1) and the FPR was 0% (0 of 181). In the prospective study in 68 twin pregnancies, which are included in the current study, there was test failure of 13.2% after first sampling and this was reduced to 7.4% after second sampling. Both cases of trisomy 21 and the one case of trisomy 18 were detected at FPR of 0% (0 of 60); although at the time of publication most euploid pregnancies were continuing they have now delivered and the result of the cfDNA test was correct.

One multicenter study, which included some of the data 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 of 1,847 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 *in vitro* fertilization.

In 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 of 31) for trisomy 21, 63% (5 of 8) for trisomy 18 or 13 and the FPR was 0.15% (1 of 658).

Implication 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 invasive testing this can easily be carried 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 dizogotic and they pose major challenges in screening and diagnosis of trisomies and subsequent management of pregnancies discordant for such aneuploidies. In the last 20 years the rate of twinning has increased, mainly due to the increasing maternal age of the population and the widespread use of *in vitro* fertilization. The consequence of increased maternal age is that the proportion of twin pregnancies that are screen positive by the traditional methods of screening is considerably higher than in singleton pregnancies. The consequence of increased conception by *in vitro* fertilization is the high risk of cfDNA test failure. It could be argued that in twin pregnancies conceived by *in vitro* fertilization in women with high body mass index 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 fetocide, the subsequent risk of miscarriage or early preterm birth increase with gestational age at fetocide [26]. The high failure rate of cfDNA testing would shift the option of prenatal diagnosis and selective

fetocide from the first to the second trimester with consequent increase in the rate of miscarriage. The counter argument 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 *in vitro* fertilization 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 low FPR as in singleton pregnancies. The number of cases of trisomies 18 and 13 examined is very 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 *in vitro* fertilization.

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Figure legend

Figure 1. Frequency distribution of log_{10} fetal fraction in maternal blood cfDNA in singleton (top) and twin (bottom) pregnancies.

Figure 2. Failure rate of cell-free DNA testing (%) plotted against maternal body mass index in singleton (solid lines) and twin (dotted lines) pregnancies with conception by *in vitro* fertilization (red lines) or spontaneously or through ovulation induction (blue lines). In these estimates it was assumed that maternal age was 35 years, the racial origin was Caucasian and the fetal crown-rump length was 55 mm.

Table 1. Maternal and pregnancy characteristics of the study population.

Maternal and pregnancy characteristic	**Singleton (n=10,698)**	**Twin (n=438)**
Maternal age in years, median (IQR)	36.3 (33.2-39.3)	37.3 (34.6-40.0)***
Maternal body mass index in kg/m2, median (IQR)	23.3 (21.1-26.5)	23.5 (21.0-26.9)
Racial origin		
Caucasian, n (%)	8,751 (81.8)	358 (81.7)
African, n (%)	698 (6.5)	26 (5.9)
South Asian, n (%)	663 (6.2)	30 (6.8)
East Asian, n (%)	386 (3.6)	19 (4.3)
Mixed, n (%)	200 (1.9)	5 (1.1)
Parity		
Nulliparous, n (%)	4,760 (44.5)	261 (59.6)***
Cigarette smoker, n (%)	263 (2.5)	3 (0.7)**
Conception		
Spontaneous or ovulation induction, n (%)	9683 (90.5)	192 (43.8)
In vitro fertilization, n (%)	1015 (9.5)	246 (56.2)***
Origin of oocyte		
Self	904 (89.1)	216 (87.8)
Donor	111 (10.9)	30 (12.2)
Gestation at sampling, median (IQR)	11.9 (10.6-12.9)	11.7 (10.4-12.9)*
Crown-rump length in mm, median (IQR)	53.7 (38.5-65.7)	54.2 (38.9-66.3)

IQR = interquartile range; CRL = crown-rump length; Significance value * $p<0.05$; ** $p<0.01$; *** $p<0.0001$

Table 2. Univariate and multivariate logistic regression analysis demonstrating factors from maternal and pregnancy characteristics providing significant contribution to prediction of failed cfDNA testing

Independent variable	Univariate analysis		Multivariate analysis	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
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, kg/m^2	1.138 (1.121-1.155)	<0.0001	1.174 (1.154-1.194)	<0.0001
Race 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		
Smoking	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 CRL in mm	0.991 (0.983-0.998)	0.009	0.985 (0.977-0.993)	<0.0001
Pregnancy type				
Singleton pregnancy	(Reference)	<0.0001		
Twin pregnancy	3.393 (2.414-4.769)	<0.0001	1.477 (1.006-2.170)	0.040

CI = confidence interval; CRL = crown-rump length; MoM = multiple of the median; NT = nuchal translucency

Table 3. Estimated rate of failure of cell-free DNA (cfDNA) testing in singleton and twin pregnancies stratified according to method of conception in women with a body mass index from 15 to 60 kg/m^2 given a fixed maternal age of 35 years, Caucasian racial origin and a fetal crown-rump length of 55 mm.

BMI (kg/m^2)	Rate of failure of cell free DNA testing [% (95%CI)]			
	Singleton pregnancy		Twin pregnancy	
	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)
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 - 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)

BMI= Body mass index; IVF = *In vitro* fertilization; CI = confidence interval

Supplementary Table 1. Univariate and multivariate regression analysis demonstrating factors from maternal and pregnancy characteristics providing significant contribution to prediction of log_{10} transformed fetal fraction

Independent variable	Univariate analysis		Multivariate analysis	
	Coefficient (95% CI)	P value	Coefficient (95% CI)	P value
Intercept			1.423 (1.394 to 1.452)	<0.0001
Age in years	-0.002 (-0.003 to -0.001)	<0.0001	-0.002 (-0.002 to -0.001)	<0.0001
Body mass index in kg/m^2	-0.015 (-0.016 to -0.014)	<0.0001	-0.015 (-0.016 to -0.014)	<0.0001
Race origin				
Caucasian	(Reference)			
African	-0.055 (-0.070 to -0.040)	<0.0001		
South Asian	-0.019 (-0.034 to -0.004)	0.013	-0.014 (-0.026 to -0.001)	0.034
East Asian	0.026 (0.006 to 0.045)	0.009		
Mixed	0.009 (-0.018 to 0.036)	0.500		
Smoking	0.010 (-0.013 to 0.034)	0.400		
In vitro fertilization	-0.107 (-0.119 to -0.096)	<0.0001	-0.092 (-0.102 to -0.081)	<0.0001
Fetal CRL in mm	$9.7e^{-05}$ ($-1.4e^{-04}$ to $3.4e^{04}$)	0.423	0.001 ($4.5e^{-04}$ to 0.001)	<0.0001
Pregnancy type				
Singleton pregnancy	(Reference)			
Twin pregnancy	-0.133 (-0.151 to -0.115)	<0.0001	-0.091 (-0.108 to -0.075)	<0.0001
Delta nuchal translucency	-0.009 (-0.015 to -0.004)	0.001		
Log_{10} PAPP-A MoM	0.160 (0.146 to 0.173)	<0.0001	0.128 (0.116 to 0.140)	<0.0001
Log_{10} free β-hCG MoM	0.170 (0.157 to 0.182)	<0.0001	0.138 (0.126 to 0.149)	<0.0001
Fetal karyotype				
Euploid	(Reference)			
Trisomy 21	-0.006 (-035 to 0.024)	0.706		
Trisomy 13/18	-0.156 (-0.201 to -0.111)	<0.0001		

CI = confidence interval; CRL = crown-rump length; MoM = multiple of the median; NT = nuchal translucency

Supplementary Table 2. Univariate and multivariate regression analysis demonstrating factors from maternal and pregnancy characteristics providing significant contribution to prediction of log_{10} transformed fetal fraction in twin pregnancies

Independent variable	Univariate analysis		Multivariate analysis	
	Coefficient (95% CI)	P value	Coefficient (95% CI)	P value
Intercept			1.182 (1.084 to 1.280)	<0.0001
Age in years	-0.004 (-0.008 to -7.7e-^{05})	0.046		
Body mass index in kg/m^2	-0.010 (-0.014 to -0.006)	<0.0001	-0.011 (-0.015 to -0.007)	<0.0001
Race origin				
Caucasian	(Reference)			
African	-0.035 (-0.115 to 0.046)	0.396		
South Asian	-0.040 (-0.115 to 0.035)	0.299		
East Asian	-0.054 (-0.147 to 0.039)	0.257		
Mixed	0.017 (-0.161 to 0.195)	0.852		
Smoking	-0.012 (-0.242 to 0.218)	0.918		
In vitro fertilisation	-0.081 (-0.118 to -0.043)	<0.0001	-0.065 (-0.100 to -0.029)	<0.0001
Origin of oocyte				
Self	(Reference)			
Donor	0.032 (-0.050 to 0.114)	0.444		
Fetal CRL in mm	0.001 (-0.0002 to 0.002)	0.102		
Delta nuchal translucency	-0.011 (-0.035-0.014)	0.388		
Chorionicity				
Dichorionic	(Reference)			
Monochorionic	0.139 (0.088 to 0.191)	<0.0001	0.118 (0.068 to 0.167)	<0.0001
Log_{10} PAPP-A MoM	0.137 (0.054 to 0.912)	0.001	0.133 (0.055 to 0.212)	0.001
Log_{10} free β-hCG MoM	0.119 (0.048 to 0.190)	0.001	0.106 (0.039 to 0.173)	0.002

CI = confidence interval; CRL = crown-rump length; MoM = multiple of the median; NT = nuchal translucency

Supplementary Table 3. Univariate and multivariate logistic regression analysis demonstrating factors from maternal and pregnancy characteristics providing significant contribution to prediction of failed cfDNA testing in twin pregnancies

Independent variable	Univariate analysis		Multivariate analysis	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
Constant			-5.054	<0.0001
Age in years	1.097 (1.017-1.184)	0.016		
Body mass index in kg/m^2	1.093 (1.030-1.160)	0.003	1.142 (1.066-1.224)	<0.0001
Race origin				
Caucasian	(Reference)			
African	0.422 (0.055-3.220)	0.405		
South Asian	2.110 (0.754-5.900)	0.155		
East Asian	1.978 (0.546-7.163)	0.299		
Mixed	2.637 (0.286-24.330)	0.392		
Smoking	-	-		
In vitro fertilization	3.563 (1.606-7.908)	0.002	3.898 (1.644-9.241)	0.002
Origin of oocyte				
Self	(Reference)			
Donor	0.992 (0.323-3.050)	0.989		
Fetal CRL in mm	0.974 (0.952-0.997)	0.027	0.971 (0.947-0.995)	0.020
Chorionicity				
Dichorionic	(Reference)			
Monochorionic	0.272 (0.064-1.155)	0.078		

CI = confidence interval; CRL = crown-rump length; MoM = multiple of the median