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First-trimester screening for trisomies by cfDNA testing of maternal blood in singleton and twin pregnancies: factors affecting test failure

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

ABSTRACT

Objective *To examine factors affecting the rate of failure to obtain a result from cell-free DNA (cfDNA) testing of maternal blood for fetal trisomies 21, 18 and 13 in singleton and twin pregnancies in the first trimester.*

Methods *This was a prospective study of 23 495 singleton and 928 twin pregnancies undergoing screening for fetal trisomy by targeted cfDNA testing at 10 + 0 to 14 + 1 weeks' gestation. Multivariate logistic regression analysis was used to determine significant predictors of failure to obtain a result after first sampling.*

Results *There was no result from cfDNA testing after first sampling in 3.4% (798/23 495) of singletons, 11.3% (91/806) of dichorionic twins and 4.9% (6/122) of monochorionic twins. Multivariate logistic regression analysis demonstrated that the risk of test failure, first, increased with increasing maternal age (odds ratio (OR), 1.02; 95% CI, 1.01–1.04) and weight (OR, 1.05; 95% CI, 1.04–1.05), decreasing gestational age (OR, 0.85; 95% CI, 0.79–0.91), serum pregnancy-associated plasma protein-A (PAPP-A) multiples of the median (MoM) (OR, 0.56; 95% CI, 0.49–0.65) and free β-human chorionic gonadotropin (β-hCG) MoM (OR, 0.67; 95% CI, 0.60–0.74), second, was higher in women of black (OR, 1.72; 95% CI, 1.33–2.20) and South Asian (OR, 1.99; 95% CI, 1.56–2.52) than those of white racial origin, in dichorionic twin than in singleton pregnancy (OR, 1.75; 95% CI, 1.34–2.26) and in pregnancies conceived by* in-vitro *fertilization than in those conceived naturally (OR, 3.82; 95% CI, 3.19–4.55) and, third, was lower in parous than in nulliparous women (OR, 0.63; 95% CI, 0.55–0.74).*

Conclusions *Maternal age, weight, racial origin and parity, gestational age, dichorionicity, method of conception and serum levels of free β-hCG and PAPP-A are independent predictors of cfDNA test failure. The risk of test failure is higher in dichorionic twin than in singleton pregnancies, mainly because a higher proportion of twins are conceived by* in-vitro *fertilization and more of the women are nulliparous. Copyright © 2019 ISUOG. Published by John Wiley & Sons Ltd.*

INTRODUCTION

In singleton pregnancy, cell-free DNA (cfDNA) analysis of maternal blood provides effective screening for trisomies 21, 18 and 13[1]. A meta-analysis of clinical validation and implementation studies has reported that the performance of cfDNA testing for trisomy 21 in twin pregnancy is similar to that in singleton pregnancy; the number of cases of trisomies 18 and 13 was too small for accurate assessment of predictive performance of the test for these trisomies[2].

One issue with cfDNA testing as a method of screening for aneuploidy is failure to provide a result. There are essentially three reasons for such failure. First, problems with blood collection and transportation of the samples to the laboratory, including inadequate blood volume, hemolysis, incorrect labeling of tubes and delay in arrival to the laboratory. Second, low fetal fraction, usually below 4%, and, third, assay failure for a variety of

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reasons, including failed DNA extraction, amplification and sequencing[3]. The most common reason for test failure is low fetal fraction and the main contributors to low fetal fraction in both singleton and twin pregnancies are, first, small placental mass, reflected in early gestational age and low serum free β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A), because the likely source of fetal cfDNA in maternal plasma is dying cells in the placenta, and, second, high maternal weight, which could be attributed to a dilution effect[4–7]. Impaired placentation has also been considered to be a likely reason for the low fetal fraction and high test failure rate in pregnancies conceived by *in-vitro* fertilization[7–10]. In twin pregnancy, the targeted approach to cfDNA testing with estimation of fetal fraction for each twin, which aims to minimize the risk of providing a false-negative result 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.

The objective of this expanded series of 23 495 singleton and 928 twin pregnancies undergoing screening for fetal trisomy by targeted cfDNA testing at 10 + 0 to 14 + 1 weeks' gestation was to explore further the relationship between test failure rate and maternal and pregnancy characteristics. This study also explored potential differences in failure rate between two methods of targeted analysis, one based on next-generation sequencing and another based on microarray.

METHODS

Study design and participants

The data for this study were derived from prospective screening for trisomies 21, 18 and 13 in singleton and twin pregnancies at 10 + 0 to 14 + 1 weeks' gestation. Two populations were included; first, self-referred women to the Fetal Medicine Centre in London, England, which is a private clinic[3,11] and, second, women selected for the cfDNA test after routine first-trimester combined testing in one of two National Health Service hospitals in England[12,13]. The patients were examined between October 2012 and January 2018. The study was approved by the National Research Ethics Committee (REC reference 13/LO/0885, 19/HRA/0576).

We recorded maternal characteristics and medical history, including maternal age and racial origin (white, black, South Asian, East Asian or 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 ≥ 24 weeks' gestation). We also measured maternal weight and height. In all cases, free β-hCG and PAPP-A were measured at 10 + 0 to 14 + 1 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 to 14 + 1 weeks to determine gestational age from the measurement of fetal crown–rump length (CRL)[14], diagnose any major fetal abnormality and measure fetal nuchal translucency 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 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[8].

Women provided written informed consent and maternal blood (20 mL) was sent via courier to the USA for cfDNA testing (Harmony® prenatal test, Roche/Ariosa Diagnostics, Inc., San Jose, CA, USA)[16,17]. Harmony uses Digital ANalysis of Selected Regions (DANSR) assays targeting sequences on chromosomes 13, 18 and 21 for chromosome quantitation and single-nucleotide polymorphisms on chromosomes 1 to 12 for fetal-fraction measurement. Products of the DANSR assays can be quantified using either next-generation sequencing (chromosome-selective sequencing) or a custom microarray; both were used during the course of this study. The data were analyzed with the fetal fraction-optimized risk of trisomy evaluation (FORTE) algorithm, which calculates probability scores for fetal trisomy, with > 1% considered to be high probability. In cases in which the cfDNA test did not provide a result, the parents were offered repeat testing or to rely on the results of the combined test in deciding whether or not to have an invasive test. In cases with a high-risk result on the cfDNA test, the parents were advised to consider having invasive fetal karyotyping before deciding on the further management of their pregnancy.

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

Statistical analysis

Descriptive data are presented as median and interquartile range (IQR) for continuous variables and as number and percentage for categorical variables. Comparisons between groups were performed using the Mann–Whitney *U*-test for continuous variables and the χ^2 test or Fisher's exact test for categorical variables. In the combined data of singleton and twin pregnancies,

multivariate logistic regression analysis was used to determine which of the factors amongst maternal age, weight, height, racial origin, smoking status, parity, method of conception, gestational age at sampling, serum PAPP-A and free β-hCG MoM, fetal karyotype, type of pregnancy (singleton or dichorionic or monochorionic twin) and type of targeted analysis (sequencing or microarray) were significant predictors of failed cfDNA test result after first sampling. Variance inflation factor (VIF), which represents the factor by which the variance is inflated, was used to assess multicollinearity; VIF values ≥ 4 require further investigation, whereas VIF values > 10, which are a sign of serious multicollinearity, require correction.

We conducted statistical analyses using R package software[18]. VIFs were calculated using the R package 'jtools'[19].

RESULTS

Study population

A total of 24 974 singleton pregnancies had cfDNA testing, but 1479 (5.9%) of these were excluded from further analysis either because the pregnancy ended in termination, miscarriage or stillbirth with no known karyotype ($n = 356$) or was lost to follow-up ($n = 1123$). In the 23 495 cases included in the study, there were 324 with trisomy 21, 104 with trisomy 18, 28 with trisomy 13 and 23 039 without trisomy 21, 18 or 13.

A total of 991 twin pregnancies had cfDNA testing, but 63 (6.4%) of these were excluded from further analysis either because the pregnancy ended in termination, miscarriage or stillbirth with no known karyotype ($n = 45$) or was lost to follow-up ($n = 18$). Of the 928 cases included in the study, 806 (86.9%) were dichorionic and 122 (13.1%) were monochorionic. The 928 twin pregnancies included 14 with trisomy 21, seven with trisomy 18, one with trisomy 13 and 906 without trisomy 21, 18 or 13; all trisomic cases were a dichorionic pregnancy and in all cases one fetus was trisomic and the cotwin was non-trisomic.

Maternal and pregnancy characteristics of the 23 495 singleton and 928 twin pregnancies included in the study are summarized in Table 1. cfDNA testing was done by means of sequencing in 9440 of the singleton pregnancies and 313 of the twin pregnancies and by microarray in 14 055 of the singleton pregnancies and 615 of the twin pregnancies.

Factors affecting cfDNA test failure after first sampling

There was no result from cfDNA testing after first sampling in 3.4% (798/23 495) of singletons (433 because of insufficient fetal cfDNA and 365 because the sample did not meet thresholds for quality control), in 11.3%

Table 1 Maternal and pregnancy characteristics of study population of singleton and twin pregnancies undergoing cell-free DNA (cfDNA) testing

Characteristic	*Singleton pregnancy* (n = *23 495*)	*Twin pregnancy* (n = *928*)	
		DC (n = *806*)	*MC* (n = *122*)
Maternal age (years)	36.1 (32.9–39.1)	37.2 (34.2–39.6)*	36.9 (34.0–39.2)
Maternal weight (kg)	64.0 (58.0–73.0)	65.0 (58.6–75.0)*	63.0 (56.1–71.7)
Maternal height (cm)	165 (161–170)	167 (162–170)*	165 (160–170)
Racial origin			
White	19 226 (81.8)	669 (83.0)*	100 (82.0)
Black	1412 (6.0)	51 (6.3)	3 (2.5)
South Asian	1525 (6.5)	52 (6.4)	8 (6.6)
East Asian	895 (3.8)	24 (3.0)	7 (5.7)
Mixed	437 (1.9)	10 (1.2)	4 (3.3)
Parity			
Nulliparous	10 402 (44.3)	494 (61.3)*	59 (48.4)*
Parous	13 093 (55.7)	312 (38.7)*	63 (51.6)*
Cigarette smoker	640 (2.7)	10 (1.2)*	1 (0.8)
Conception			
Natural	20 978 (89.3)	310 (38.5)*	85 (69.7)*
Ovulation induction	79 (0.3)	14 (1.7)*	2 (1.6)
In-vitro fertilization	2438 (10.4)	482 (59.8)*	35 (28.7)
Gestational age at sampling (weeks)	11.9 (10.7–12.9)	11.9 (10.6–12.7)	12.0 (10.7–13.0)
Serum PAPP-A MoM	0.89 (0.58–1.32)	1.04 (0.75–1.46)*	0.99 (0.72–1.48)*
Serum β-hCG MoM	1.11 (0.72–1.73)	1.01 (0.72–1.52)*	1.02 (0.65–1.56)*
Method of cfDNA testing			
Sequencing	9440 (40.2)	269 (33.4)*	44 (36.1)
Microarray	14 055 (59.8)	537 (66.6)*	78 (63.9)
No result after first cfDNA test	798 (3.4)	91 (11.3)	6 (4.9)

Data are given as median (interquartile range) or *n* (%). Comparisons between groups were performed using Mann–Whitney *U*-test for continuous variables and χ^2 or Fisher's exact test for categorical variables, with *post-hoc* Bonferroni correction. *$P < 0.025$ on comparison with singleton pregnancies. β-hCG, β-human chorionic gonadotropin; DC, dichorionic; MC, monochorionic; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein-A.

Table 2 Univariate and multivariate logistic regression analyses of factors from maternal and pregnancy characteristics in prediction of failed cell-free DNA (cfDNA) testing in 23 495 singleton and 928 twin pregnancies

	Univariate analysis		*Multivariate analysis*	
Variable	*Odds ratio (95% CI)*	P	*Odds ratio (95% CI)*	P
Intercept			0.008 (0.003–0.022)	< 0.0001
Maternal age in years	1.048 (1.033–1.064)	< 0.0001	1.024 (1.009–1.041)	0.002
Maternal weight in kg	1.041 (1.037–1.044)	< 0.0001	1.049 (1.045–1.053)	< 0.0001
Maternal height in cm	1.001 (0.991–1.011)	0.841	—	—
Racial origin				
White	Reference			
Black	1.942 (1.544–2.415)	< 0.0001	1.716 (1.328–2.196)	< 0.0001
South Asian	1.685 (1.333–2.105)	< 0.0001	1.992 (1.557–2.521)	< 0.0001
East Asian	0.665 (0.415–1.005)	0.069	—	—
Mixed	1.261 (0.765–1.952)	0.330	—	—
Cigarette smoker	1.187 (0.790–1.710)	0.382	—	—
Conception				
Natural	Reference			
Ovulation induction	0.789 (0.130–2.500)	0.741	—	—
In-vitro fertilization	4.550 (3.944–5.242)	< 0.0001	3.815 (3.195–4.551)	< 0.0001
Parity				
Nulliparous	Reference			
Parous	0.617 (0.539–0.706)	< 0.0001	0.635 (0.546–0.737)	< 0.0001
Gestational age in weeks	0.872 (0.824–0.923)	< 0.0001	0.847 (0.792–0.906)	< 0.0001
Serum PAPP-A MoM	0.576 (0.504–0.656)	< 0.0001	0.563 (0.489–0.645)	< 0.0001
Serum free β-hCG MoM	0.606 (0.547–0.669)	< 0.0001	0.668 (0.601–0.739)	< 0.0001
Fetal karyotype				
Non-trisomic	Reference			
Trisomy 21	0.975 (0.516–1.663)	0.932	—	—
Trisomy 18 or 13	2.484 (1.298–4.316)	0.003	1.133 (0.565–2.090)	0.704
Pregnancy type				
Singleton	Reference			
Dichorionic twin	3.620 (2.862–4.529)	< 0.0001	1.748 (1.343–2.255)	< 0.0001
Monochorionic twin	1.471 (0.574–3.072)	0.358	—	—
Method of cfDNA testing				
Sequencing	Reference			
Microarray	1.083 (0.945–1.244)	0.254	—	—

β-hCG, β-human chorionic gonadotropin; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein-A.

(91/806) of dichorionic twins (70 because of insufficient fetal cfDNA and 21 because the sample did not meet thresholds for quality control) and in 4.9% (6/122) of monochorionic twins (five because of insufficient fetal cfDNA and one because the sample did not meet thresholds for quality control).

In 614 of the 798 singleton pregnancies with no result after first sampling, the cfDNA test was repeated 1–2 weeks later and in 413 (67.3%) cases a result was obtained. Of 82 of the 91 dichorionic twin pregnancies with no result after first sampling, repeat testing provided a result in 45 (54.9%) cases. Of five of the six monochorionic twin pregnancies with no result after first sampling, repeat testing provided a result in all cases.

In the combined data from singleton and twin pregnancies, multivariate logistic regression analysis demonstrated that the risk of test failure, first, increased with increasing maternal age and weight and decreasing gestational age and serum PAPP-A MoM and free β-hCG MoM, second, was higher in women of black and South Asian than those of white racial origin, in dichorionic twin than in singleton pregnancy and in pregnancies conceived by *in-vitro* fertilization than in those conceived naturally and, third, was lower in parous than in nulliparous women; there was no significant contribution from fetal karyotype or method of cfDNA testing (Table 2). VIF was < 1.5 for all variables included in the regression analysis, excluding any concerns regarding multicollinearity.

DISCUSSION

Principal findings

In this study of cfDNA testing in singleton and twin pregnancies at 10–14 weeks' gestation, we found that the most important contributor to cfDNA test failure after first sampling is conception by *in-vitro* fertilization, which, by comparison with natural conception, increases the risk by 3.8 times. Other contributors to test failure are black or South Asian racial origin, which, by comparison with white origin, increase the risk by 2.0 and 1.7 times, respectively, and dichorionicity, which, by comparison with singleton pregnancy, increases the risk by 1.7 times. The risk of test failure increases by about 5% with each additional kilogram of maternal weight and by about 2% with each additional year of maternal age. In parous

women, the risk of test failure is 37% lower than in nulliparous women and other protective factors against test failure are increasing gestational age and increasing serum PAPP-A and free β-hCG. Test failure is unrelated to method used for cfDNA analysis and fetal karyotype, once serum PAPP-A and free β-hCG are taken into account.

Test failure after first sampling in dichorionic twins was 3.3 times higher than in singletons (11.3% *vs* 3.4%) but, to a great extent, this excess failure rate can be attributed to the fact that a considerably higher proportion of twins were conceived by *in-vitro* fertilization (59.8% *vs* 10.4%) and more women were nulliparous (61.3% *vs* 44.3%).

Comparison with previous studies

The data presented in this paper were derived from the use of the Harmony prenatal test and the results may not be applicable to other methods of cfDNA testing. In a previous meta-analysis on performance of screening for aneuploidy by cfDNA testing, we noted a wide range of failure rates between studies and it was not possible to draw conclusions on the possible relationship between the no-result rate and the method used for the analysis of the samples[1].

The main cause of cfDNA test failure is reduced fetal fraction and many previous studies have reported an inverse association between fetal fraction and maternal weight, which could be attributed to a dilution effect and/or enhanced turnover of adipocytes and a secondary increase in maternal cfDNA in maternal plasma[4–7,20–23]. Similarly, our finding of an increased risk of cfDNA test failure with decreasing gestational age, PAPP-A MoM and free β-hCG MoM is consistent with findings of previous studies and is probably related to smaller placental mass and therefore decreased placental cfDNA in maternal blood[5,6,20,24,25].

In our previous studies, we also found that, in pregnancies conceived by *in-vitro* fertilization, cfDNA test failure is increased[7,26], but another study on twin pregnancies analyzed by a different cfDNA technique could not demonstrate such an association[27]. Low fetal fraction in pregnancies conceived by *in-vitro* fertilization could be the consequence of the associated impaired placentation; in such pregnancies, the serum concentration of PAPP-A is decreased by 10–25%[8–10] and the incidence of pre-eclampsia is increased[28,29]. Two likely explanations for these different results are, first, the significantly higher gestational age at sampling (median, 16 (range, 10–35) weeks) in the study of Le Conte *et al.*[27] in comparison with our study (median, 11 (range, 10–14) weeks) and, second, the different method used for cfDNA analysis (massively parallel sequencing[27] *vs* targeted approach). It has been reported that the failure rate is lowest when using methods based on massive parallel sequencing (1.58%), followed by chromosome-selective sequencing (3.56%) and single-nucleotide polymorphism-based analysis (6.39%)[30]. In our study, two different methods for targeted cfDNA analysis were used (chromosome-selective sequencing and microarray-based analysis) and no differences were found between them in relation to test failure.

In pregnancies with cfDNA test failure, there is increased risk of fetal trisomies 18 and 13 and triploidy, but not trisomy 21[7,31,32]. In our study, we also found a significantly higher risk of test failure in trisomies 18 and 13, but this association did not remain after adjusting for serum biomarkers; it is therefore likely that this association is mediated by the small placental mass related to these conditions, rather than the conditions themselves.

Implications for clinical practice

In a very high proportion of both singleton and twin pregnancies, cfDNA testing will provide a result after first sampling. In pregnancies with cfDNA test failure, repeat sampling will provide a result in half to two-thirds of cases. Most women at highest risk of test failure (advanced age, obese, black, nulliparous women with dichorionic twin pregnancy conceived by *in-vitro* fertilization) are still likely to obtain a result from cfDNA testing but they should have pretest counseling regarding the possibility of a failed test. The decision in favor or against invasive testing in the management of pregnancies with a failed result, should depend on the risk from prior screening and the results of detailed ultrasound examination looking for specific features of trisomies 18 and 13 and triploidy.

Conclusions

Maternal age, weight, racial origin and parity, gestational age, dichorionicity, method of conception and serum levels of free β-hCG and PAPP-A are independent predictors of cfDNA test failure. The risk of test failure is higher in dichorionic twin than in singleton pregnancies, mainly because a higher proportion of twins are conceived by *in-vitro* fertilization and more of the women are nulliparous.

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