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

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Short title: cfDNA test failure

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ABSTRACT

Objectives: To examine factors affecting the failure rate to obtain a result from (cf) DNA testing of maternal blood for fetal trisomies 21, 18 and 13 in singleton and twin pregnancies in the first trimester of pregnancy.

Methods: This was a prospective study in 23,495 singleton and 928 twin pregnancies undergoing screening for fetal trisomies by targeted cfDNA testing at 10^{+0}-14^{+1} weeks' gestation. Multivariate 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 DC twins and in 4.9% (6/122) of MC twins. Multivariate logistic regression analysis demonstrated that the risk of test failure first, increased with increasing maternal age (odds ratio (OR) 1.02; 95% confidence interval (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) and serum PAPP-A (OR 0.56; 95% CI 0.49, 0.64) and free ß-hCG (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 White racial origin, in dichorionic twin (OR 1.75; 95% CI 1.34, 2.25) than singleton pregnancy and in *in vitro* fertilization (OR 3.82; 95% CI 3.19, 4.55) than natural conception and third, was lower in parous (OR 0.63; 95% CI 0.55, 0.74) than nulliparous women.

Conclusions: Maternal age, weight, racial origin and parity, gestational age, dichorionicity, method of conception and serum levels of free ß-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.

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

INTRODUCTION

In singleton pregnancies, cell-free (cf) DNA 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 pregnancies is similar to that in singleton pregnancies; the number of cases of trisomies 18 and 13 was too small for accurate assessment of predictive performance of the test for these trisomies.[1]

One issue with cfDNA testing as a method of screening for aneuploidies 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 labelling of tubes and delay in arrival to the laboratory. Second, low fetal fraction, usually below 4%, and thirdly, assay failure for a variety of reasons, including failed DNA extraction, amplification and sequencing.[2] 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-hCG and 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 dilutional effect.[3-6] Impaired placentation has also been considered to be the likely reason for the low fetal fraction and high failure rate in conceptions by *in vitro* fertilization.[6-9] In twin pregnancies the targeted approach to cfDNA testing 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.

The objective of this expanded series of 23,495 singleton and 928 twin pregnancies undergoing screening for fetal trisomies by targeted cfDNA testing at 10^{+0}-14^{+1} weeks' gestation was to explore further the relationship between failure rate and maternal and pregnancy characteristics. The study also explores 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 the study were derived from prospective screening for trisomies 21, 18 and 13 in twin pregnancies at 10^{+0}-14^{+1} weeks' gestation. Two populations were included; first self-referred women to the Fetal Medicine Centre in London, which is a private clinic[2,10] and second, women selected for the cfDNA test after routine first-trimester combined testing in one of two National Health Service hospitals in England.[11,12] 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, racial origin (White, Black, 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 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),[13] 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.[14] 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.[7]

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).[15,16] Harmony® uses DANSR assays targeting sequences on chromosomes 13, 18, and 21 for chromosome quantitation and single nucleotide polymorphisms on chromosomes 1 to 12 for fetal fraction measurement. Products of the DANSR assays can be quantified using either next-generation sequencing (chromosome selective sequencing) or a custom microarray; both were used during the course of this study. The data are analysed with the fetal fraction optimized FORTE algorithm, which calculates probability scores for fetal trisomy, with greater than 1% considered to be high probability. In cases where the cfDNA test did not provide results the parents were offered repeat testing or to rely on the results of the combined test in deciding whether to have an invasive test or not. In cases with a high-risk result from the cfDNA test, the parents were advised to consider having invasive fetal karyotyping before deciding on the further management of their pregnancy.

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

Statistical analyses

Descriptive data were presented in median and interquartile range (IQR) for continuous variables and in numbers and percentages for categorical variables. Comparisons between groups were performed using the Mann–Whitney U-test for continuous variables and the $\chi 2$-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 ß-hCG MoM, fetal karyotype, type of pregnancy (singleton, DC and MC twins) and type of targeted analysis (sequencing and 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 $\geq$ 4 require further investigation, whereas VIF values exceeding 10, which are signs of serious multicollinearity, require correction.

We conducted statistical analyses using package R software.[17] VIFs were calculated using the R package "jtools".[18]

RESULTS

Study population

A total of 24,974 singleton pregnancies had cfDNA testing, but 1,479 (5.9%) of these were excluded from further analysis either because the pregnancies ended in termination, miscarriage or stillbirth with no known karyotype (n=356) or they were lost to follow up (n=1,123). In the 23,495 cases included in the study, there were 324 with trisomy 21, 104 with trisomy 18, 28 of 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 pregnancies ended in termination, miscarriage or stillbirth with no known karyotype (n=45) or they were lost to follow up (n=18). In 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, 7 with trisomy 18, 1 of trisomy 13 and 906 without trisomy 21, 18 or 13; all trisomic cases were in the DC pregnancies 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 9,440 of 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% (91/806) of DC 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 MC twins (5 because of insufficient fetal cfDNA and 1 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. In 82 of the 91 dichorionic twin pregnancies with no result after first sampling repeat testing provided a result in 45 (54.9%) cases. In 5 of the 6 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, decreasing gestational age and serum PAPP-A and free ß-hCG, second, was higher in women of Black and South Asian than White racial origin, in dichorionic twin than singleton pregnancy and in *in vitro* fertilization than natural conception and third, was lower in parous than nulliparous women; there was no significant contribution from fetal karyotype or method of cfDNA testing (Table 2). VIFs for all variables included in the regression analysis were <1.5, excluding any concerns regarding multicollinearity.

DISCUSSION

Principal findings of the study

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 to natural conception, increases the risk by 3.8 times. Other contributors to test failure are Black or South Asian racial origin, which by comparison to White origin, increase the risk by 2.0 and 1.7 times, respectively, and dichorionicity, which by comparison to singleton pregnancies, increases the risk by 1.7 times. The risk of test failure increases by about 5% with each additional kilogram in maternal weight and by about 2% with each additional year in 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 ß-hCG. Test failure is unrelated to method used for cfDNA analysis and fetal karyotype, once serum PAPP-A and free ß-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 to findings in 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 aneuploidies by cfDNA testing we noted a wide range in 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 dilutional effect and / or enhanced turnover of adipocytes and a secondary increase in maternal cfDNA in maternal plasma.[3-6,19-22] Similarly, our finding of an increased cfDNA test failure with decreasing gestational age, PAPP-A and free β-hCG MoMs is consistent with the findings from previous studies and it is probably related to smaller placental mass and therefore decreased placental cfDNA in maternal blood.[4,13,19,23,24]

In our previous studies we also found that in pregnancies conceived by *in vitro* fertilization cfDNA test failure is increased,[6,25] but another study on twin pregnancies analysed by a different cfDNA technique could not demonstrate such an association.[26] Low fetal fraction in conceptions 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%[7-9] and the incidence of preeclampsia is increased.[27,28] Two likely explanations for these different results are first, the significantly higher gestational age at sampling (median 16 weeks, range 10-35) in the study of Le Conte et al.,[26] by comparison to our study (median 11 weeks, range 10-14) and second, the different method used for cfDNA analysis (massively parallel sequencing[26] vs. targeted approach). It has been reported that the failure rate is lowest in methods based on massive parallel sequencing (1.58%), followed by chromosome selective sequencing (3.56%) and single-nucleotide polymorphism based analysis (6.39%).[29] 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, 13 and triploidy, but not trisomy 21.[6,29,30] 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 counselling 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, 13 and triploidy.

Conclusions

Maternal age, weight, racial origin and parity, gestational age, dichorionicity, method of conception and serum levels of free ß-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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Table 1. Maternal and pregnancy characteristics of the study population.

Maternal and pregnancy characteristic	**Singleton (n=23,495)**	**DC twins (n=806)**	**MC twins (n=122)**
Maternal age in years, median (IQR)	36.1 (32.9, 39.1)	37.2 (34.2, 9.6)*	36.9 (34.0, 39.2)
Maternal weight in kg, median (IQR)	64.0 (58.0, 73.0)	65.0 (58.6, 75.0)*	63.0 (56.1, 71.7)
Maternal height in cm, median (IQR)	165 (161, 170)	167 (162, 170)*	165 (160, 170)
Racial origin			
White, n (%)	19,226 (81.8)	669 (83.0)*	100 (82.0)
Black, n (%)	1,412 (6.0)	51 (6.3)	3 (2.5)
South Asian, n (%)	1,525 (6.5)	52 (6.4)	8 (6.6)
East Asian, n (%)	895 (3.8)	24 (3.0)	7 (5.7)
Mixed, n (%)	437 (1.9)	10 (1.2)	4 (3.3)
Parity			
Nulliparous, n (%)	10,402 (44.3)	494 (61.3)*	59 (48.4)*
Parous, n (%)	13,093 (55.7)	312 (38.7)*	63 (51.6)*
Cigarette smoker, n (%)	640 (2.7)	10 (1.2)*	1 (0.08)
Conception			
Natural, n (%)	20,978 (89.3)	310 (38.5)*	85 (69.7)*
Ovulation induction, n (%)	79 (0.3)	14 (1.7)*	2 (1.6)
In vitro fertilization, n (%)	2,438 (10.4)	482 (59.8)*	35 (28.7)
Gestation at sampling in weeks, median (IQR)	11.9 (10.7, 12.9)	11.9 (10.6, 12.7)	12.0 (10.7, 13.0)
Serum PAPP-A MoM, median (IQR)	0.89 (0.58, 1.32)	1.04 (0.75, 1.46)*	0.99 (0.72, 1.48)*
Serum ß-hCG MoM, median (IQR)	1.11 (0.72, 1.73)	1.01 (0.72, 1.52)*	1.02 (0.65, 1.56)*
Method of cfDNA test			
Sequencing	9,440 (40.2)	269 (33.4)*	44 (36.1)
Microarray	14,055 (59.8)	537 (66.6)*	78 (63.9)
No result after first cfDNA testing	798 (3.4)	91 (11.3)	6 (4.9)

DC = dichorionic; MC = monochorionic; IQR = interquartile range; MoM = multiple of the median

Comparisons between groups were performed using Mann–Whitney U-test for continuous variables and the $\chi 2$ or Fisher's exact test for categorical variables, with post-hoc Bonferroni corrections. *P< 0.025.

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
Intercept			0.008 (0.003, 0.022)	<0.0001
Age in years	1.048 (1.033, 1.064)	<0.0001	1.024 (1.009, 1.041)	0.002
Weight in kg	1.041 (1.037, 1.044)	<0.0001	1.049 (1.045, 1.053)	<0.0001
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	-	
Smoking	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				
Nulliparity	(Reference)			
Multiparity	0.617 (0.539, 0.706)	<0.0001	0.635 (0.546, 0.737)	<0.0001
Gestational age	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 ß-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 twins	3.620 (2.862, 4.529)	<0.0001	1.748 (1.343 (2.255)	<0.0001
Monochorionic twins	1.471 (0.574, 3.072)	0.358	-	
Method of cfDNA test				
Sequencing	(Reference)			
Microarray	1.083 (0.945, 1.244)	0.254	-	

CI = confidence interval; MoM = multiple of the median