Received: 24 May 2017 | Revised: 8 August 2017 | Accepted: 2 September 2017

DOI: 10.1002/pd.5152

ORIGINAL ARTICLE

The association between anticoagulation therapy, maternal characteristics, and a failed cfDNA test due to a low fetal fraction

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Abstract

Objectives: The objective of this study was to identify maternal characteristics associated with a failed cell-free DNA (cfDNA) test due to a low fetal fraction (FF).

Method: Retrospective cohort study of women with singleton pregnancies who had cfDNA screening at 10–25 weeks gestation between October 2011 and January 2016. cfDNA screening was performed using methylation techniques until October 2013; thereafter, samples were run with massively parallel sequencing. Multivariable logistic regression was performed to identify maternal characteristics associated with no cell free DNA result secondary to low FF.

Results: Thirty-three (1.2%) of 2890 eligible women had a failed cfDNA test, including 18 (0.6%) cases with a low FF. A failed cfDNA test due to a low FF was associated with obesity (aOR 1.11, CI 1.05–1.18, $p = 0.0003$) and treatment with enoxaparin (aOR 37.5, 11.19–125.87, $p < 0.0001$). 5 of 28 (18%, 95% CI: 6.1%–36.9%) women on enoxaparin had a failed cfDNA test secondary to a low FFx.

Conclusion: Enoxaparin therapy and obesity were associated with an increased incidence of a failed cfDNA test due to low FF. Further research is needed to determine the mechanism by which anticoagulation therapy alters cfDNA test functionality and identify approaches to improve test performance in these women.

1 | INTRODUCTION

The existence of fetal cell-free DNA (cfDNA) in maternal plasma was first reported in 1997.[1] Cell-free DNA testing is increasingly being used to screen pregnant women for fetal aneuploidy since becoming clinically available in 2011. Cell-free DNA screening can detect over 99% of cases of trisomy 21 and has high sensitivity and specificity for the detection of trisomy 13 and 18 as well as sex chromosome aneuploidies.[2]

Despite numerous technological advances, there continue to be a small proportion of women who do not receive a result after undergoing cfDNA screening for fetal aneuploidy. This is often secondary to a low fraction of fetal DNA in the maternal circulation. Previous reports have suggested that a fetal fraction (FF) of approximately 2–4% is required to receive an accurate result.[3,4] Identification of maternal and fetal factors associated with reduced fetal fraction has been the subject of ongoing investigation over the past several years.[5] Obesity, maternal hypertension, early gestational age and fetal aneuploidy have been associated with a low fetal fraction.[6-9] There is scant literature reporting an association between anticoagulation with low molecular weight heparin (LMWH) and failed cfDNA results.[7,10,11]

The goal of this study was to identify maternal characteristics and comorbidities associated with a failed cfDNA test result due to low fetal fraction.

2 | METHODS

This is a retrospective cohort study of women with singleton pregnancies who underwent cfDNA screening at 10–25 weeks gestation between October 2011 and January 2016 at the University of Pennsylvania. The University of Pennsylvania Institutional Review

The University of Pennsylvania has sponsored research by Progenity. Dr. Dugoff serves as the primary investigator for that project. The remaining authors do not have funding sources to disclose.

Board (IRB) approved the study. Women who had a spontaneous loss at <20 weeks or a fetal demise at ≥20 weeks, elective termination of pregnancy, or unavailable pregnancy outcome data were excluded. If a woman had more than one cfDNA test in a pregnancy, only the first test result was included in the analysis. Results of subsequent testing, if performed, are included in Figure 1 for those women whose initial test did not yield a result. If a woman had more than one pregnancy during the study period, only the first pregnancy was included.

All maternal blood samples were analyzed by the same laboratory; fetal fraction data were provided by the laboratory for the purpose of this study. Fetal fraction was determined using methylation techniques as previously described for samples drawn prior to 10/24/2013 (n = 1196).[12] Fetal fraction estimation was determined using a multivariable model derived by machine learning approaches using regional read depth counts from autosomes generated by whole genome low coverage massively parallel single-end sequencing for samples drawn beginning on 10/24/2013 (n = 1741).[13] Both techniques were verified on an independent cohort prior to application to this cohort. Despite the change in laboratory technique, the fetal fraction threshold of 4% remained constant.

Maternal age, gestational age, and BMI were recorded at the time of cfDNA blood draw. Demographic, prenatal, obstetric and neonatal information was abstracted from the electronic medical record.

The sample size was fixed based on the number of women presenting for cfDNA screening within the study period. Mean values and standard deviations were calculated for continuous variables. These variables were compared across cfDNA groups by the Student t test. Pearson chi-square test was utilized for associations between tests results and categorical variables. Multivariate logistic regression was performed to assess the relationship between maternal characteristics and comorbidities and a failed cell free DNA test due to a low fetal fraction. Confounders were initially identified from unadjusted associations (p < 0.2) with final confounders selected using a backward selection strategy.

3 | RESULTS

A total of 3602 women underwent cfDNA screening during the study period (Figure 1). Ultimately, 2890 women met inclusion criteria. The vast majority of women (98.8%) received a cfDNA test result. Thirty-three women had a failed test on initial screening. In eighteen cases the test failure was due to a low fetal fraction, and the remaining 15 cases did not have a result secondary to technical error. Characteristics of repeat testing, if performed, are presented in Figure 1.

Characteristics of the entire study cohort and the characteristics of the women who received and did not receive a cfDNA test result are presented in Table 1. There were no significant differences in the age, race, or parity of the patients between the groups. There was no difference in the gestational age at time of test. Women who underwent testing with the initial method using methylation technique[10] were more likely to not receive a result (21 of1196, 1.76% v. 12 of 1694, 0.71%, p = 0.009). However, the number of cases with no result due a low fetal fraction was similar using both techniques (10 of 1196, 0.84% v. 8 of 1694, 0.47%, p = 0.215). The mean BMI for women with a failed test due to a low fetal fraction was 31.1 kg/m^2 in contrast to 26.5 kg/m^2 in those women who received a test result (p < 0.0001). A history of hypertension was more prevalent in women who received a failed test result secondary to a low fetal fraction (4 of 18, 22% v.190 of 2857, 7%, p = 0.008). Twenty-eight percent (5 of 18) of women with a failed test secondary to a low fetal fraction were being treated with anticoagulation at the time of their cfDNA blood draw. In contrast, only one percent (23 of 2857) of women who received a test result were on anti-coagulation (p < 0.0001).

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- A low fetal fraction may result in a failed cell free DNA test; obesity, early gestational age, maternal hypertension, and fetal aneuploidy are associated with a low fetal fraction

WHAT DOES THIS STUDY ADD?

- This is a large retrospective study confirming the effect of obesity on fetal fraction and identifying a significant effect of enoxaparin therapy on fetal fraction.

Table 2 shows the characteristics of all patients (n = 28) receiving anticoagulation. Twenty-seven of the women were treated with enoxaparin, and one woman with a mechanical heart valve was treated with therapeutic dalteparin. In this subgroup, there was no significant difference between the women who received a result on cfDNA testing and those who had a failed test with regard to age or BMI. Women with a failed test were on average of later gestational age at the time of their blood draw (12.5 (2.5) weeks v. 15.9 (5.5) weeks, p = 0.036). There was no difference with regard to indication for anticoagulation. Four of the five women were being treated for a current or past venous thromboembolism while one was receiving enoxaparin for anti-phospholipid syndrome. The majority of women who received a cfDNA test result were receiving prophylactic enoxaparin. In contrast, women with a failed cfDNA test were more likely to be prescribed therapeutic enoxaparin (p = 0.024). The one woman on dalteparin received a negative cfDNA result.

Of the five women on enoxaparin who did not receive a result, four of them had a repeat test. Three did not receive a result on their second test secondary to a low fetal fraction. The repeat tests were performed four to five weeks after the initial test. The fourth patient had a negative test on repeat three weeks after her initial test.

Recognizing that obesity, hypertension, and thromboembolic disorders may occur more frequently in the same women, a multivariable logistic regression was performed to assess the relationship of each of these disorders to the primary outcome. Because fetal fraction increases as pregnancy progresses,[12] gestational age at time of cfDNA test was also included in this analysis (Table 3). The adjusted odds ratio (aOR) for a failed cfDNA test while on anticoagulation was 37.53 (CI 11.19–125.87; p < 0.0001). Increasing BMI also remained a

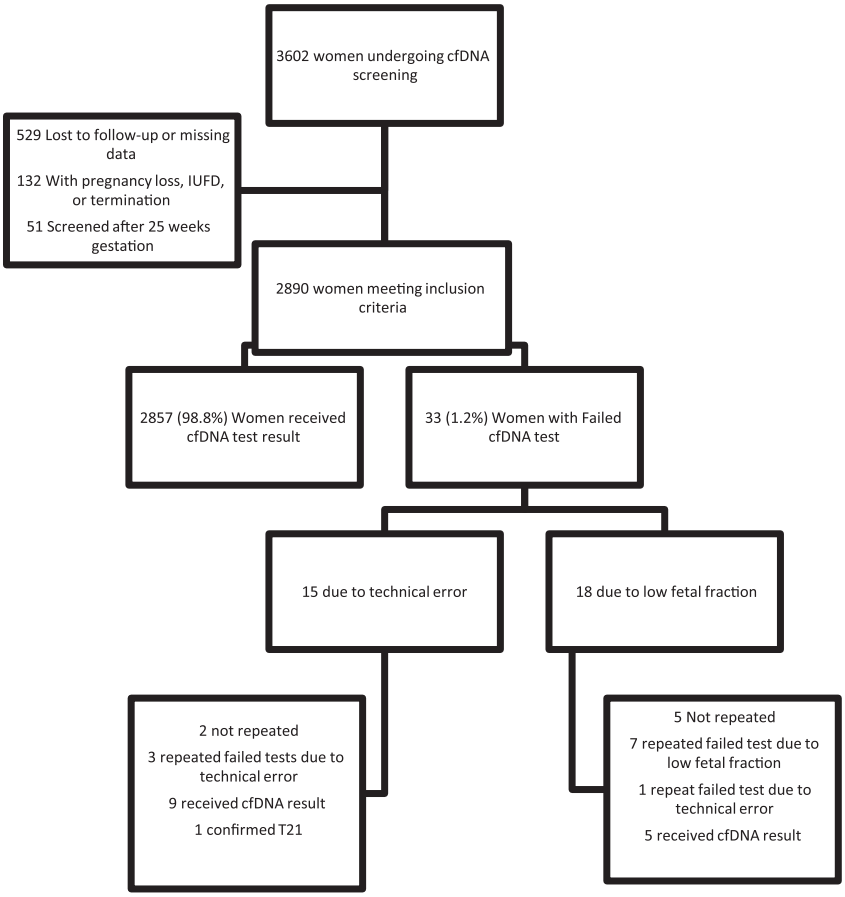

FIGURE 1 Study Population

significant risk factor for a failed cfDNA test (aOR 1.11, CI 1.05–1.18, $p = 0.0003$). Hypertension was no longer significantly associated after adjusting for BMI, anticoagulation therapy, and gestational age at screening (aOR 1.34, CI 0.33–5.41, $p = 0.681$). This effect was largely driven by BMI alone. The adjusted odds ratio for hypertension and BMI together was 1.52, which closely approximates the aOR for hypertension when all covariates are considered.

4 | DISCUSSION

In this retrospective cohort study, maternal obesity and anticoagulation therapy with enoxaparin were associated with an increased incidence of a failed cfDNA test due to a low fetal fraction. Prior research has demonstrated that obese patients have increased apoptosis and necrosis in their adipose cells, resulting in elevated plasma levels of maternal cfDNA in comparison to fetal cfDNA and resulting in a decreased fetal fraction.[14] There is specific information available to counsel women regarding the chance of obtaining a cfDNA result based on BMI values and gestational age at the time of the cfDNA test.[9,15,16] While the association between obesity and a low fetal fraction is well-established, there is scant literature on the association between anticoagulant use and cfDNA test results.

Gromminger et al. published the first report on the association between low molecular weight heparin (LMWH) use and failed cfDNA tests.[5] In a cohort of 1614 women who had cfDNA screening, 12 cases had no reportable result due to elevated guanosine-cytosine (GC) content. Nine of these twelve women were receiving LMWH prophylaxis. Of note, the specific type of LMWH received was not provided in the study. Five of the women receiving LMWH prophylaxis had an additional blood sample just prior to the next pending dose of LMWH when the plasma level of the drug was the lowest. The increased incidence of small cfDNA fragments with increased GC content was not observed in the repeat samples, and results were reported in all five cases. Unlike our study, Gromminger et al. did not observe a decreased fetal fraction in the women treated with LMWH who did not receive a result. Although the fetal fractions were not consistently lower in the initial samples with no cfDNA result, the total amount of cfDNA (maternal and fetal) and the fetal DNA alone were higher in the initial samples with no result than in the pre-LMWH dose samples. Gromminger et al. concluded that the higher proportion of smaller GC-rich DNA fragments supports the hypothesis that LMWH might induce apoptosis.

There are isolated case reports in the literature involving failed cfDNA screening due to a low fetal fraction in women treated with LMWH. A fetal fraction of 1.60% at 19 weeks gestation was reported in a woman receiving therapeutic anticoagulation with enoxaparin due to a history of Protein S deficiency.[10] She had a normal BMI of 25.9. At 21 weeks gestation a second maternal blood sample drawn 24 hours after the last enoxaparin dose reported a true positive result for trisomy 21. The fetal fraction from the second sample was 5.76%. A second case report described a woman receiving daily treatment with

TABLE 1 Patient demographics

Characteristic	Entire cohort mean (±SD) or N (%) N = 2890	cfDNA result* mean (±SD) or N (%) N = 2857	No cfDNA result due to low fetal fraction mean (±SD) or N (%) N = 18	No cfDNA result due to technical error mean (±SD) or N (%) N = 15	p-value°
Age (y)	39.1 (11.6)	39.2 (11.6)	37.3 (5.90)	35.4 (5.02)	0.359
Race					0.679
Black	666 (23)	661 (23)	5 (28)	2 (13)	
White	1726 (60)	1714 (60)	12 (67)	10 (67)	
Other	483 (17)	482 (17)	1 (6)	3 (20)	
BMI (kg/m^2)	26.4 (6.30)	26.4 (6.25)	34.6 (8.90)	27.2 (7.99)	<0.0001
Gestational age	13.4 (3.33)	13.4 (3.33)	14.3 (3.78)	13.5 (2.91)	0.548
Indication					0.338
AMA	2265 (79)	2251 (79)	14 (78)	11 (73)	
Abnormal U/S	111 (4)	110 (4)	1 (6)	0 (0)	
Pos sequential screen	286 (10)	285 (10)	1 (6)	1 (7)	
Other	91 (3)	91 (3)	0 (0)	1 (7)	
Missing	122 (4)	120 (4)	2 (11)	2 (13)	
Fetal fraction	12.2 (5.81)	12.2 (5.78)	2.96 (1.88)	9.84 (6.69)	<0.0001
Type of test					
Methylation	1196 (41)	1175 (41)	10 (56)	11 (73)	0.019
Single end sequencing	1694 (59)	1682 (59)	8 (44)	4 (27)	
Gravidity	2.87 (1.91)	2.86 (1.91)	3.33 (2.11)	3.33 (2.11)	0.42
Parity	0.99 (1.20)	0.99 (1.20)	1.17 (1.20)	0.93 (1.16)	0.815
HTN	194 (7)	190 (7)	4 (22)	3 (20)	0.008
Diabetes	78 (3)	77 (3)	1 (6)	0 (0)	0.597
Anticoagulation therapy	28 (1)	23 (1)	5 (28)	0 (0)	<0.0001

*Category includes patients with a negative or positive (Trisomy 21, 13, or 18) result.

°*p* values calculated from Fischer's exact test for categorical variables and type III ANOVA for continuous variables and are reported for comparison of cfDNA result v. No cfDNA result secondary to Low Fetal Fraction.

TABLE 2 Descriptive statistics of patients on anticoagulation (N = 28)

Characteristic	Patients on anticoagulation mean (±SD) or N (%) N = 28	cfDNA result* mean (±SD) or N (%) N = 23	No cfDNA result† mean (±SD) or N (%) N = 5	p-value°
Age (y)	39.6 (4.34)	39.7 (4.53)	38.8 (3.58)	0.6691
BMI (kg/m^2)	29.5 (8.47)	28.7 (8.83)	33.2 (5.96)	0.2979
Gestational age at time of cfDNA screening	13.1 (3.34)	12.5 (2.48)	15.9 (5.48)	0.0358
Type				0.0244
Prophylactic enoxaparin	22 (79)	20 (87)	2 (40)	
Therapeutic enoxaparin	5 (18)	2 (9)	3 (60)	
Therapeutic Dalteparin	1 (4)	1 (4)	0 (0)	
Indication				0.1082
H/o PE, DVT	20 (71)	18 (78)	2 (40)	
Current PE, DVT	3 (11)	1 (4)	2 (40)	
APLS	4 (14)	3 (13)	1 (20)	
Mechanical valve	1 (4)	1 (4)	0 (0)	
Fetal fraction	7.74 (3.54)	8.73 (2.88)	3.19 (2.67)	0.0005

*Category includes patients with a negative or positive (Trisomy 21, 13, or 18) result. (1 patient with true positive for Trisomy 21).

†Category includes only patients with a failed test secondary to low fetal fraction.

°*p* values calculated from chi-square analysis for categorical variables and t-test for continuous variables.

aspirin 100 mg, prednisone 20 mg, and LMWH secondary to immune thrombocytopenia and antiphospholipid syndrome.[11] She had failed SNP-based cfDNA tests at 11w5d, 13w6d, and 16w1d gestation due to an insufficient fetal fraction. The authors hypothesized that the multiple failed attempts at obtaining a sufficient fetal fraction may have been secondary to increased maternal cell destruction in women with autoimmune disorders diluting the cfDNA in the maternal sample or the use of LMWH.

TABLE 3 Bivariable and multivariable analysis of effect of patient characteristics on failed cfDNA result

	cfDNA result vs no cfDNA result					
	Unadjusted OR	95% CI	*p*-value	aOR*	95% CI	*p*-value
BMI (kg/m^2)	1.13	1.08–1.18	<0.0001	1.11	1.05–1.18	0.0003
HTN	4.01	1.31–12.31	0.015	1.34	0.33–5.41	0.681
Anticoagulation use	47.39	15.62–143.81	<0.0001	37.53	11.19–125.87	<0.0001

*aOR adjusting for BMI, HTN, anticoagulation use, and gestational age at cfDNA blood draw.

Although we did not investigate the mechanism by which enoxaparin lowered the fetal fraction, we speculate that low molecular weight heparin resulted in low fetal fractions due to reduction of placental apoptosis. Heparin has been demonstrated to reduce trophoblast apoptosis and enhance trophoblast survival through complex mechanisms including reduction of cytokeratin neoepitope and nucleosomal DNA formation[17] and E-cadherin expression.[18] Our study has several limitations. We were unable to confirm compliance with anticoagulation dosing. Additionally, we did not have data regarding the timing of LMWH administration with respect to the cfDNA blood draw. The study was also limited by the small number of no result cases secondary to a low fetal fraction.

5 | CONCLUSION

The use of cfDNA as a modality for aneuploidy screening has increased in popularity since its commercial release in 2011. The incidence of a failed cfDNA test varies from 1 to 12% depending upon the approach used.[5] Understanding the risk factors associated with a failed cfDNA test is, therefore, an important element for patient counseling, and optimizing the likelihood of successfully obtaining a cfDNA test result is a patient-care goal. This study identifies obesity and treatment with enoxaparin as risk factors for a failed test secondary to low fetal fraction. The effect of enoxaparin appears to be more clinically relevant. Further research is necessary to elucidate the mechanisms by which these conditions affect the fetal fraction in the maternal plasma, confirm if this finding varies across different labs, and determine if there is a means to enhance test performance in women who are obese or on anticoagulation. Prospective studies of women treated with LMWH are warranted to study the impact of gestational age and dosage timing with respect to the maternal blood collection on fetal fraction and the likelihood of obtaining a cfDNA result.

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How to cite this article: Burns W, Koelper N, Barberio A, et al. The association between anticoagulation therapy, maternal characteristics, and a failed cfDNA test due to a low fetal fraction. *Prenatal Diagnosis*. 2017;37:1125–1129. https://doi.org/10.1002/pd.5152