Received: 22 January 2020 | Revised: 11 July 2020 | Accepted: 13 July 2020

DOI: 10.1002/pd.5797

ORIGINAL ARTICLE

PRENATAL DIAGNOSIS WILEY

Cell-free DNA screening in twin pregnancies: A more accurate and reliable screening tool

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Abstract

Objective: Outcome data from cell-free DNA (cfDNA) screening in twin gestations are limited. This study adds an appreciable number of confirmed outcomes to the literature, and assesses performance of cfDNA screening in twins over a 4.5-year period at one large clinical laboratory.

Method: Prenatal cytogenetic and SNP microarray results were cross-referenced with cfDNA results for twin pregnancies, yielding 422 matched cases. Using diagnostic results as truth, performance of cfDNA screening in this population was assessed.

Results: Of the 422 twin pregnancies with both cfDNA and diagnostic results, 3 specimens failed amniocyte analysis, and 48 samples (11.5%) were nonreportable from the initial cfDNA draw. Analysis of the 371 reportable samples demonstrated a collective sensitivity of 98.7% and specificity of 93.2% for trisomies 21/18/13. Positive predictive values (PPVs) in this study population, which is enriched for aneuploidy, were 78.7%, 84.6%, and 66.7% for trisomy 21, 18, and 13, respectively.

Conclusion: CfDNA screening in a cohort of twin pregnancies with matched diagnostic results showed superior performance compared to traditional serum biochemical screening in twins. This study adds to a growing body of evidence suggesting that cfDNA is an accurate and reliable screening tool for the major trisomies in twin pregnancies.

1 | INTRODUCTION

Historically, twin gestations have posed unique challenges to aneuploidy detection during pregnancy. Traditional serum screening is complicated by the presence of two fetuses contributing varying levels of analytes in maternal blood, resulting in a less sensitive screening test in twins compared to singleton gestations.[1] Additionally, traditional screening methods are often focused solely on the detection of Down syndrome, at the exclusion of other chromosome abnormalities. Cell-free DNA (cfDNA) screening for aneuploidy in multifetal gestations has been validated and clinically available since 2012.[2] This screening approach allows for the evaluation of multiple aneuploidies, and validation studies suggest that this testing may offer increased sensitivity and specificity compared to traditional serum screening.

Despite the growing use of this test by clinicians, professional societies have not yet broadly supported cfDNA screening for multifetal pregnancies. In a recent Practice Bulletin, the American College of Obstetricians and Gynecologists (ACOG) stated: "Noninvasive prenatal testing that uses cell free fetal DNA from the plasma of pregnant

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women offers potential as a screening tool for fetal aneuploidy. However, more information is needed before use of this test can be recommended in women with multifetal gestations."[1] The American College of Medical Genetics (ACMG) does not overtly endorse cfDNA screening for multiples, rather, ACMG recommends "In pregnancies with multiple gestations and/or donor oocytes, testing laboratories should be contacted regarding the validity of NIPS [noninvasive prenatal screening] before it is offered to the patient as a screen option."[3] In light of these society recommendations, it is important for laboratories to continue to report information about test performance and monitor outcome data to assist in determining the efficacy of this screening tool in multifetal gestations.

Since 2012, over 30 000 cfDNA samples from twin and higher-order multifetal pregnancies have been analyzed at Sequenom Laboratories.[4] Data from these samples suggest that test performance over time has matched or exceeded test performance from original validation studies. Furthermore, these data suggest that cfDNA screening performs similarly in singleton and multifetal gestations. However, outcome data to confirm the cfDNA findings have typically relied upon voluntary feedback from providers, and therefore, have been limited.

As a large clinical laboratory offering both cfDNA screening as well as diagnostic testing, there exists a unique opportunity to compare the results obtained from patients who have submitted samples for both cfDNA screening and diagnostic testing in the same pregnancy. The goal of this study is to analyze the performance of a genome-wide cfDNA screening test for common aneuploidies in twin gestations in a cohort of patients with matched karyotype and/or microarray results from diagnostic specimens.

What's already known about this topic?

- Serum biochemical screening for fetal aneuploidy is a less sensitive screening tool in twin gestations compared to singleton pregnancies.
- Cell-free DNA offers an alternative to traditional serum aneuploidy screening in multifetal gestations, and has been validated and clinically available since 2012.
- Despite its growing use by clinicians, professional societies have not broadly supported cfDNA screening for multifetal pregnancies, citing the need for more data.

What does this study add?

- This study analyzes outcome data of cfDNA screening in twin gestations by matching cfDNA results with cytogenetic and/or microarray outcomes from patients pursing both cfDNA screening and diagnostic testing during the same pregnancy at a large clinical laboratory.
- These data suggest that cfDNA screening performs similarly in twin and singleton gestations, and offers higher sensitivities and positive predictive values for aneuploidy screening compared to traditional serum biochemical screening in twins.

2 | METHODS

Cytogenetic and SNP microarray diagnostic results from chorionic villus samples (CVS), amniocentesis samples, and products of conception (POC) specimens submitted to LabCorp and Integrated Genetics Laboratories from September 2013 through June 2018 were included for analysis. Diagnostic results were cross-referenced with cfDNA results for twin gestation pregnancies during the same time period. The process of consolidation and comparison of data across the three datasets (cfDNA results, cytogenetic results and microarray results) was approved by AspireIRB under clinical protocol SCMM-RND-402.

Extracted cfDNA fragments from maternal plasma were subjected to genome-wide sequencing and algorithmic analysis for chromosomal aneuploidies. Both fetal (placental) and maternal fragments were sequenced and mapped to unique regions of the genome. The unique reads were assigned to 50 kb bins, normalized across the genome, and counted. An under- or over-representation of fragments in a 50 kb bin were indicative of a loss or gain in the genome profile, respectively. For autosomal trisomy analysis, this technique analyzed for over-representation of DNA along the entire chromosome as previously described.[5]

For a pregnancy to be considered a twin gestation, the cfDNA test requisition had to be accessioned with a fetal number of "2" indicated. For a cfDNA sample to be "reportable" for a multifetal gestation, fetal fraction requirements were adjusted in proportion to fetal number (ie, the fetal fraction threshold for twins was twice the minimum fetal fraction requirement for a singleton gestation). While the methodology used to estimate fetal contribution and minimum thresholds has evolved over time, the proportional stringency increase for amplified fetal fraction to fetal signal has remained constant.

Data from all samples were reviewed by a laboratory director prior to the final reporting of results to the ordering provider. Samples with insufficient fetal DNA were classified as quantity not sufficient (QNS) using a previously described method.[6] Samples failing other laboratory quality metrics including library and sequencing passing criteria were classified as technical nonreportable.

Amniotic fluid and chorionic villus specimens were cultured, harvested, and G-banded according to standard methods. For in situ amniocyte analysis, at least 15 primary colonies from two or more independent cultures were examined. If fewer than 15 primary colonies were available, a total of 20 cells from both primary and trypsinized cultures were examined. For chorionic villus specimens, at least 20 metaphase cells from two or more independent cultures were

examined. In cases of mosaicism, abnormal cell lines were present in at least two independent cultures. For products of conception, at least 20 metaphase cells were examined.

All chromosomal microarray studies were performed utilizing the ThermoFisher Cytoscan HD array [ThermoFisher and CytoScan are Registered Trademarks of ThermoFisher, Inc.]. This array contains approximately 2 695 000 markers across the entire human genome. There are approximately 743 000 single nucleotide polymorphic probes (SNPs) and 1 953 000 structural non-polymorphic probes. On the average there is approximately 0.88 kb between each marker. DNA was extracted utilizing standard methods and 250 ng of total genomic DNA extracted was digested with Nsp1, ligated to Nsp1 adaptors, and then amplified using Titanium Taq with a GeneAmp PCR System 9700. PCR products were purified using AMPure beads and quantified using NanoDrop 8000. Purified DNA was fragmented, biotin labeled, and hybridized to the ThermoFisher Cytoscan HD GeneChip. Data were analyzed using Chromosome Analysis Suite. The analysis was based on the GRCh37/hg19 assembly.

For a cfDNA sample to be considered a match to a cytogenetic and/or microarray specimen, the diagnostic and screening results were required to have identical patient identifiers (name and date of birth), and the collection date for the diagnostic test had to be within 90 days of the patient's cfDNA screening date. Samples outside of the 90-day window (n = 13) underwent additional scrutiny to ensure that both samples (cfDNA and diagnostic specimens) were collected during the same pregnancy. Using a pregnancy dating wheel, the patient's gestational age at the time of cfDNA sample collection was compared to the patient's gestational age at time of diagnostic testing. Only samples for which these two dates aligned were included in data analysis (n = 12/13). When multiple diagnostic results (eg, two diagnostic samples for twins, or a cytogenetic result with a microarray result) were available for the same patient, results were combined under one final characterization.

Analysis was focused exclusively on trisomies 21, 18, and 13. Data consolidation resulted in 422 correlated cases, but there were three cases in which the amniocentesis analysis failed to yield results, so these cases were excluded from analysis of test performance since no diagnostic information was available to confirm the cfDNA finding. Using the diagnostic results as truth, reportable cfDNA results were then classified into one of four categories (true positive, true negative, false positive, and false negative) for each aneuploidy.

Test performance metrics were calculated for individual conditions and the overall cohort using the following formulas:

Sensitivity = true positives/(true positives + false negatives).

Specificity = true negatives/(true negatives + false positives).

Positive predictive value (PPV) = true positives/(true positives + false positives).

Negative predictive value (NPV) = true negatives/(true negatives + false negatives).

Patient demographics and laboratory metrics of this cohort were compared with a larger, previously-published cohort of twin cfDNA samples analyzed during a partially-overlapping time period in the same laboratory. Mean maternal age, gestational age, and maternal BMI were compared using a two-sample, two-sided *t* test. Non-reportable rates of the two cohorts were compared using the Fisher Exact test.

There were 23 samples submitted for cfDNA screening with no indication for referral provided on the test requisition. For the purpose of data analysis in this study, if the patient's date of birth qualified her as "advanced maternal age" at the time of delivery, the reason for referral was classified as such (n = 15/23).

3 | RESULTS

3.1 | Laboratory experience

From September 2013 through June 2018, 422 twin gestations were identified as having both cfDNA screening and diagnostic testing at the laboratory. In the majority of cases (407/422), only a karyotype was ordered for diagnostic testing. There were 7 cases in which only a SNP microarray was ordered, and 8 cases where both karyotype and SNP microarray results were identified for the same patient. The majority of samples (74%, n = 313) were from amniotic fluid specimens, 25% (n = 105) from chorionic villus specimens, and 1% (n = 4) from products of conception.

The most common indication for cfDNA screening in this cohort was advanced maternal age (73%), followed by ultrasound findings (14%), multiple indications (8%), personal or family history (3%), indication not provided (2%), and abnormal serum biochemical screening (<1%). (Table 1).

The patient demographic and laboratory metrics for the 422 matched cases in this study were analyzed and compared to a larger published cohort of over 23 000 twin cfDNA specimens analyzed during a partially-overlapping time frame in the same laboratory.[4] The average maternal age in the matched cohort was 36.4 years, which is older than the average maternal age seen in the larger screening group (35.0 years) ($P < .001$). On average, samples were submitted earlier in pregnancy for the matched cases than the broader screening group (12.1 vs 13.7 weeks; $P < .001$), and the

TABLE 1 Testing indication for cfDNA screening in the 422 matched twin cases

Reason for referral	n	Percentage of total
Maternal age	307[a]	73%
Ultrasound findings	59	14%
Multiple indications	33	8%
Personal/Family history	11	3%
Not provided	8	2%
Abnormal serum biochemical screening	4	<1%

[a]Includes 15 cases in which no reason for testing was indicated on the test requisition, but the patient's date of birth qualified her as advanced maternal age at the time of delivery.

TABLE 2 Sample characteristics and demographic breakdown of the 422 matched twin cases compared to broader twin population screened at the same laboratory

	Current data set (n = 422)	Dyr et al, 2019 (n = 23 986)	*P*-value
Mean maternal age	36.4 years (range: 16.8–49.8 years)	35.0 years (range: 14.5–61.5 years)	$P < .001$
Mean gestational age	12w1d (range: 9–34 weeks)	13w5d (range: 9–38 weeks)	$P < .001$
Mean maternal body mass index (BMI)	26.3 kg/m^2	27.6 kg/m^2	$P < .001$
Average fetal fraction	11.6%	12.3%	$P < .001$

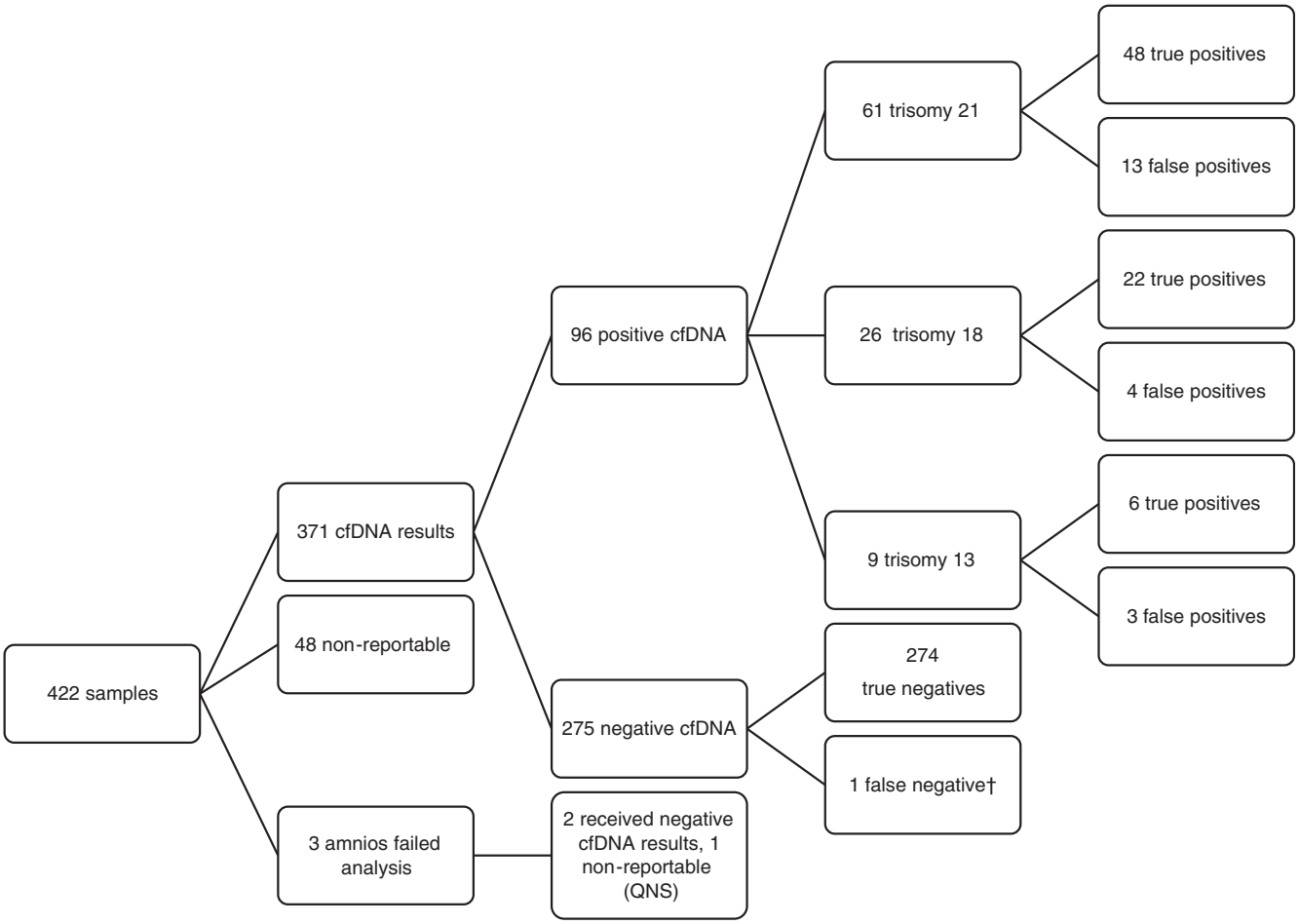

FIGURE 1 Results of cfDNA screening after initial blood draw in 422 matched twin cases

average maternal body mass index (BMI) was lower in the matched cohort than the larger screening group (26.3 vs 27.6 kg/m^2; $P < .001$). (Table 2) The average fetal fraction (FF) in the matched cohort was 11.6%, which is lower than the 12.3% average FF witnessed in the broader screening group ($P < .001$).

3.2 | Test performance from matched outcomes

Three hundred seventy-one of the 422 matched cases yielded a reportable result from cfDNA screening on the first draw. Forty-eight cases were non-reportable on the first draw. Three matched cases failed analysis of amniocytes and were excluded from analysis of test performance, as no diagnostic outcome information was available to confirm the cfDNA results. Of the 371 reportable cases, there were 96 positive cfDNA results, and 275 negative cfDNA results. (Figure 1).

A review of the 96 positive cfDNA results yielded 76 concordant (true positive, "TP") results and 20 discordant (false positive, "FP") results. There were 61 cases of trisomy 21 (48 TP, 13 FP), 26 cases of trisomy 18 (22 TP, 4 FP), and 9 cases of trisomy 13 (6 TP, 3 FP). The overall positive predictive value (PPV) for the three core trisomies in the matched cohort was 79.2% (95% CI: 69.4, 86.5). For individual

TABLE 3 Calculation of sensitivity, specificity, positive predictive value, and negative predictive value (with 95% confidence intervals) for 371 matched twin cases reportable for aneuploidy after one blood draw

Condition (n = 371)	True positive (TP)	False positive (FP)	True negative (TN)	False negative (FN)	Sensitivity TP/(TP + FN) (95% CI)	Specificity TN/(FP + TN) (95% CI)	PPV TP/(TP + FP) (95% CI)	NPV TN/(FN + TN) (95% CI)
Trisomy 21	48	13	309	1	98.0% (87.8-99.9%)	96.0% (93.0-97.7%)	78.7% (66.0-87.7%)	99.7% (97.9-100%)
Trisomy 18	22	4	345	0	100% (81.5-100%)	98.9% (96.9-99.6%)	84.6% (64.3-95.0%)	100% (98.6-100%)
Trisomy 13	6	3	362	0	100% (51.7-100%)	99.2% (97.4-99.8%)	66.7% (30.9-91.0%)	100% (98.7-100%)
Overall (21/18/13 combined)	76	20	274	1	98.7% (92.0-99.9%)	93.2% (89.5-95.7%)	79.2% (69.4-86.5%)	99.6% (97.7-100%)

Note: Each sample received a final characterization of TP, TN, FP, or FN for each trisomy. For example, a sample positive for trisomy 21 received a classification for trisomy 21 (true positive or false positive), as well classifications for trisomy 18 and trisomy 13 (true negative or false negative). No samples in this cohort were positive for more than one aneuploidy.

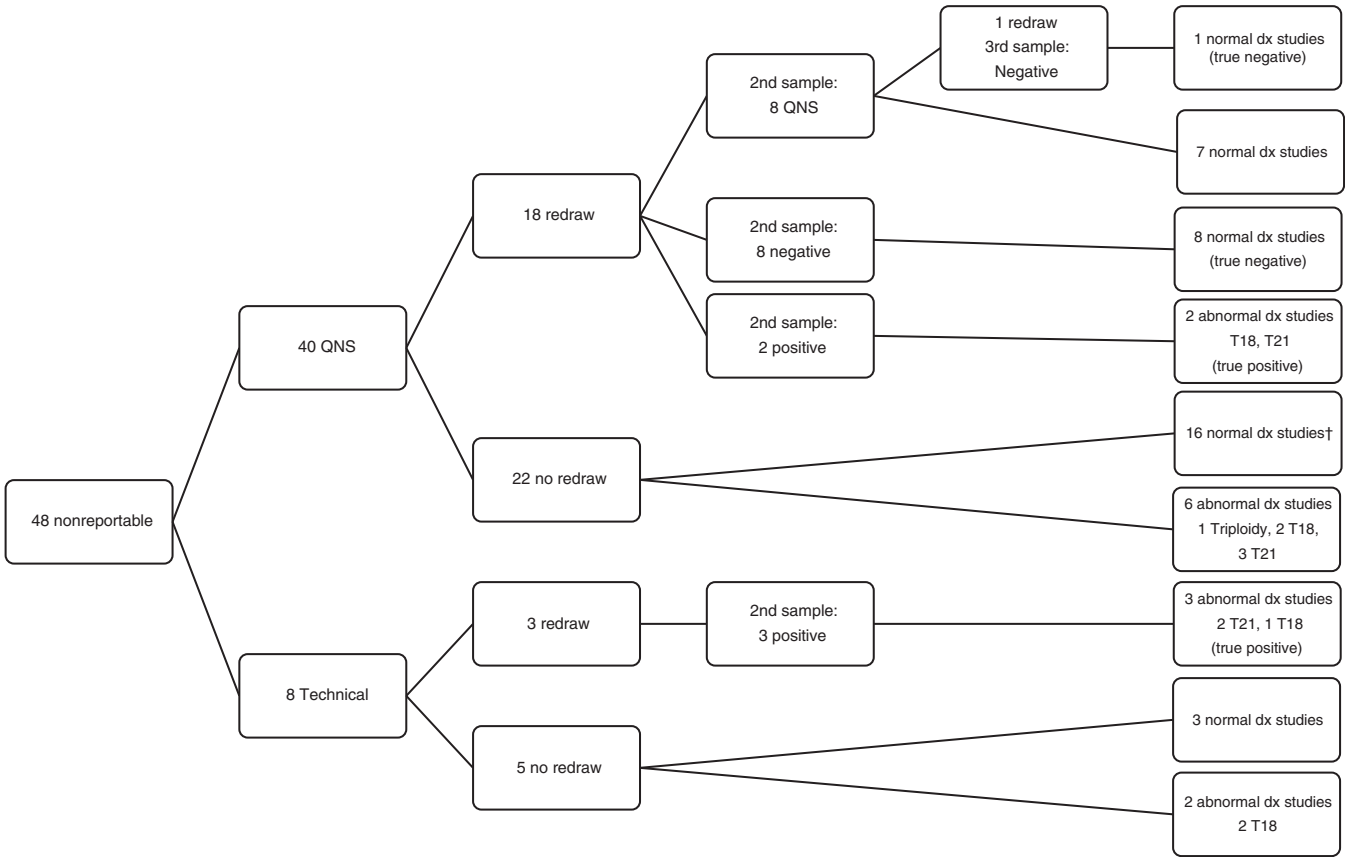

FIGURE 2 Outcomes of samples non-reportable after one blood draw

aneuploidies, the PPV was: 78.7% (95% CI: 66.0, 87.7) for trisomy 21, 84.6% (95% CI: 64.3, 95.0) for trisomy 18, and 66.7% (95% CI: 30.9, 91.0) for trisomy 13. (Table 3).

As a point of reference, the 96 positive results identified in the matched cohort represent approximately 20% of the 482 total positive results that the laboratory issued for twin gestations during the study time frame (September 2013 through June 2018). Interestingly, the breakdown of positive results by aneuploidy in the matched cohort is comparable to the distribution seen in the larger screening population. Approximately 64% of the positive results in the matched cohort were for trisomy 21, compared to 66% in the overall cohort. The numbers appear similar for trisomy 18 (27% vs 23%) and trisomy 13 (9% vs 11%).

Among the 275 cfDNA cases that were negative for all aneuploidies, there were 274 concordant (true negative) results and 1 discordant (false negative) result. Therefore, the negative predictive value (NPV) for patients who received a negative result for all three aneuploidies in this cohort was 99.6% (95% CI: 97.7, 100). The false

negative case involved a pregnancy with negative cfDNA results, mosaic CVS results for trisomy 21, and abnormal amniocentesis results confirming trisomy 21 in one fetus, with a normal co-twin.

Analysis of the results that were reportable after a single draw allows for calculation of sensitivity and specificity values for the overall matched cohort, as well as for individual conditions. Table 3 summarizes this data and includes 95% confidence intervals.

Forty-eight diagnostic specimens were matched to non-reportable cfDNA samples on the initial blood draw. The majority (n = 40) of these cases were non-reportable due to low fetal fraction ("NR-QNS" or quantity not sufficient) and 8 were non-reportable due to failure to meet laboratory quality metrics ("NR-tech"). Of the 40 NR-QNS samples, 18 redraws were submitted, of which 8 were negative on the second draw (all true negatives), 2 were positive (one trisomy 18, one trisomy 21- both true positives), and 8 samples were NR-QNS on the second draw. Of these 8, 7 were associated with normal karyotypes, and 1 case submitted a second redraw which resulted in a negative result (true negative). For the 8 NR-tech cases, 3 redraws were submitted. All 3 redraws resulted in a positive result on the second draw (two trisomy 21 and one trisomy 18 - all true positives). (Figure 2).

4 | DISCUSSION

This retrospective study analyzed 422 cases of cfDNA screening tests matched with diagnostic results from CVS, amniocentesis, or products of conception from September 2013 through June 2018.

As noted above, three cfDNA cases were associated with amniocentesis specimens that failed to return a result and these cases were excluded from analysis of test performance as there was no diagnostic outcome information to confirm the cfDNA results. Additionally, there were 48 cases that were non-reportable after the initial draw. This number decreased was reduced to 34 nonreportable results after redraw.

In 2019, Gil et al and The Fetal Medicine Foundation (FMF) published a meta-analysis of cfDNA screening for trisomies in twin pregnancies.[7] They concluded that: "The performance of cfDNA testing for trisomy 21 in twin pregnancy is similar to that reported in singleton pregnancy and is superior to that of the first-trimester combined test or second-trimester biochemical test." They went on to say: "The number of cases of trisomy 18 and 13 is too small for accurate assessment of the predictive performance of the cfDNA test." Data from the current study support their conclusions regarding trisomy 21 screening and provide additional information regarding cfDNA screening for trisomy 18 and trisomy 13 in twin gestations.

4.1 | Sensitivity and PPV of cfDNA in twin vs singleton gestations

The overall sensitivity observed in the current study was 98.7% for the three core trisomies (21/18/13). This is similar to the overall sensitivity observed for the core trisomies in singleton pregnancies using the same assay (98.9%).[8] When focusing specifically on trisomy 21, the sensitivity observed in the current study (98.0%) is consistent with the sensitivity observed in the FMF twin meta-analysis (98.2%), and is comparable to the sensitivity documented in the original trisomy 21 validation study in singletons (99.1%).[7,8]

The positive predictive values observed in the current study of twin gestations were similar to the PPVs observed in previous studies of cfDNA screening in singleton gestations.[9] Twins were found to have a 78.7% PPV for trisomy 21 in the current study compared to an 80.9% PPV previously documented in singletons. Similarly, trisomy 18 was associated with an 84.6% PPV in twins vs 90.0% in singletons, and trisomy 13 PPV was 66.7% in twins vs 50.0% in singletons. It should be noted that the PPVs obtained from the current study are expected to be impacted by the inherently higher rate of aneuploidy in the cohort of patients pursuing diagnostic testing compared to the general screening population.

4.2 | Sensitivity of cfDNA vs traditional serum screening in twin gestations

Current standard-of-care screening tests, including first trimester maternal serum screening (with nuchal translucency and serum biochemical analytes) and second-trimester maternal serum screening are quoted to have a 75%-85% detection rate and 63% detection rate, respectively, for Down syndrome in twin gestations.[1] In the current study, of the 371 cfDNA cases that were reportable after a single draw, a 98.0% sensitivity was observed when screening for trisomy 21. Based on data from this study and other publications,[7] cfDNA is expected to offer an increased detection rate for Down syndrome compared to current screening methodologies.

Furthermore, when considering the scope of testing, most traditional serum screens do not provide information about chromosome abnormalities other than trisomy 21 in multifetal gestations. The current study demonstrates 100% sensitivity for cfDNA screening for trisomies 18 and 13 in twins. Though not considered in the current analysis, but an area of possible future study, cfDNA is also available to screen for select microdeletion syndromes in multifetal gestations.

4.3 | Specificity and positive likelihood ratio calculations

By nature of the patient population pursing both cfDNA screening and diagnostic testing, the cohort analyzed in the current study contains a large proportion of aneuploid results. The number of euploid cases in the study population, though appreciable, is low compared to the proportion of euploid gestations presenting for screening in the general pregnancy population. Despite the proportionally small number of euploid cases in this study, the specificity of cfDNA screening in twins (96.0% for trisomy 21, 98.9% for trisomy 18, and 99.2% for trisomy 13) still compares favorably to the current standard-of-care

screening methods which quote false positive rates between 5% and 10.8%.[1] It should be noted that cfDNA screening is expected to have a higher specificity (>99%)[2] when utilized for screening in a general pregnancy population with a more robust distribution of euploid gestations.

Even when considering the reduced specificity that results from the proportionally small number of euploid cases in the current study, the positive likelihood ratio (calculated by dividing sensitivity by false positive rate) remains notable at 24.5 for trisomy 21. To put this into context, if a provider were to set a risk threshold of 1 in 200 for offering diagnostic testing to a patient, then any twin gestation with an a priori risk for trisomy 21 of 1 in 4900 or higher who receives a positive NIPT result for that condition would meet the threshold to be offered diagnostic testing.

4.4 | Discordant and nonreportable cases

There were 20 cases classified as "false positives" in the matched cohort. Experience with cfDNA screening over the past several years has demonstrated that results initially classified as "false positives" often have an underlying biological explanation for the discordance between the cfDNA and diagnostic results, including but not limited to: mosaicism, maternal copy number variation, or the demise of a co-twin.[10-13] These biological phenomena are not exclusive to singleton pregnancies, and can be an etiology of discordant results in multifetal gestations, as well. Of the 20 discordant cases in the current data set, there was a plausible biological explanation for 17 of the results. In nine cases, the cfDNA data appeared mosaic,[14,15] suggestive of one affected fetus, confined placental mosaicism, or an undiagnosed co-twin/co-triplet demise. In all 9 of these cases, only a single prenatal diagnostic specimen was submitted from the twin gestation for analysis (6 amniocenteses, 3 CVS), which could be consistent with typical clinical care in the case of a co-twin demise. There was a documented co-twin demise or selective reduction in 7 cases prior to prenatal diagnosis. Additionally, one case was suspected to have a complex abnormality involving chromosome 13 suspected to be maternal in origin in a patient with autoimmune lymphoproliferative syndrome. There were three cases for which follow-up was limited and no biological explanation could be provided for the discordant results. Inclusion of these 17 cases with a plausible biologic explanation into positive predictive value calculations would raise the upper PPV limit in this cohort to 96.9%.

Of the 419 total specimens with diagnostic results, 11.5% (n = 48) of the matched cases were non-reportable from the initial blood draw. The majority of these samples (n = 40) were non-reportable due to low fetal fraction. The overall nonreportable rate in this matched cohort is significantly higher than the non-reportable rate (6.05%) observed in a much larger cohort of twin specimens submitted to the laboratory for screening.[4] This current data set represents patients who elected to pursue diagnostic testing as opposed to the general twin cfDNA testing population and therefore is enriched for non-reportable cfDNA results when compared to the general cfDNA screening population. This enrichment suggests that clinicians are following ACOG and ACMG guidance which recommend offering diagnostic testing for women who receive non-reportable results from cfDNA screening, particularly in cases with low fetal fraction.[3,12]

If the outcomes of the non-reportable results from the current study population were factored into calculations of test performance (ie, considering any patient with abnormal diagnostic results and failed cfDNA test as a "false negative," and any patient with a normal diagnostic result and failed cfDNA test as a "false positive"), cfDNA screening would offer an overall sensitivity of 91.0% (95% CI: 82.6, 95.8), specificity of 85.8% (95% CI: 81.4, 89.3), PPV of 63.3% (95% CI: 54.3, 71.5), and NPV of 97.3% (95% CI: 94.4, 98.7) combined for the three trisomies. As mentioned earlier, this cohort of patients is enriched for nonreportable samples compared to the broader screening population (ie, the nonreportable rate in the current study is almost double that seen in the broader twin screening population). Despite factoring in this significant population of nonreportable results, cfDNA screening performance still compares favorably to the performance offered by traditional serum screening tests in twin gestations.

Lastly, 5 of the 8 technical non-reportable results in the study population resulted in an abnormal outcome (2 cases of trisomy 21 and 3 cases of trisomy 18) from second draw/diagnostic testing. What appears to be an "enrichment" for aneuploidy may be explained, in part, by the small sample size (n = 8). Additionally, the patients in this group who elected to pursue diagnostic testing following a technical non-reportable result likely had additional clinical factors which influenced their decision to pursue such testing (eg, ultrasound findings in the pregnancy may have prompted these patients to pursue prenatal diagnosis). Analysis of aneuploidy in non-reportable results is outside the scope of the current study; however, future research could focus on this topic.

4.5 | Limitations

At a high-volume laboratory offering both screening and diagnostic tests there exists a unique opportunity to compare data from patients that have pursued both tests during the same pregnancy. Though this novel approach allows for data mining from unsolicited cases submitted for analysis, helping to reduce the opportunity for selection bias, this methodology is not without limitations.

Samples included in this study were from patients who opted to pursue diagnostic testing. Therefore, the cohort is expected to be enriched with cases positive for aneuploidy from cfDNA screening. A patient receiving negative cfDNA results may be less likely, in general, to pursue diagnostic testing during pregnancy, particularly in the case of a high-risk pregnancy involving twins. As professional societies recommend offering prenatal diagnosis in the event of a non-reportable cfDNA result, this study cohort is also enriched with cases that were non-reportable from cfDNA screening. Given these factors, the number of euploid samples in this matched cohort is relatively small. Despite the proportionally small number of euploid cases in the study population, specificity values calculated from this cohort were still

higher than those associated with traditional serum screening tests for twins, though these values were lower than previously observed for this assay.[2,4] Further studies focusing on larger numbers of euploid twin gestations are needed to support more robust calculations of specificity and NPV for cfDNA screening.

Another limitation of this analysis involves the filtering of cfDNA specimens for inclusion in the study. Samples were selected based upon the number of fetuses indicated on the test requisition by the ordering provider. This information may not accurately reflect the gestational status of the ongoing pregnancy. There are documented cases in this cohort of pregnancies that began as a twin gestation and spontaneously or selectively reduced to a singleton fetus prior to screening or diagnostic testing. Likewise, the data set may contain cases documented as twins that had reduced from a triplet gestation at some point prior to testing. These scenarios could increase the chance of discordant results, translating to a reduced specificity in this cohort.

As previously discussed, the cohort of patients analyzed in this study were a subset of patients from a much larger population presenting for cfDNA screening, and it is important to note that the findings from this study may not necessarily represent the larger screening group. In order to determine test performance in the broader screening group, outcome data on all cfDNA tests would be needed. For instance, from voluntary clinician feedback it is known that additional false positive and false negative results were reported in a previously published study of over 23 000 twin samples submitted to the same laboratory for cfDNA screening during a partially-overlapping time frame,[4] and that these cases may not have been fully captured in the current study derived solely from laboratory data.

Additionally, analysis of patient demographics from the current study vs the previously-published larger twin cohort showed significant differences which may impact test performance metrics. It was noted that the patients in the current data set were significantly older than the patients in the larger testing cohort. Also, the gestational age of the pregnancies at the time of cfDNA screening was significantly lower than the patients in the broader screening cohort. Older maternal age and earlier gestational age could translate to an increased disease prevalence which could artificially enhance the PPV seen in the current study group compared to the broader screening cohort. The mean maternal body mass index of patients in the current study was significantly lower than observed in the broader screening cohort, which would typically translate to higher fetal fractions and lower non-reportable rates. However, the average fetal fraction in the current data set was significantly lower than the broader screening cohort. Therefore, BMI is unlikely to unlikely to contribute to a significant difference between the matched and broader cohort.

5 | CONCLUSION

According to current clinical practice, twin pregnancies are offered maternal serum screening (with or without ultrasound evaluation) for aneuploidy risk assessment. These tests offer limited sensitivity and specificity, and a relatively low positive predictive value. The current study, along with previous publications,[2,7] suggest that cfDNA screening may offer superior performance to the current standard-of-care tests for Down syndrome screening in twin gestations. Furthermore, cfDNA screening may offer a reliable screening option for conditions beyond trisomy 21, such as trisomy 18 and trisomy 13.

Prior to this study, outcome data from cfDNA screening in twin gestations were limited. This unique data set was generated by matching cfDNA results with diagnostic studies from patients pursing both tests during the same pregnancy at a large clinical laboratory. The data presented here adds to a growing body of evidence suggesting that cfDNA is an accurate and reliable screening tool for the major trisomies in twin pregnancies.

ACKNOWLEDGEMENTS

Stuart Schwartz, PhD and his dedicated team at the microarray laboratory at LabCorp's Center for Molecular Biology and Pathology.

The team of hardworking laboratory directors, cytogeneticists, and genetic counselors at LabCorp and Integrated Genetics. The authors state that no external funding was received to support the research in connection with this article.

CONFLICT OF INTEREST

All authors are full-time employees of Laboratory Corporation of America Holdings.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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How to cite this article: Chibuk J, Rafalko J, Boomer T, et al. Cell-free DNA screening in twin pregnancies: A more accurate and reliable screening tool. *Prenatal Diagnosis*. 2020;40: 1321–1329. https://doi.org/10.1002/pd.5797