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Cell-free DNA Analysis for Noninvasive Examination of Trisomy

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ABSTRACT

BACKGROUND

Cell-free DNA (cfDNA) testing for fetal trisomy is highly effective among high-risk women. However, there have been few direct, well-powered studies comparing cfDNA testing with standard screening during the first trimester in routine prenatal populations.

METHODS

In this prospective, multicenter, blinded study conducted at 35 international centers, we assigned pregnant women presenting for aneuploidy screening at 10 to 14 weeks of gestation to undergo both standard screening (with measurement of nuchal translucency and biochemical analytes) and cfDNA testing. Participants received the results of standard screening; the results of cfDNA testing were blinded. Determination of the birth outcome was based on diagnostic genetic testing or newborn examination. The primary outcome was the area under the receiver-operating-characteristic curve (AUC) for trisomy 21 (Down's syndrome) with cfDNA testing versus standard screening. We also evaluated cfDNA testing and standard screening to assess the risk of trisomies 18 and 13.

RESULTS

Of 18,955 women who were enrolled, results from 15,841 were available for analysis. The mean maternal age was 30.7 years, and the mean gestational age at testing was 12.5 weeks. The AUC for trisomy 21 was 0.999 for cfDNA testing and 0.958 for standard screening ($P=0.001$). Trisomy 21 was detected in 38 of 38 women (100%; 95% confidence interval [CI], 90.7 to 100) in the cfDNA-testing group, as compared with 30 of 38 women (78.9%; 95% CI, 62.7 to 90.4) in the standard-screening group ($P=0.008$). False positive rates were 0.06% (95% CI, 0.03 to 0.11) in the cfDNA group and 5.4% (95% CI, 5.1 to 5.8) in the standard-screening group ($P<0.001$). The positive predictive value for cfDNA testing was 80.9% (95% CI, 66.7 to 90.9), as compared with 3.4% (95% CI, 2.3 to 4.8) for standard screening ($P<0.001$).

CONCLUSIONS

In this large, routine prenatal-screening population, cfDNA testing for trisomy 21 had higher sensitivity, a lower false positive rate, and higher positive predictive value than did standard screening with the measurement of nuchal translucency and biochemical analytes. (Funded by Ariosa Diagnostics and Perinatal Quality Foundation; NEXT ClinicalTrials.gov number, NCT01511458.)

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SCREENING FOR FETAL ANEUPLOIDY WITH the use of cell-free DNA (cfDNA) obtained from maternal plasma was introduced in 2011. Such screening has been reported to have a detection rate for trisomy 21 (Down's syndrome) of more than 99%, with a false positive rate as low as 0.1%.¹ Thus, cfDNA testing appears to represent a substantial improvement over traditional multiple-marker screening. In practice, the use of this test could result in a significant reduction in diagnostic procedures.

Although several large proof-of-principle studies have confirmed the high sensitivity and specificity of cfDNA testing for the detection of trisomy 21, most of these studies have included only selected populations of high-risk women who were sampled before invasive testing. There are more limited data available on the performance of cfDNA testing in the general pregnancy population.²⁻⁴

In this blinded, prospective study, called the Noninvasive Examination of Trisomy (NEXT) study, we tested the hypothesis that cfDNA testing has better performance than standard first-trimester screening (with measurement of nuchal translucency and biochemical analytes) in risk assessment for trisomy 21 in a large, unselected population of women presenting for aneuploidy screening. We also evaluated the performance of cfDNA testing and standard screening in the assessment of risk for trisomies 18 and 13.

METHODS

STUDY CONDUCT

From March 2012 through April 2013, we enrolled pregnant women undergoing first-trimester aneuploidy screening at 35 centers in six countries. At enrollment, maternal blood was drawn, locally deidentified, and sent for risk assessment for trisomy 21 with the use of cfDNA testing (Harmony Prenatal Test, Ariosa Diagnostics). We submitted the results of cfDNA testing and standard screening to an independent data-coordinating center (Veristat). We then collected pregnancy outcomes for all participants who met the eligibility criteria and completed standard screening. The institutional review board at each participating site approved the study. Written informed consent was obtained from all the participants.

STUDY OVERSIGHT

The study was a collaboration between the clinical investigators and the sponsors (Ariosa Diagnostics

and the Perinatal Quality Foundation). The first and last authors designed the protocol in collaboration with the sponsor. Representatives of the sponsor performed the analyses and interpretation of cfDNA data; data regarding maternal and gestational age were required. Laboratory personnel performed their analyses in a blinded fashion with respect to all other clinical data, including results of ultrasonographic and standard screening. Research staff members at the clinical sites entered clinical and laboratory data into an electronic case-report form, which was stored in a secure database. The data-coordinating center compiled and analyzed the laboratory and clinical data. Ariosa supervised data accrual, participated in the preparation of the manuscript, and approved the final version of the manuscript. Veristat performed the primary analysis; secondary analyses were conducted by Ariosa. The first author wrote the first draft of the manuscript. All the authors vouch for the accuracy of the data and fidelity of the study to the protocol (available with the full text of this article at NEJM.org) and approved the submission of the manuscript for publication. There were no confidentiality agreements among the authors, sites, or sponsor.

STUDY POPULATION AND SAMPLE COLLECTION

Eligible patients were at least 18 years of age and had a singleton pregnancy between 10.0 and 14.3 weeks of gestation at the time of the study blood-sample collection. Gestational age was determined according to the crown-rump length at the time of the measurement of nuchal translucency.

Patients were ineligible if they were outside the gestational-age window, had no standard screening result, had known maternal aneuploidy or cancer, had conceived with the use of donor oocytes, or had a twin pregnancy or an empty gestational sac that was identified on ultrasonography. Peripheral blood was collected into two Cell-free DNA BCT tubes (Streck) that were labeled with a unique patient identifier. Samples were sent to the Ariosa clinical laboratory, which is certified according to the Clinical Laboratory Improvement Amendments, without further processing. Results for cfDNA testing were not available to providers or participants.

TESTING METHODS

All patients underwent standard screening (including the measurement of serum pregnancy-associated plasma protein A, total or free beta

subunit of human chorionic gonadotropin, and nuchal translucency) with the use of local laboratories. All providers of nuchal translucency were certified by the Nuchal Translucency Quality Review program, the Fetal Medicine Foundation, or other national quality-review programs. All measurements of nuchal translucency were performed and serum samples collected within the gestational age range required by the local laboratory.

For clinical risk assessment, we used local risk algorithms and cutoffs according to standard clinical practice. For study purposes, one of the authors used a standard algorithm⁵ to recalculate risk using serum multiples of the median (MoM) and measurements of nuchal translucency and crown-rump length. A positive result on standard screening was defined as a mid-trimester risk of at least 1 in 270 for trisomy 21 and at least 1 in 150 for trisomy 18 and trisomy 13, cutoffs that are commonly used by laboratories in the United States.

Details on Ariosa laboratory testing methods have been described previously.⁶⁻⁸ For cfDNA testing, samples were rejected if they were not collected in Cell-free DNA BCT tubes; if the tubes were broken, unfilled, or not labeled; or if the sample was grossly hemolyzed or arrived in the laboratory more than 7 days after collection. Each acceptable sample underwent plasma separation and cfDNA isolation, followed by ligation of locus-specific oligonucleotides to produce a template from selected genomic loci (Fig. S1 in the Supplementary Appendix, available at NEJM.org). We estimated the risk of aneuploidy using a previously described algorithm, including chromosome cfDNA counts, fetal fraction of cfDNA, and a priori trisomy risk based on maternal and gestational age⁸ (Fig. S2 in the Supplementary Appendix). A risk of 1 in 100 or higher was the laboratory-designated threshold for classifying a sample as high risk. Samples were not included in the analyses if they did not pass laboratory quality control because of a low fraction of fetal cfDNA (<4%), an inability to measure the fraction of fetal cfDNA, a high variation in cfDNA counts, or an assay failure.

PREGNANCY AND NEWBORN OUTCOMES

We recorded all pregnancy outcomes, including miscarriage, termination, and delivery. Results of invasive prenatal diagnostic testing and testing of products of conception (i.e., miscarriages) were collected when available. Newborn outcomes were determined by medical-record review of the

physical examination at birth and any genetic testing performed. In the absence of genetic testing, a newborn with a normal physical examination was considered to be euploid. The results for women who had a miscarriage, chose to terminate the pregnancy, or had a stillbirth were included only if confirmatory genetic testing was performed; those without genetic analysis were excluded. In a blinded fashion, the first and last authors reviewed medical records of all neonates with congenital anomalies and excluded those with phenotypes suggestive of aneuploidy if no confirmatory genetic testing was performed. Results of fetal and newborn genetic testing were adjudicated by two clinical geneticists, categorized as euploid or aneuploid, and classified according to the type of abnormality.

DATA HANDLING

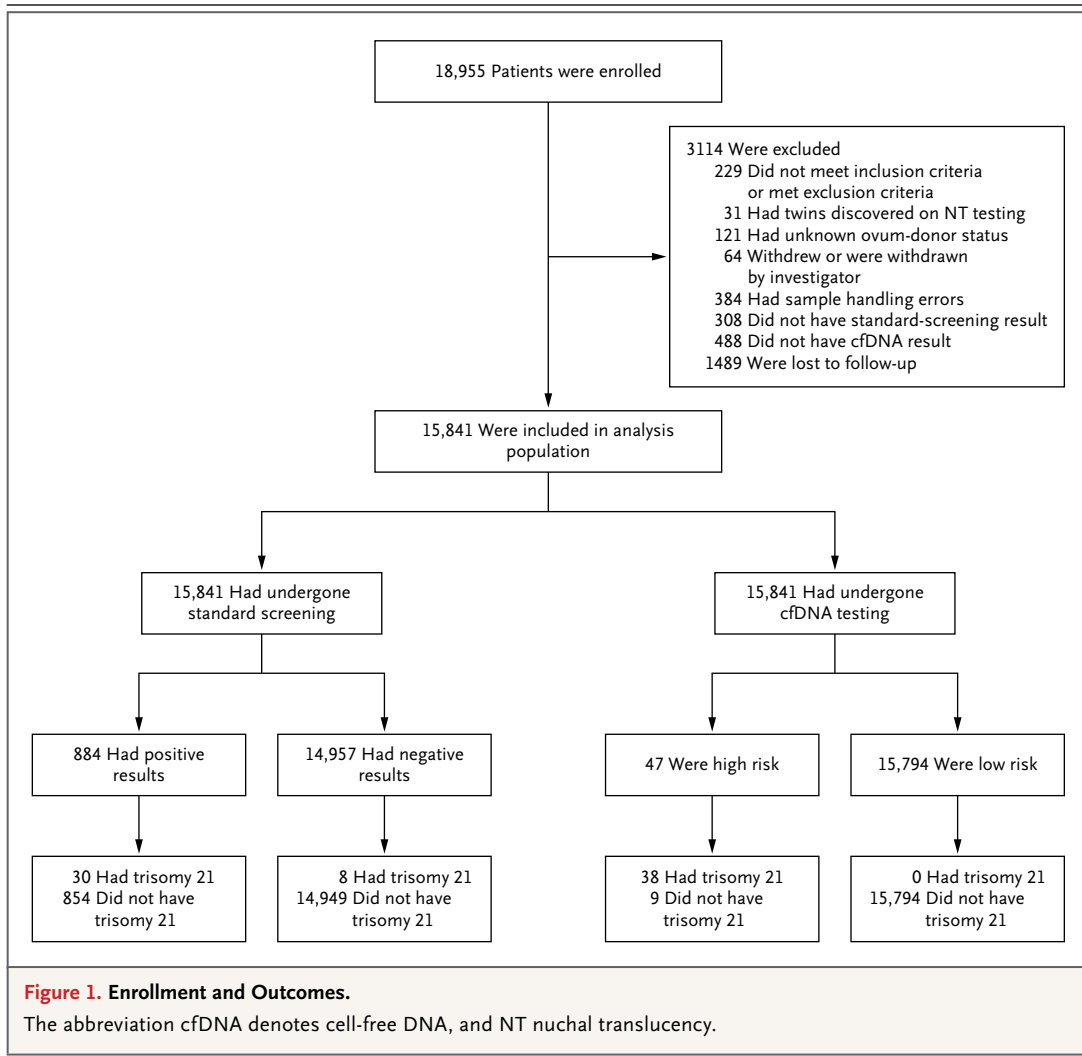
We transferred the results of cfDNA testing, standardized risk scores for standard screening, and clinical data to the independent data-coordinating center for consolidation and unblinding. The primary-analysis population included all eligible participants who had results on both cfDNA testing and standard screening and a documented normal or adjudicated newborn examination or results of prenatal or postnatal genetic testing.

STUDY OUTCOMES

The primary outcome was the area under the receiver-operating-characteristic (ROC) curve (AUC) for trisomy 21 screening with cfDNA testing versus standard screening in women with complete results for the two tests. Secondary outcomes included the evaluation of cfDNA testing and standard screening to assess the risk of trisomies 18 and 13. The evaluation of the performance of cfDNA testing for trisomy 13 included only patients who were enrolled after the introduction of the analysis in September 2012. We also evaluated the performance of cfDNA testing in low-risk patients, who were defined as having a maternal age of less than 35 years or a risk of trisomy 21 of less than 1 in 270 on standard screening.

STATISTICAL ANALYSIS

Standard screening and cfDNA testing each produces a measured value representing the risk of each aneuploidy. The ROC curve was generated by computing sensitivity and specificity at each observed cutoff for risk score. We calculated the differences between the ROC curves, taking into account the paired nature of the data. AUC values



were compared with the use of a z-test according to the method of DeLong et al.⁹ A P value of less than 0.05 was considered to indicate statistical significance. Confidence intervals were computed with the use of the Clopper–Pearson method. We used the exact binomial test¹⁰ for paired comparisons in sensitivity and specificity and used the generalized score statistic¹¹ to analyze positive and negative predictive values. We compared the sensitivity, specificity, positive and negative predictive values, and likelihood ratios of standard screening and cfDNA testing for the detection of trisomy 21.

On the basis of results of previous studies^{12,13} and assumptions with respect to the performance of cfDNA testing, we determined that a

sample size of 32 cases of trisomy 21 and 1500 negative controls would provide a power of 80% to determine the primary outcome at an alpha level of 0.05. To detect an increase to an AUC of 0.95 for cfDNA testing at a power of 80% and with a prevalence of 1 in 700 for trisomy 21, we estimated that 22,400 participants would be required. To account for loss to follow-up, we planned to enroll 25,000 participants. Using the maternal age of enrolled participants mid-trial, we revised the estimate of the prevalence of trisomy 21 at 1 in 500, and we reduced the required sample size to 18,700. Interim study outcomes were not unblinded or considered in the decision to stop enrollment before achieving the planned sample size.

RESULTS

STUDY PARTICIPANTS

From March 2012 through April 2013, a total of 18,955 women at 35 centers in the United States, Canada, and Europe were enrolled. Of these women, 445 were excluded because they did not meet the eligibility criteria, were discovered to be carrying twins during ultrasonography to measure nuchal translucency, had undergone in vitro fertilization with unknown ovum-donor status, or withdrew from the study or were withdrawn by an investigator. In addition, 384 women were excluded because of a blood-collection or labeling error, 308 because of the absence of a result on standard screening, 488 because of the absence of a result on cfDNA screening, and 1489 because they were lost to follow-up. After all exclusions, the primary analysis cohort included 15,841 women (Fig. 1).

Baseline characteristics of the primary analysis cohort are outlined in Table 1. The mean maternal age was 31 years (range, 18 to 48), and the mean gestational age was 12.5 weeks (range, 10.0 to 14.3). In all, 557 women underwent invasive prenatal diagnostic testing, 52 underwent postnatal genetic testing, and 16 underwent testing on products of conception from miscarriages. For the remainder of the women, the outcome was based on examination of the newborn.

Among the 15,841 pregnancies in the primary analysis population, there were 68 chromosomal abnormalities (1 in 236 pregnancies). Of these abnormalities, 38 were trisomy 21, 10 were trisomy 18, 6 were trisomy 13, 3 were 45,X, 3 were marker chromosomes, 2 were unbalanced translocations, 2 were balanced translocations, and 1 each was deletion 7p, deletion/duplication 5p, 1q41 deletion, and isochromosome Yp. Trisomy 21 was identified in 38 of 15,841 women, for a prevalence of 1 in 417.

PRIMARY ANALYSIS

The AUC for trisomy 21 was 0.999 for cfDNA testing and 0.958 for standard screening ($P=0.001$) (Fig. 2). Of the 38 participants with trisomy 21 with a result on cfDNA testing, cfDNA identified all 38 cases, for a sensitivity of 100% (95% confidence interval [CI], 90.7 to 100). Standard screening identified 30 of 38 cases as positive, a sensitivity of 78.9% (95% CI, 62.7 to 90.4; $P=0.008$).

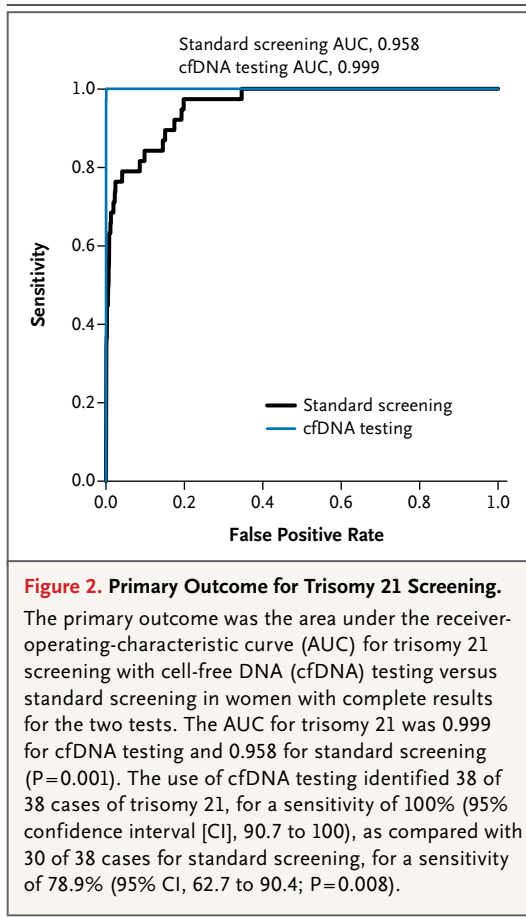
Table 1. Demographic and Clinical Characteristics of the Patients.

Characteristic	Value
No. of patients	15,841
Mean maternal age (range) — yr	31 (18–48)
Mean gestational age at sample collection (range) — wk	12.5 (10.0–14.3)
Race or ethnic group — no. (%) [*]	
White	11,235 (70.9)
Black	1,295 (8.2)
Asian	1,659 (10.5)
Native American	93 (0.6)
Multiracial	422 (2.7)
Other	1,060 (6.7)
Missing data	77 (0.5)
Hispanic ethnic group — no. (%) [*]	
Hispanic	3,202 (20.2)
Non-Hispanic	12,639 (79.8)
Median maternal weight (range) — kg	65.8 (31.8–172.4)
Pregnancy through assisted reproductive technology — no. (%)	480 (3.0)
Current smoker — no. (%)	432 (2.7)
Insulin-dependent diabetes — no. (%)	188 (1.2)
Genetic testing — no./total no. (%)	
Any	625/15,841 (3.9)
Chorionic villus sampling	135/625 (21.6)
Amniocentesis	422/625 (67.5)
Products of conception	16/625 (2.6)
Newborn	52/625 (8.3)
Pregnancy outcome — no. (%)	
Live birth	15,715 (99.2)
Termination	62 (0.4)
Stillbirth	17 (0.1)
Miscarriage	24 (0.2)
Unknown [†]	23 (0.1)

^{*} Race and ethnic group were self-reported.

[†] The birth outcome was unknown, but results of invasive prenatal testing were available.

There were 9 false positives among the 15,803 women in the cfDNA-testing group without trisomy 21, for a false positive rate of 0.06% (95% CI, 0.03 to 0.11). There were 854 false positive results for trisomy 21 on standard screening, for a false positive rate of 5.4% (95% CI, 5.1 to 5.8; $P<0.001$). The positive predictive value was



80.9% (95% CI, 66.7 to 90.9) for cfDNA testing and 3.4% (95% CI, 2.3 to 4.8) for standard screening ($P<0.001$) (Table 2). The median nuchal translucency for the entire cohort was 0.98 MoM, and the standard deviation of the \log_{10} MoM was 0.09.

SECONDARY ANALYSES

Trisomy 21

Among the 11,994 women with low-risk pregnancies on the basis of a maternal age under 35 years, cfDNA testing identified 19 of 19 women with trisomy 21, with 6 false positive results. Among the 14,957 women for whom standard screening showed a risk of less than 1 in 270, cfDNA testing identified 8 of 8 women with trisomy 21, with 8 false positive results. The positive predictive value for cfDNA testing was 76.0% (95% CI, 54.9 to 90.6) for women under the age of 35 years and 50.0% (95% CI, 24.7 to 75.3) for those with a negative result on standard screening (Table 2).

Trisomy 18

There were 10 cases of trisomy 18 in the primary analysis population. Of these cases, cfDNA testing identified 9 and standard screening identified 8; cfDNA testing had 1 false positive result, for a false positive rate of 0.01% (95% CI, 0 to 0.04) and a positive predictive value of 90.0% (95% CI, 55.5 to 99.7), as compared with 49 false positive results on standard screening, for a false positive rate of 0.31% (95% CI, 0.23 to 0.41) and a positive predictive value of 14.0% (95% CI, 6.3 to 25.8) ($P<0.001$ for both comparisons).

Trisomy 13

Among the 11,185 women who underwent both cfDNA testing and standard screening for trisomy 13, there were 2 confirmed cases; of these cases, cfDNA testing identified 2 and standard screening identified 1. There was 1 false positive result on cfDNA testing and 28 false positive results on standard screening, for false positive rates of 0.02% (95% CI, 0 to 0.06) and 0.25% (95% CI, 0.17 to 0.36), respectively ($P<0.001$) (Table 3).

Findings among Excluded Participants

Of the 16,329 otherwise eligible women, 488 (3.0%) were excluded from the primary analysis because of a lack of results on cfDNA testing. In the group of 16,329 women, 192 (1.2%) had a fetal fraction of less than 4%, 83 (0.5%) had a fetal fraction that could not be measured, and 213 (1.3%) had a high assay variance or an assay failure. The median maternal weight in women with a low fetal fraction was 93.7 kg, as compared with 65.8 kg in the women with a successful result on cfDNA testing ($P<0.001$).

In the group with no results on cfDNA testing, there were 13 aneuploidies: 3 with trisomy 21, 1 with trisomy 18, 2 with trisomy 13, 4 with triploidy, 1 with trisomy 16 mosaic, 1 with deletion 11p, and 1 with a structurally abnormal chromosome. The prevalence of aneuploidy in this group (1 in 38 [2.7%]) is higher than the prevalence of 1 in 236 (0.4%) in the overall cohort ($P<0.001$). Specifically, for women with a fetal fraction of less than 4%, 9 in 192 (4.7%) had aneuploidy. Among the women with the six common aneuploidies for which there was no result on cfDNA testing, each case was detected on standard screening, with risks ranging from 1 in 26 to 1 in 2.

Table 2. Test Performance for Trisomy 21 in the Primary Analysis Cohort, According to Maternal Age and Risk.*

Variable	Standard Screening		Cell-free DNA Testing	
	All Patients (N=15,841)	All Patients (N=15,841)	Maternal Age <35 Yr (N=11,994)	Low Risk (N=14,957)†
True positive — no.	30	38	19	8
True negative — no.	14,949	15,794	11,969	14,941
False positive — no.	854	9	6	8
False negative — no.	8	0	0	0
Sensitivity (95% CI) — %	78.9 (62.7–90.4)	100 (90.7–100)‡	100 (82.4–100)	100 (63.1–100)
Specificity (95% CI) — %	94.6 (94.2–94.9)	99.9 (99.9–100)§	99.9 (99.9–100)	99.9 (99.9–100)
Positive predictive value (95% CI) — %	3.4 (2.3–4.8)	80.9 (66.7–90.9)¶	76.0 (54.9–90.6)	50.0 (24.7–75.3)
Negative predictive value (95% CI) — %	99.9 (99.9–100)	100 (99.9–100)¶¶	100 (99.9–100)	100 (99.9–100)
Positive likelihood ratio	14.6	1755.9	1995.8	1868.6
Negative likelihood ratio	0.22	0	0	0

* P values are for the comparison between standard screening and cell-free DNA screening in the primary analysis cohort.

† Low risk was defined as a mid-trimester risk of trisomy 21 of less than 1 in 270 on standard screening.

‡ P=0.008

§ P<0.001

¶ P=0.005.

Table 3. Test Performance for Trisomy 18 and Trisomy 13.*

Metric	Trisomy 18		Trisomy 13	
	Standard Screening (N=15,841)	Cell-free DNA Testing (N=15,841)	Standard Screening (N=11,185)	Cell-free DNA Testing (N=11,185)
True positive — no.	8	9	1	2
True negative — no.	15,782	15,830	11,155	11,181
False positive — no.	49	1	28	2
False negative — no.	2	1	1	0
Sensitivity (95% CI) — %	80.0 (44.4–97.5)	90.0 (55.5–99.7)	50.0 (1.2–98.7)	100 (15.8–100)
Specificity (95% CI) — %	99.7 (99.6–99.8)	100 (99.9–100)†	99.7 (99.6–99.8)	100 (99.9–100)†
Positive predictive value (95% CI) — %	14.0 (6.2–25.8)	90.0 (55.5–99.7)†	3.4 (0.1–17.8)	50.0 (6.8–93.2)
Negative predictive value (95% CI) — %	100 (99.9–100)	100 (99.9–100)	100 (99.9–100)	100 (99.9–100)

* Included in the trisomy 13 analysis are patients who were enrolled after September 2012.

† P<0.001 for the comparison with standard screening.

DISCUSSION

In this large, multicenter cohort study, we found that cfDNA testing had a higher sensitivity and specificity than did standard screening for the detection of trisomy 21 in a general prenatal-screening population. The false positive rate of cfDNA testing was nearly 100 times lower than

that of standard screening. Our study included pregnant women of all risk levels, and 76% were under the age of 35 years. We found that cfDNA testing was more sensitive than standard screening and yielded lower false positive rates, regardless of maternal age.

Approximately 3% of cfDNA tests did not yield a result because of assay variation or a low fetal

fraction. In previous studies, obesity was associated with a low fetal fraction,^{14,15} and we too found that such samples were obtained from participants with a higher body weight. We also observed a high frequency of aneuploidy among patients with no result on cfDNA testing. This association has been reported previously^{16,17} and strongly suggests that “no results” cases should be taken into account when reporting results and calculating test performance. If we had included in the “not detected” category participants with trisomy 21 who had no result on cfDNA testing, it would have lowered the detection rate of cfDNA testing. Alternatively, if we had categorized participants with no result on cfDNA testing as being high risk and requiring further investigation, it is possible that we could have determined their true status, but the percentage of women with positive results on cfDNA testing would have been higher. Further study is needed to determine the best approach in such cases, including the value of repeat testing, adjusting the initial test for maternal weight, additional screening by another approach, or the recommendation of invasive diagnostic testing to women with no results on cfDNA testing.

Although the strength of our study is the large sample size in a general prenatal screening population, a limitation is the comparison between cfDNA testing and only standard first-trimester screening, since methods such as integrated first- and second-trimester screening with nuchal translucency and biochemical analytes have higher sensitivity and specificity.¹³ The detection rate of standard screening for trisomy 21 was 79%, somewhat lower than the rate of 82 to 87% (at a false positive rate of 5%) that has been reported previously.¹³ It is possible that standard screening has lower performance in clinical practice than under the stringent experimental conditions in which previously reported data were collected. Finally, the study was powered only to compare the detection of trisomy 21 in the two study groups. Nevertheless, the lower false positive rate and higher positive predictive value support the use of cfDNA testing in risk assessment for trisomies 18 and 13.

Before cfDNA testing can be widely implemented for general prenatal aneuploidy screening, careful consideration of the screening method and costs is needed. Although the sensitivity and specificity of cfDNA testing are higher than those of standard screening, these

benefits are lower when cases with no results on cfDNA testing are considered. It has been noted that the marginal cost for each additional detected case of trisomy 21 is high.¹⁸ In our study, among women with negative results on standard screening, 1868 would have needed to undergo cfDNA testing to identify one additional case of trisomy 21. However, the false positive rate of cfDNA testing is far lower than that of standard screening, which means that fewer invasive tests would have been performed to detect each case.

Clinical implementation of cfDNA testing requires consideration of expectations regarding prenatal genetic testing. For trisomy 21 and other common aneuploidies, cfDNA testing represents a highly accurate screening option, especially since it can also detect some sex chromosomal aneuploidies that are not identified on standard screening.^{19,20} However, maternal serum and nuchal translucency screening can identify risk for a broad array of abnormalities that are not detectable on cfDNA testing.^{21,22} As in other studies, cases of trisomy 21 comprised just over 50% of aneuploidies present in this population. Women who desire a comprehensive assessment may prefer diagnostic testing with karyotype or chromosomal microarray analysis. Further study is needed to address the incremental value of nuchal translucency, first-trimester ultrasonography, and serum analytes for the detection of atypical aneuploidies, copy-number variants, structural anomalies, and other adverse perinatal outcomes.

In conclusion, the performance of cfDNA testing was superior to that of traditional first-trimester screening for the detection of trisomy 21 in a routine prenatal population. Although these data support the use of cfDNA testing in women regardless of age or risk status, further cost utility studies are warranted. As emphasized by professional societies,²³⁻²⁶ the use of cfDNA testing and other genetic tests requires an explanation of the limitations and benefits of prenatal test choices to the patient.

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