OBSTETRICS

Cell-free DNA screening for trisomies 21, 18, and 13 in pregnancies at low and high risk for aneuploidy with genetic confirmation

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BACKGROUND: Cell-free DNA noninvasive prenatal screening for trisomies 21, 18, and 13 has been rapidly adopted into clinical practice. However, previous studies are limited by a lack of follow-up genetic testing to confirm the outcomes and accurately assess test performance, particularly in women at a low risk for aneuploidy.

OBJECTIVE: To measure and compare the performance of cell-free DNA screening for trisomies 21, 18, and 13 between women at a low and high risk for aneuploidy in a large, prospective cohort with genetic confirmation of results

STUDY DESIGN: This was a multicenter prospective observational study at 21 centers in 6 countries. Women who had single-nucleotide-polymorphism-based cell-free DNA screening for trisomies 21, 18, and 13 were enrolled. Genetic confirmation was obtained from prenatal or newborn DNA samples. The test performance and test failure (no-call) rates were assessed for the cohort, and women with low and high previous risks for aneuploidy were compared. An updated cell-free DNA algorithm blinded to the pregnancy outcome was also assessed.

RESULTS: A total of 20,194 women were enrolled at a median gestational age of 12.6 weeks (interquartile range, 11.6—13.9). The genetic outcomes were confirmed in 17,851 cases (88.4%): 13,043 (73.1%) low-risk and 4808 (26.9%) high-risk cases for aneuploidy. Overall, 133 trisomies were diagnosed (100 trisomy 21; 18 trisomy 18; 15 trisomy 13). The cell-free DNA screen positive rate was lower in the low-risk vs the high-risk group (0.27% vs 2.2%; $P<.0001$). The sensitivity and specificity were similar between the groups. The positive predictive value for the low- and high-risk groups was 85.7% vs 97.5%; $P=.058$ for trisomy 21; 50.0% vs 81.3%; $P=.283$ for trisomy 18; and 62.5% vs 83.3; $P=.58$ for trisomy 13, respectively. Overall, 602 (3.4%) patients had no-call result after the first draw and 287 (1.61%) after including cases with a second draw. The trisomy rate was higher in the 287 cases with no-call results than patients with a result on a first draw (2.8% vs 0.7%; $P=.001$). The updated algorithm showed similar sensitivity and specificity to the study algorithm with a lower no-call rate.

CONCLUSION: In women at a low risk for aneuploidy, single-nucleotide-polymorphism-based cell-free DNA has high sensitivity and specificity, positive predictive value of 85.7% for trisomy 21 and 74.3% for the 3 common trisomies. Patients who receive a no-call result are at an increased risk of aneuploidy and require additional investigation.

Key words: aneuploidy, cell-free DNA, prenatal screening, trisomy

Introduction

Noninvasive prenatal testing using cell-free DNA (cfDNA) to screen for fetal chromosomal aneuploidy has seen rapid uptake since 2011.[1,2] It was demonstrated to have high sensitivity and specificity[3,4] and be superior to standard maternal serum analyte-based screening. Currently, most professional societies recommend cfDNA as an option for primary aneuploidy screening.[4–8]

Despite this, the routine offer of cfDNA screening to all patients has not been uniformly adopted. Cost, loss of benefits associated with ultrasound-based screening, and limitations of existing studies in particular are a concern. In addition, some providers may feel that a benefit of primary cfDNA screening over contingency screening in low-risk patients has not been clearly demonstrated. Initial validation studies using genetic confirmation were conducted on small cohorts of pregnancies at a high previous risk for aneuploidy.[9,10] Conversely, studies on large cohorts that included all-risk populations have been limited by a lack of genetic confirmation.[11–13] This left some doubt as to whether there was underreporting of trisomies and whether the measurement of sensitivity and positive predictive value, particularly in women at a low risk of aneuploidy, was accurate enough.[2–4,12,13] In addition, previous studies have generally excluded cases with a noninterpretable ("no-call") result, leaving questions about how this impacts the overall test performance.[4,14]

The Single-nucleotide-polymorphism-based Microdeletion and Aneuploidy RegistTry (SMART) was a large prospective study designed to evaluate cfDNA performance for the 22q11.2 deletion syndrome and the common trisomies (trisomy 21 [T21], trisomy 18 [T18], and trisomy 13 [T13]) in a

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AJOG at a Glance

Why was this study conducted?

There are limited data on the performance of cell-free DNA (cfDNA) screening for aneuploidy in low-risk populations.

Key findings

In women at low previous risk for aneuploidy, cfDNA has high sensitivity and specificity and a positive predictive value of 85.7% for trisomy 21 and of 74% for trisomies 21, 18, and 13 combined. Patients who receive a failed (no-call) result are at an increased risk of aneuploidy. An updated algorithm has a lower no-call rate while maintaining performance.

What does this add to what is known?

This is the first study to assess cfDNA screening performance using genetic confirmation in a prospective obstetrical population. It adds valuable information on test performance in women at a low risk for aneuploidy and in cases with failed cfDNA tests.

general referral population. A unique aspect of the SMART study was the confirmatory genetic testing requested in all cases through cytogenetic or cytogenomic analysis of fetal samples or chromosome microarray analysis (CMA) of newborn DNA samples, including analysis of cases with no-call cfDNA results. Here we report the results of the SMART study for the prenatal detection of T21, T18, and T13 in women at low vs high previous risk for aneuploidy.

Materials and Methods

Study design and participants

We enrolled pregnant women undergoing cfDNA screening for aneuploidy and 22q11.2DS at 21 centers in 6 countries (Supplement #1). The study was approved by each site's institutional review board or ethics committee, and all the participants provided written consent. Eligible women who requested and underwent screening for aneuploidy and 22q11.2 deletion syndrome were ≥18 years old, ≥9 weeks' gestation, had a singleton pregnancy, and planned to deliver at a study site-affiliated hospital. Women were excluded if they received a cfDNA result before enrollment, had a history of organ transplantation, conceived using ovum donation, had a vanishing twin, or were unwilling or unable to provide a newborn sample. Women who had a serum screening result for aneuploidy or sonographic detection of fetal anomalies were eligible for inclusion. Women were considered to be at a high risk for aneuploidy if they had a previous positive serum-based (first trimester combined or second trimester triple or quadruple) screen for aneuploidy, fetal nuchal translucency (NT) ≥3.0 mm, an ultrasound-detected anomaly before enrollment, or if the maternal age was ≥35 years at delivery and no other screening results (eg, serum) were available. The participants did not receive remuneration for enrolling. The results of cfDNA screening were utilized by the providers and patients as part of clinical care.

Outcomes

The primary outcome was the test performance of single nucleotide polymorphism (SNP)-based cfDNA for detecting T21, T18, and T13 in participants with a low previous risk for aneuploidy than those at a high risk. The secondary outcomes included the rates of trisomies in cfDNA no-call cases and the test performance of an updated algorithm that was made available after enrollment completion.

Procedures

The sample preparation and analysis of cfDNA were performed as previously described (Natera Inc, Austin, TX).[15] Noninvasive prenatal testing results indicating a risk of ≥1/100 for a trisomy were categorized as high-risk and those <1/100 were categorized as low-risk. In cases that did not yield a result, the patients were offered repeat testing and results after a second draw were included for analysis. During enrollment, the cfDNA laboratory protocol was modified once[10]; the results from both the periods were combined for analysis (original algorithm).

Independent of the study, the laboratory developed an updated algorithm optimized to improve the no-call rate at a low fetal fraction using a deep neural network, which utilizes an artificial intelligence approach. A deep learning (Tensorflow v1.15 [Google Inc., Mountain View, CA])[16] approach was used to optimally model noise using a deep mixture-of-experts neural network with multiple independent networks, combining the results into a probability score. This self-supervised algorithm leveraged 1.6 million sequenced mixtures of mother and fetus cfDNA samples, learning to harness linkage among the SNPs to make high-confidence calls for a larger proportion of samples. Deeper sequencing of high-risk calls was applied to lower false positive rates. This updated protocol was assessed after enrollment completion and was blinded to the outcomes.

The genetic outcomes were assessed by CMA through the analysis of DNA from fetal (chorionic villus sampling, amniocentesis, or products of conception) or infant (cord blood, buccal swab, or newborn blood spot obtained for state newborn screening) samples. Postnatal confirmatory samples were obtained at the end of pregnancy in all the cases regardless of the availability of previous prenatal diagnostic genetic testing.

CMA was performed by an independent laboratory (Center for Applied Genomics, Children's Hospital of Philadelphia, Philadelphia, PA) and was blind to the clinical findings and cfDNA results. For CMA analysis, the DNA was prepared from cord blood, buccal smear, or a dried blood spot. Copy number variants were identified using the Illumina (San Diego, CA) SNP-based Infinium Global Screening Array (GSA)

platform. The samples were genotyped on standard versions GSA-V1.0, GSA-V2.0, GSAMD-V1.0, or GSAMD-V2.0, which contain >700,000 SNPs from chromosome 1 to 22 or a custom-designed SMARTArray in which additional SNPs were added to the GSA backbone. In addition, positive samples underwent confirmation on the Omni 2.5-8V1-3 array and were reviewed by a clinical molecular cytogeneticist before results were generated.

If a postnatal sample for CMA confirmation was not available, results from pre- or postnatal clinical testing with karyotype, quantitative fluorescent polymerase chain reaction (QF-PCR), fluorescence in situ hybridization (FISH), or CMA were used for genetic confirmation, if available.

The cases with mosaicism were considered affected if >80% of cells were trisomic on confirmatory testing. Mosaicism identified only by chorionic villus sampling (CVS) was not considered as confirmation of genetic outcome. The study steering committee reviewed any discordance between the confirmatory tests blinded to the clinical outcome to adjudicate how the results should be interpreted and included in the analysis.

The neonatal DNA samples were mostly obtained in the form of dry blood spots from the States' health departments; they were collected as a part of neonatal screening programs. For quality assurance, a concordance test was developed and designed to confirm that cfDNA results and newborn samples were correctly paired using alignment between SNPs in the 2 samples; any samples that could not be paired were excluded.

Data collection

Research coordinators at each site recorded clinical data using a secured computerized tracking system developed and managed by the Data Coordinating Center at The Biostatistics Center at the George Washington University, Washington, DC. We collected patient and obstetrical data, imaging reports, and aneuploidy serum screening and prenatal diagnosis results. In addition, information on pregnancy complications; genetic testing or ultrasound findings; and newborn features suggestive of genetic abnormality, major malformations, and other adverse outcomes was collected after delivery.

Study oversight

The study was a collaboration between the clinical investigators and the sponsor (Natera, Inc, Austin, TX). The first and last authors designed the protocol with the sponsor and had a majority vote in study design and data interpretation. All the laboratory analyses were blinded to the outcome data. The clinical and laboratory results were managed by the Data Coordinating Center, which independently matched the deidentified information and analyzed the results only after the pregnancy outcomes were available and testing was complete.

Statistical analysis

The trisomy analysis was a secondary analysis, and the sample size was calculated on the basis of confidence intervals for the 22q11.2 deletion syndrome, with a prevalence range of 1 per 1000 to 1 per 5000. This was more than adequate to assess the detection of T21, with an expected prevalence of 1 per 425, and it would provide a reasonable assessment of the detection rates of T18 (prevalence of 1/1000) and T13 (prevalence of 1/3000). The sensitivity, specificity, positive likelihood ratio, and positive and negative predictive values of cfDNA results were assessed in the entire cohort and within the risk groups. When appropriate, exact (Clopper–Pearson) 95% confidence intervals (CIs) were reported. Low- and high-risk groups were compared for test performance using the Fisher's exact test. Participants without genetic confirmation were excluded from the analysis. The SAS Studio 9.04 software (SAS Institute, Cary, NC) was used for analysis. The MedCalc software was used to calculate the CIs for the positive likelihood ratios.[17] Continuous variables were compared using the Wilcoxon test and categorical variables were compared using the chi-square or Fisher exact test. The McNemar test was used for paired analyses.

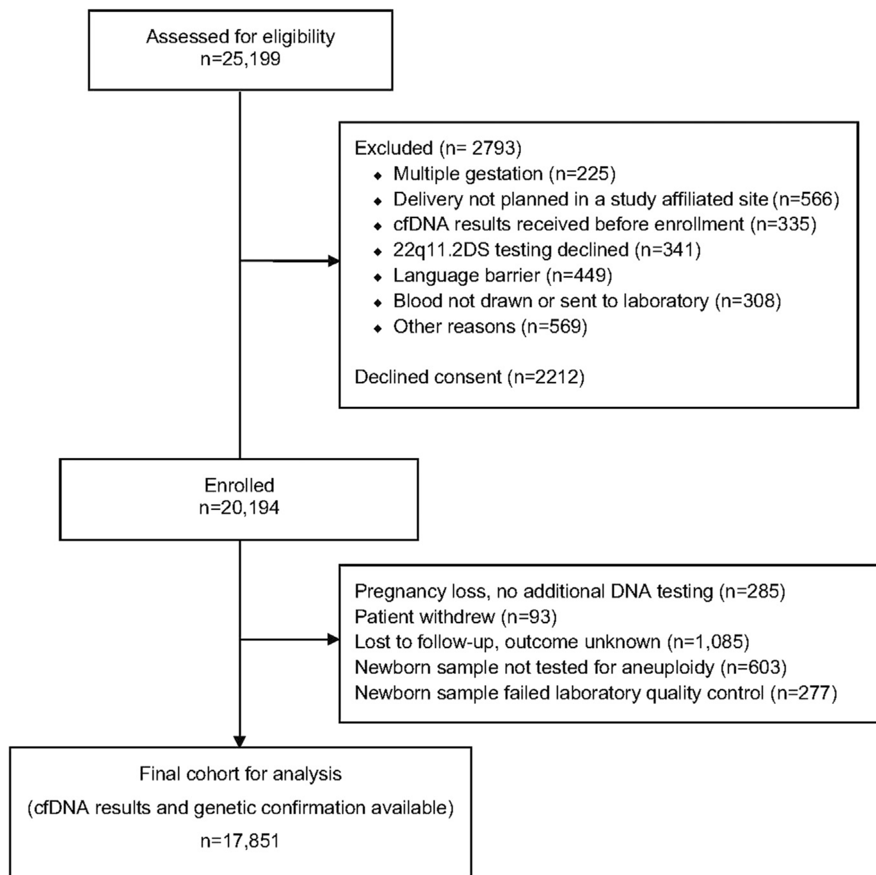
Results

Study participants

A total of 25,199 pregnant individuals were assessed for eligibility, and 20,194 (80.1%) were enrolled (Figure); 56.6% were enrolled in the US and 43.4% in Europe or Australia. Of the enrolled participants, 285 (1.4%) had pregnancy loss without genetic confirmation, 93 (0.5%) withdrew consent, 1085 (5.4%) were lost to follow-up; in 603 (3.0%), a sample for genetic confirmation of aneuploidy was not obtained, and in 277 (1.4%) the confirmation test failed laboratory quality control. The latter group included 48 cases in which the neonatal sample could not be genetically paired with a cfDNA sample. After all exclusions, the study cohort included 17,851 (88.4%) women for whom both cfDNA results and DNA analysis of the fetus or newborn were available.

The baseline characteristics of the entire study cohort stratified by risk groups are outlined in Table 1. The median maternal age was 34.3 years (interquartile range [IQR], 30.2–37.4), and the median gestational age was 12.6 weeks (IQR, 11.4–13.9). A total of 13,043 cases (73.1%) were considered low-risk for aneuploidy, including 3,873 that were ≥35 years old but had a low-risk result on a screening test before enrollment. The remaining 4808 (26.9%) were categorized as high-risk (Table 1). Most high-risk women (4010, 83.4%) were ≥35 years old with no previous serum screening; 616 (12.8%) were high-risk on the basis of the results of traditional serum analyte-based screening, 112 (2.3%) had cfDNA screening following the detection of a fetal abnormality on ultrasound, and 101 (2.1%) had a cystic hygroma or a NT ≥3 mm. Participants at a high risk for aneuploidy were enrolled at an earlier gestational age, were more likely to be enrolled in Europe, and were more likely to have conceived using in vitro fertilization. Compared with non-US participants, the US participants were younger (median 32.6 vs 35.9; *P*<.0001), had a higher median body mass index (BMI) (26.1 vs 24.1; *P*<.0001), and enrolled at a later mean gestational age (13.7 week vs 12.8 week; *P*<.0001).

FIGURE
Patient enrollment flowchart



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Primary and secondary outcomes

Among the 17,851 pregnancies in the primary analysis population, 133 (0.8%) targeted chromosomal abnormalities were identified in the cohort as follows: 100 T21 (1 in 179), 18 T18 (1 in 992), and 15 T13 (1 in 1195). In most cases (17,533, 98.2%), the genetic outcome was confirmed after birth: 17,548 (98.3%) by postnatal CMA, 28 (0.16%) by neonatal karyotype, and 2 (0.01%) by placental karyotype. Of the remaining 288 cases, in 232 (1.3%), confirmation was done by prenatal diagnostic testing and in 56 (0.3%) it was confirmed from miscarriages or termination specimens. Four cases with mosaicism, all < 80% trisomic, were identified in the cohort, including 3 T21 cases and 1 T18 case. (Supplemental Table 1). These 4 cases were considered as unaffected for the purpose of the analysis.

The cfDNA results were reported as high risk for aneuploidy in 138 patients (0.77%); 123 (89.1%) of these were confirmed whereas 15 (10.8%) were false positive results (n=5 T21, n=6 T18 and n=4 T13). There were 2 false negative results (0.01%): 1 T21 and 1 T18. Test performance for the entire cohort, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and positive likelihood ratios, are represented in Table 2.

Overall, 29/133 (21.8%) trisomies were in the group with a low previous risk for aneuploidy, whereas 104/133 (78.1%) were in high-risk cases (P<.001). The cfDNA screen positive rate was lower in the low-risk group than the high-risk group (0.27% vs 2.2%; P<.0001). The performance of cfDNA screening for the different trisomies in both risk groups is presented in Table 3.

The groups were similar for sensitivity and specificity. The PPV for all 3 trisomies was 74.3% (26/35) in the low-risk cohort and 94.2% (97/103) in the high-risk cohort (P=.003). The PPVs for the individual trisomies among low vs high-risk cases were 85.7% vs 97.5% (P=.06) for T21, 50.0% vs 81.2% (P=.28) for T18, and 62.5% vs 83.3%, (P=.58) for T13, respectively. Within the low-risk group, the PPV for T21 was 81.8% (95% CI, 59.0–100) in women ≥35 years old with a low previous risk screen and 90.0% (95% CI, 71.4–100) in women <35 years old (Table 3).

In 602 (3.4%) women, cfDNA did not yield a result after a first draw and 10 (1.66%) of these had a trisomy. These included 5/15 (33.3%) T13s, 2/18 (11.1%) T18s, and 3/100 (3%) T21s. In this group, the mean BMI was higher (31.3 vs 26.2; P<.001) and the median fetal fraction was lower (4.5% vs 9.4%; P<.001) than those who received a result after the first draw. Of the 427 women who attempted a second draw, 112 (26.2%) participants remained without a result and 2 (1.8%) of these had a trisomy, T13 in both cases, comprising a total of 1.5% of all trisomy cases. The rate of trisomy in the 287 patients with failed results after a first or second draw was higher than those with a result (2.8% vs 0.7%; P=.001). The no-call rates were similar between the high- and low-risk patients after the first draw (3.8% vs 3.2%, respectively; P=.051) and after 2 draws (1.7% vs 1.6%, respectively; P=.717).

Of the enrolled participants (n=20,237), 352 (1.7%) had pregnancy loss either before 20 weeks (201, 1.0%) or a later fetal or neonatal demise (151, 0.7%). Of those, 27 (7.7%) had a high-risk cfDNA result for a trisomy (13 T21, 10 T18, and 4 T13). Genetic confirmation was available for 108/352 (30.7%), and these cases were included in the analysis cohort. Of the 108 with genetic confirmation, 18 (16.7%) had a trisomy (7 cases with T21, 9 T18, and 2 T13).

The overall performance of the updated algorithm was similar to that of the original algorithm in the entire cohort (Supplemental Table 2) and in the different risk groups (Supplemental Table 3). In the 4808 patients with a

TABLE 1
Patient and gestational characteristics in the entire cohort and low- and high-risk groups

Variable	Full cohort (n=17,851)	Low risk (n=13,043)	High risk (n=4808)	*P* value Low vs high risk
Maternal and gestational characteristics				
Median maternal age (IQR) - y	34.3 (30.2–37.4)	32.5 (28.8–35.7)	37.6 (35.8–39.7)	<.001
Nulliparity	7876 (44.2)	6283 (48.2)	1593 (33.4)	<.001
Median BMI (kg/m^2) (IQR)	25.0 (22.3–29.1)	25.0 (22.3–29.3)	25.0 (22.4–28.8)	.699
Race/ethnicity				<.001
Asian	1532 (8.6)	1260 (9.7)	272 (5.7)	
Black	1569 (8.8)	1300 (10.0)	269 (5.6)	
White	10,811 (60.6)	7283 (55.8)	3528 (73.4)	
Hispanic	3331 (18.7)	2704 (20.7)	627 (13.0)	
Other/unknown	608 (3.4)	496 (3.8)	112 (2.3)	
Median gestational age at enrollment (IQR)—wk	12.6 (11.4–13.9)	12.7 (11.9–14.0)	11.7 (10.4–13.6)	<.001
Pregnancy through assisted reproductive technology	904 (5.1)	582 (4.5)	323 (6.7)	<.001
Current smoker	314 (1.8)	257 (2.0)	57 (1.2)	<.001
Enrollment site				<.001
United States	10,105 (56.6)	8345 (64.0)	1760 (36.6)	
Europe	7331 (41.1)	4401 (33.7)	2930 (60.9)	
Australia	415 (2.3)	297 (2.3)	118 (2.5)	
Prenatal screening and testing				
Positive first trimester screen before cfDNA testing	509 (2.9)		509 (10.6)	
NT>3 mm before cfDNA testing	101 (0.9)		101 (2.1)	
Positive second trimester before cfDNA testing	107 (0.6)		107 (2.2)	
Major anomaly before cfDNA testing	112 (0.6%)		112 (2.3%)	
No call - %	287 (1.6)	207 (1.6)	80 (1.7)	.717
Mean cfDNA fetal fraction (SD)	9.9 (4.1)	9.9 (4.1)	9.7 (4.2)	<.001
Diagnostic testing (CVS and amniocentesis)—%	544 (3.1%)	283 (2.2)	261 (5.4)	<.001
Any trisomy (T13, 18, 21)	133 (0.8%)	29 (0.2)	104 (2.2)	<.001
Pregnancy and delivery outcome				
Delivery outcome				<.001
Miscarriage	49 (0.3%)	15 (0.1)	34 (0.7)	
Elective abortion	159 (0.9%)	64 (0.5)	95 (2.0)	
Live birth	17,600 (98.7%)	12,935 (99.3)	4665 (97.1)	
Stillbirth	30 (0.2%)	19 (0.2)	11 (0.2)	
Neonatal death	29 (0.2%)	16 (0.1)	13 (0.3)	.036
Median gestational age at delivery (IQR) - wk	39.4 (38.4–40.3)	39.4 (38.6–40.3)	39.3 (38.3–40.1)	<.001
PTB<34 wk	459 (2.6%)	262 (2.0)	197 (4.1)	<.001
Preeclampsia	711 (4.1%)	519 (4.1)	192 (4.1)	.846
Small for gestational age	1546 (8.9%)	1158 (9.1)	388 (8.3)	.135
Mean birthweight (SD) g	3353 (555)	3347 (544)	3371 (586)	<.001
Apgar 1 < 7	797 (5.1%)	587 (4.9)	210 (6.2)	.002

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(continued)

TABLE 1
Patient and gestational characteristics in the entire cohort and low- and high-risk groups *(continued)*

Variable	Full cohort (n=17,851)	Low risk (n=13,043)	High risk (n=4808)	*P* value Low vs high risk
Apgar 5 < 7	154 (1.0%)	106 (0.9)	48 (1.4)	.006
Median days to newborn discharge (IQR)—d	2.0 (2.0—3.0)	2.0 (2.0—3.0)	3.0 (2.0—4.0)	<.001

BMI, body mass index; *cfDNA*, cell-free DNA; *CVS*, chorionic villus sampling; *IQR*, interquartile range; *NT*, nuchal translucency; *PTB*, preterm birth; *SD*, standard deviation.

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priori risk for aneuploidy, there were 104 trisomies (2.16%), and 100/104 (96.1%) were detected by cfDNA. In the 13,043 low-risk patients, 29 (0.22%) had a trisomy and 27/29 (93.1%) were detected. The no-call rate was lower with the updated algorithm than the original protocol (1.4% vs 3.4% after the first draw and 0.5% vs 1.6% after an optional second draw; $P<.001$). In the group with a no-call result on the first blood draw, there were 5 trisomies (2.0%) including 1 of 100 (1.0%) T21, 3 of 15 (20.0%) T13s, and 1 of 18 (5.6%) T18s. In the group with 2 sequential no-call results (N=28), there were 2 (7.1%), both cases of T13.

Discussion

Summary of the key findings

In a large prospective cohort with genetic confirmation, we found that SNP-based cfDNA has high sensitivity, specificity, and PPV for the common trisomies in women who are at a high or low risk for aneuploidy. The findings in this study are in broad agreement with previous reports that have shown high sensitivity and specificity of cfDNA screening for T21, T18, and T13 in women of all risk categories.[4,12,13] Because the PPV depends on disease prevalence, it is expected to be lower in the group of women with a lower previous risk. In this study, the PPVs for T21, T18, and T13 for the low previous risk cohort were 85.7%, 50.0%, and 62.5%, respectively. Although the PPV for the low-risk group was somewhat lower than the PPV reported in those with a previous high risk (97.5%, 81.2%, and 83.3%, respectively), these differences were not statistically significant, possibly because of a small sample size. Norton et al reported a PPV of 76% for T21 in women under 35 years and 50% for those with low-risk first trimester screening, with the ascertainment of aneuploidy done mainly through clinical assessment.[4] Zhang et al[13] reported a PPV of 81.3% in women at a low risk for T21 in a large prospective cohort, although confirmation was only available for 76.7% of cases in their report. Although the PPV of > 85% for T21 and at least 50% for T18 and T13 are lower in both low- and high-risk patients, they are substantially higher than the PPV associated with other accepted conventional aneuploidy screening tests,[18] further supporting the recommendation that patients with positive cfDNA results should be followed with confirmatory testing, regardless of their previous risk status.[6,7] Although all of the positive likelihood ratios were high, it is important to note that cfDNA should not be considered diagnostic because of confined placental mosaicism and other potentially unexpected chromosomal anomalies.

Approximately 3.4% of the cfDNA tests in the cohort did not yield a result after a first draw, and 1.6% after an

TABLE 2
Cell-free DNA test performance to screen for trisomies 21, 18, and 13

Variable	T21	T18	T13	T21/18/13
	Full cohort (n=17,564)			
Sensitivity	97/98 98.98% (96.99—100)[a]	16/17 94.12% (82.93—100)	10/10 100% (69.15—100)	123/125 98.40% (96.20—100)
Specificity	17,461/17,466 99.97% (99.95—100)	17,541/17,547 99.97% (99.94—99.99)	17,550/17,554 99.98% (99.96—100)	17,424/17,439 99.91% (99.87—99.96)
PPV	97/102 95.10% (90.91—99.29)	16/22 72.73% (54.12—91.34)	10/14 71.43% (47.76—95.09)	123/138 89.13% (83.94—94.32)
NPV	17,461/17,462 99.99% (99.98—100)	17,541/17,542 99.99% (99.98—100)	17,550/17,550 100% (99.98—100)	17,424/17,426 99.99% (99.97—100)
Likelihood ratio (+)[b]	3458 (1439—8308)	2752 (1226—6180)	4388 (1647—11,692)	1144 (689—1898)

NPV, negative predictive value; *PPV*, positive predictive value; *T*, trisomy.

[a] 95% Confidence interval; [b] Likelihood ratio (+) — Se/(100-Sp).

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TABLE 3
Comparison of cell-free DNA test performance for trisomies 21, 18, and 13 between women at a low and high risk for aneuploidy[a]

Variable	High risk[b] n=4728	Low risk n=12,836	*P* value (High vs low risk)	Low risk ≥35 n=3803	Low risk <35 n=9033
Trisomy 21 (n=98)					
Sensitivity	98.8[c] 79/80 (96.3–100)	100 18/18 (81.5–100)	1.00	100 9/9 (66.37–100)	100 9/9 (66.37–100)
Specificity	99.96 4646/4648 (99.90–100)	99.98 12,815/12,818 (99.95–100)	.61	99.95 3792/3794 (99.89–100)	99.99 9023/9024 (99.97–100)
PPV	97.53 79/81 (94.15–100)	85.71 18/21 (70.75–100)	.06	81.82 9/11 (59.03–100)	90.00 9/10 (71.41–100)
NPV	99.98 4646/4647 (99.94–100)	100 12,815/12,815 (99.97–100)	.27	100 3792/3792 (99.90–100)	100 9023/9023 (99.96–100)
Prevalence	1.69[c]	0.14		0.24	0.10
Likelihood ratio (+)[d]	2295 (574–9176)	4273 (1378–13246)		1897 (475–7582)	9024 (1271–64,057)
Trisomy 18 (n=17)					
Sensitivity	100 13/13 (75.3–100)	75.0 3/4 (32.6–100)	.24	100 2/2 (15.81–100)	50.00 1/ 2 (1.26–98.74)
Specificity	99.94 4712/4715 (99.86–100)	99.98 12,829/12,832 (99.95–100)	.20	99.95 3799/3801 (99.89–100)	99.99 9030/9031 (99.97–100)
PPV	81.25 13/16 (62.13–100)	50.00 3/6 (10.00–90.01)	.28	50.00 2/4 (6.76–93.24)	50.00 1/ 2 (1.26–98.74)
NPV	100 4712/4712 (99.92–100)	99.99 12,829/12,830 (99.98–100)	1.00	100 3799/3799 (99.90–100)	99.99 9030/9031 (99.97–100)
Prevalence	0.27	0.03		0.05	0.02
Likelihood ratio (+)	1572 (507–4971)	3208 (905–11,367)		1900 (475–7596)	4516 (409–49,796)
Trisomy 13 (n=10)					
Sensitivity	100 5/5 (47.8–100)	100 5/5 (47.8–100)	1.00	100 1/1 (2.50–100)	100 4/4 (39.76–100)
Specificity	99.98 4722/4723 (99.94–100)	99.98 12,828/12,831 (99.95–100)	1.00	99.97 3801/3802 (99.93–100)	99.98 9027/9029 (99.95–100)
PPV	83.3 5/6 (53.51–100)	62.50 5/8 (28.95–96.05)	.58	50.00 1/ 2 (1.26–98.74)	66.67 4/6 (28.95–100)
NPV	100 4722/4722 (99.92–100)	100 12,828/12,828 (99.97–100)	1.00	100 3801/3801 (99.92–100)	100 9027/9027 (99.96–100)
Prevalence	0.11	0.04		0.03	0.04

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(continued)

TABLE 3
Comparison of cell-free DNA test performance for trisomies 21, 18, and 13 between women at a low and high risk for aneuploidy[a] *(continued)*

Variable	High risk[b] n=4728	Low risk n=12,836	*P* value (High vs low risk)	Low risk ≥35 n=3803	Low risk <35 n=9033
Likelihood ratio (+)	4723 (665–33,523)	4227 (1380–13,260)		3802 (536–26,985)	4515 (1129–18,048)
Trisomy 21,18, & 13 (n=125)					
Sensitivity	99.0 97/98 (96.9–100)	96.3 26/27 (89.2–100)	.39	100 12/12 (73.54–100)	93.33 14/15 (80.71–100)
Specificity	99.87 4624/ 4630 (99.77–99.97)	99.93 12,800/12,809 (99.88–99.98)	.25	99.87 3786/3791 (99.79–99.99)	99.96 9014/9018 (99.91–100)
PPV	94.17 97/103 (89.65–98.70)	74.29 26/35 (59.81–88.77)	<.01	70.59 12/17 (48.93–99.25)	77.78 14/18 (58.57–100)
NPV	99.98 4624/4625 (99.94–100)	99.99 12,800/12,801 (99.98–100)	.46	100 3786/3786 (99.92–100)	99.99 9014/9015 (99.97–100)
Prevalence	2.07	0.21		0.32	0.17
Likelihood ratio (+)	763 (343–1700)	1371 (710–2644)		758 (316–1821)	2104 (783–5658)

NPV, negative predictive value; *PPV*, positive predictive value.

[a] Excluding no-call results; [b] Women were considered as high-risk for aneuploidy if they had a previous positive serum-based (first trimester combined or second trimester triple or quadruple) screen for aneuploidy, fetal nuchal translucency ≥3.0 mm, an ultrasound-detected anomaly before enrollment, or if the maternal age was ≥35 years at delivery and no other screening results (eg, serum) were available; [c] percent; [d] Likelihood ratio (+) - Se/(100-Sp).

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optional second draw was included, a rate that is comparable to previous studies.[3] The inability of cfDNA to make a high-confidence call relates most commonly to a low proportion of cfDNA of fetal origin, or "fetal fraction,"[19] which is associated with early gestational age and high maternal weight.[20,21] Previous studies have also demonstrated an association between a low fetal fraction and trisomies 13 and 18, which may be explained by a small placenta leading to reduced fetal DNA in the maternal plasma.[22,23] Data suggesting an increased risk for fetal trisomy 21 among low fetal fraction cases have been conflicting.[4,24] In this study, over 7.5% of the trisomies were in the no-call group and there was a 3- to 4-fold increase in the aneuploidy risk. The increased risk, particularly after 2 failed draws, was mainly attributable to T13 and to a lesser extent to T18, whereas for T21, the association did not seem to be clinically significant. It is still important to note that although the no-call rate was not higher in the T21 group, 2 cases of T21 did have no-call results after the first draw.

Clinical and research implications

Two trisomy cases, 1 T21 and 1 T18, were reported as negative by cfDNA. Both were subsequently identified at 19 weeks after the sonographic detection of growth restriction and ventricular septal defect in the T18 fetus and ventriculomegaly and unbalanced atrioventricular septal defect in the T21 fetus. Although our findings confirm that false positive and negative results, particularly for T21, are uncommon, their presence clearly indicates that cfDNA is a screening and not a diagnostic test. Pre- and post-test patient education are therefore important, and patients with a negative cfDNA result should be aware of the possibility of false negative results. The focus of this study is the performance of cfDNA screening for trisomies. However, patients should be aware that though other aneuploidies and microdeletions or duplications are individually rare, they are more common than the common trisomies in aggregate, and

options for prenatal detection, primarily through diagnostic testing, are available. Therefore, those who have a sonographic finding of a structural anomaly should be offered diagnostic testing despite a previous negative cfDNA result.

Technology continued to evolve during the 38 months of patient enrollment, and the laboratory developed an updated algorithm near the end of the project; we assessed this updated algorithm after enrollment was completed. Although sensitivity and specificity were comparable to the original algorithm, the no-call rate was significantly lower. Nevertheless, though the total number of trisomies in the no-call group was also lower, the proportion of these trisomies among the no-call cases increased, reinforcing the recommendation to follow up no-call reports with an additional evaluation.[7]

Strengths and limitations

The main strength of this study is the application of genetic analysis to confirm outcome in a large prospective cohort undergoing prenatal screening. The requirement for confirmatory genetic testing in all cases assured a complete and accurate assessment of screening performance in all risk groups and outcome of cases with no-call results. Nevertheless, this study is not without limitations. The study was designed to include an unselected population, but with a median maternal age of 34.3 years, the cohort was somewhat older than expected[25] and likely indicates the use of cfDNA screening in a higher risk population. Consequently, the prevalence of aneuploidy was also higher than expected.[4,26] Again, this difference likely represents a referral bias associated with recommendations of professional societies at the time of enrollment to only offer cfDNA to women at a high risk. It is also possible that there was a higher demand for cfDNA, which may be viewed as an alternative to diagnostic testing by older women. Despite the overall older maternal age, subgroup analyses enabled us to assess the test performance for each of the prior risk subgroups. The inability to obtain confirmatory samples after the spontaneous loss of fetuses, likely disproportionately aneuploid, represents an unavoidable biologic limitation to any assessment of prenatal screening. The lack of genetic confirmation in the 1.4% of cases because of fetal loss or neonatal demise may result in an underestimation of the actual prevalence of chromosomal abnormalities in the cohort. The rate of trisomy was 16.7% in those cases of fetal or neonatal demise that did have genetic confirmation, which is consistent with evidence that common trisomies continue to play a significant role in pregnancy loss after 10 weeks' gestation.[27,28] Finally, the results of this study may not apply to all patients or be generalizable to all cfDNA laboratories, as the exclusion criteria and cfDNA analysis techniques differ.

Conclusions

The findings in this study demonstrate that SNP-based cfDNA screening for the common trisomies performs similarly well in both high- and low-risk groups. Although technological advancements have decreased the rate of no-call results, such cases are at an increased risk of specific aneuploidies and require additional investigation. The data from this study will be helpful to patients and providers when considering their prenatal screening and diagnostic testing options. ■

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B.J. reports research clinical diagnostic trials with Ariosa (completed), Vanadis (completed), Natera (ongoing), and Hologic (completed), with institutional expenditures reimbursed per patient and no personal reimbursements. He also reports clinical probiotic studies with products provided by FukoPharma (ongoing, no funding) and BioGaia (ongoing; also provided a research grant for the specific study), coordination of scientific conferences and meetings with commercial partners as such as ESPBC 2016 and a Nordic educational meeting about noninvasive prenatal testing and preeclampsia screening. B.J. and Y.C. collaborated in the IMPACT study where Roche, Perkin Elmer, and Thermo Fisher provide reagents to placental growth factor analyses. R.J.W. receives research funding from the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development and receives support from Illumina for research reagents. M.N. is a consultant to Invitae. All the other authors report no conflict of interest.

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The trial was registered on ClinicalTrials.gov with identifier NCT02381457, Single-nucleotide-polymorphism-based Microdeletion and Aneuploidy RegisTry (SMART).

Data sharing: Data sharing requests should be submitted to the corresponding author (P.D.) for consideration. The requests will be considered by the study publication committee. The study protocol and statistical analysis plan will be available on request. Individual patient data will not be available. Access to deidentified data may be granted following submission of a written proposal and a signed data sharing agreement. Files will be shared using a secure file transfer protocol.

Ethical approval: This study was designed in compliance with an investigational-review-board-approved protocol (Ethical and Independent Review Services Study ID, 17113; date of certification, August 28, 2017, date of renewal August 20, 2020). Written informed consent was obtained from all the study participants.

This study was presented as an oral presentation at the 41st annual meeting of the Society of Maternal and Fetal Medicine, held virtually, January 25—30, 2021.

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Supplemental information

Methods

Procedures

Sample preparation and analysis of cell-free DNA (cfDNA) were performed as previously described (Natera Inc, Austin, TX).[14] Noninvasive prenatal testing (NIPT) results indicating a risk of ≥1/100 for a trisomy were categorized as high risk and those at <1/100 as low risk. In the cases that did not yield a result, the patients were offered repeat testing, and results after a second draw were included for analysis. During enrollment, the cfDNA laboratory protocol was modified once[15]; the results from both periods are combined for analysis (original algorithm).

Independent of the study, the laboratory developed an updated algorithm optimized to improve the no-call rate at a low fetal fraction using a deep neural network component, which utilizes an artificial intelligence approach. A deep learning (Tensorflow v1.15) approach was used to optimally model noise using a deep mixture-of-experts neural network with multiple independent networks, combining the results into a probability score. This self-supervised algorithm leveraged 1.6 million sequenced mixtures of mother and fetus cfDNA samples, learning to harness linkage among the single-nucleotide-polymorphisms (SNPs) to make high-confidence calls for a larger proportion of samples. Deeper sequencing of high-risk calls was applied to lower false positive rates. This updated protocol was assessed after enrollment completion and was blinded to the outcomes.

The genetic outcomes were assessed by chromosome microarray analysis (CMA) through analysis of DNA from fetal (chorionic villus sampling, amniocentesis, or products of conception) or infant (cord blood, buccal swab, or newborn blood spot obtained for state newborn screening) samples. Postnatal confirmatory samples were obtained at the end of the pregnancy in all cases, regardless of the availability of previous prenatal diagnostic genetic testing.

CMA was performed by an independent laboratory (Center for Applied Genomics, Children's Hospital of Philadelphia, Philadelphia, PA) and was blind to clinical findings and cfDNA results. For CMA analysis, DNA was prepared from cord blood, buccal smear, or a dried blood spot. The copy number variants were identified using the Illumina (San Diego, CA) SNP-based Infinium GSA platform. Samples were genotyped on standard versions GSA-V1.0, GSA-V2.0, GSAMD-V1.0, or GSAMD-V2.0 that contain >700,000 SNP from chromosome 1−22 or a custom-designed SMARTArray in which additional SNPs were added to the GSA backbone. In addition, positive samples underwent confirmation on the Omni 2.5-8V1-3 array and were reviewed by a clinical molecular cytogeneticist before generated results.

If a postnatal sample for CMA confirmation was not available, results from pre or postnatal clinical testing with karyotype, quantitative fluorescent polymerase chain reaction (QF-PCR), FISH or CMA were used for genetic confirmation, if available.

Cases with mosaicism were considered affected if >80% of cells were trisomic on confirmatory testing. Mosaicism identified only by CVS was not considered as confirmation of genetic outcome. The study steering committee reviewed any discordance between confirmatory tests, blinded to the clinical outcome, to adjudicate how results should be interpreted and included in the analysis.

For quality assurance purposes, a concordance test was developed to confirm that cfDNA results and newborn samples were correctly paired using alignment between SNPs in the 2 samples; any samples that could not be paired were excluded.

Study design and participants

Full information on study dates, including enrollment and completion are provided on ClinicalTrials.gov, Identifier NCT02381457. The relevant dates are as follows: periods of recruitment: April 8, 2015 to December 12, 2019; follow-up: April 8, 2015 to July 18, 2019; data collection: April 8, 2015 to September 18, 2019.

This study involved 21 locations including the following: University of California San Francisco, San Francisco, California, United States; Cooper University Hospital, Camden, New Jersey, United States; Virtua, Mount Laurel, New Jersey, United States; St. Peter's University, New Brunswick, New Jersey, United States; Complete Women's Healthcare, Garden City, New York, United States; North Shore University Hospital, Manhasset, New York, United States; Madonna Perinatal, Mineola, New York, United States; Long Island Jewish Medical Center New Hyde Park, New York, United States; New York University, New York, New York, United States; Icahn School of Medicine Mt Sinai, New York, New York, United States; Columbia University, New York, New York, United States; Montefiore Medical Center, New York, New York, United States; Suffolk OB, Port Jefferson, New York, United States; North Austin Maternal Fetal Medicine, Austin, Texas, United States; Zeid Women's Health Center, Longview, Texas, United States; University of Utah, Salt Lake City, Utah, United States; Royal Prince Alfred, Camperdown, New South Wales, Australia; Royal College Surgeons in Ireland, Dublin, Ireland, 1; Dexeus, Barcelona, Spain; Sahlgrenska University Hospital, Gothenburg, Sweden; St. George University Hospital, London, United Kingdom.

This multicenter prospective observational study enrolled pregnant women who presented clinically at or after 9 weeks gestation and elected Panorama microdeletion and aneuploidy screening as part of their routine care. The primary objective was to evaluate the performance of SNP-based NIPT for 22q11.2 microdeletion in a large cohort of pregnant women. Data collection began at enrollment and continued until patients delivered and their child was discharged from the hospital. Biospecimens were obtained from infants after birth to perform genetic diagnostic testing for 22q11.2 deletion. The results from the follow-up specimens were compared with those obtained by the Panorama screening test to determine test performance. In the event a newborn sample could not be obtained before discharge from the hospital, the participants were mailed a saliva buccal swab kit for testing at home. The samples were then shipped to Natera for testing.

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SUPPLEMENTAL TABLE 1

Outcome of cases confirmed as mosaicism

Case	Cytogenetics (pre- or postnatal)	Array (pre- or postnatal)	Clinical outcome	Original Alg T13	Original Alg T18	Original Alg T21	Updated Alg T13	Updated Alg T18	Updated Alg T21
1	Prenatal amnio: M21/Ti21 50%/50% Postnatal: • Direct buccal FISH: T21/M21 50%/50% • Karyotype blood: T21 • Karyotype skin T21/M21 50%/50%	Prenatal amnio: Mosaic T21 (30%) Postnatal: Mosaicism with majority T21.	DORV diagnosed prenatally. Neonatal demise.	No call	No call	No call	Low risk	Low risk	Low risk
2	Prenatal amnio: M18p/T18q 50%/50%	none	Pregnancy Termination	Low risk	High risk	Low risk	Low risk	High risk	Low risk
3	Prenatal CVS: T21 Mosaicism Prenatal amnio FISH: 37% T21	Prenatal: 70% T21	Pregnancy Termination	No call	No call	No call	Low risk	Low risk	Low risk
4	Prenatal amnio: 30% T21	none	Pregnancy Termination	Low risk	Low risk	High risk	Low risk	Low risk	High risk

Alg, algorithm; *Amnio*, Amniocentesis; *CVS*, Chorionic Villus Sampling; *DORV*, double outlet right ventricle; *FISH*, fluorescence in situ hybridization.

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SUPPLEMENTAL TABLE 2

Cell-free DNA test performance to screen for trisomies 21, 18, and 13 with the updated algorithm

	T21	T18	T13	T21/18/13
Updated algorithm	Full cohort (n=17,737)			
Sensitivity	99/100 99.00% (97.05–100)[a]	16/17 94.12% (82.93–100)	12/12 100% (73.54–100)	127/129 98.45% (96.32–100)
Specificity	17,630/17,637 99.96% (99.93–99.99)	17,716/17,720 99.98% (99.96–100)	17,722/17,725 99.98% (99.96–100)	17,594/17,608 99.92% (99.88–99.96)
PPV	99/106 93.40% (88.67–98.12)	16/20 80.00% (62.47–97.53)	12/15 80.00% (59.76–100)	127/141 90.07% (85.13–95.01)
NPV	17,630/17,631 99.99% (99.98–100)	17,716/17,717 99.99% (99.98–100)	17,722/17,722 100% (99.98–100)	17,594/17,596 99.99% (99.97–100)

NPV, negative predictive value; *PPV*, positive predictive value.

[a] 95% confidence interval.

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SUPPLEMENTAL TABLE 3

Cell-free DNA test performance to screen for trisomies 21, 18, and 13 using the updated algorithm for the entire cohort and by risk groups

Variable Updated algorithm	T21	T18	T13	T21	T18	T13
	Low risk (n=12,967)			High risk (n=4770)		
Sensitivity	100[a] 18/18 (81.47—100)[b]	75.00 3/4 (32.57—100)	100 6/6 (54.07—100)	98.78 81/82 (96.40—100)	100 13/13 (75.29—100)	100 6/6 (54.07—100)
Specificity	99.97 12,945/12,949 (99.94—100)	99.98 12,961/12,963 (99.96—100)	99.98 12,958/12,961 (99.95—100)	99.94 4685/4688 (99.87—100)	99.96 4755/4757 (99.90—100)	100 4764/4764 (99.92—100)
PPV	81.82 18/22 (65.70—97.94)	60.00 3/5 (17.06—100)	66.67 6/9 (35.87—97.46)	96.43 81/84 (92.46—100)	86.67 13/15 (69.46—100)	100 6/6 (54.07—100)
NPV	100 12,945/12,945 (99.97—100)	99.99 12,961/12,962 (99.98—100)	100 12,958/12,958 (99.97—100)	99.98 4685/4686 (99.94—100)	100 4755/4755 (99.92—100)	100 4764/4764 (99.92—100)

NPV, negative predictive value; *PPV*, positive predictive value.

[a] Percent; [b] 95% confidence interval.

Dar et al. Performance of cell-free DNA screening for aneuploidy in low-risk pregnancies. Am J Obstet Gynecol 2022.