OBSTETRICS

Noninvasive prenatal screening for fetal trisomies 21, 18, 13 and the common sex chromosome aneuploidies from maternal blood using massively parallel genomic sequencing of DNA

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OBJECTIVE: The objective of this study was to validate the clinical performance of massively parallel genomic sequencing of cell-free deoxyribonucleic acid contained in specimens from pregnant women at high risk for fetal aneuploidy to test fetuses for trisomies 21, 18, and 13; fetal sex; and the common sex chromosome aneuploidies (45, X; 47, XXX; 47, XXY; 47, XYY).

STUDY DESIGN: This was a prospective multicenter observational study of pregnant women at high risk for fetal aneuploidy who had made the decision to pursue invasive testing for prenatal diagnosis. Massively parallel single-read multiplexed sequencing of cell-free deoxyribonucleic acid was performed in maternal blood for aneuploidy detection. Data analysis was completed using sequence reads unique to the chromosomes of interest.

RESULTS: A total of 3430 patients were analyzed for demographic characteristics and medical history. There were 137 fetuses with trisomy 21, 39 with trisomy 18, and 16 with trisomy 13 for a prevalence rate of the common autosomal trisomies of 5.8%. There were no false-negative results for trisomy 21, 3 for trisomy 18, and 2 for trisomy 13; all 3 false-positive results were for trisomy 21. The positive predictive values for trisomies 18 and 13 were 100% and 97.9% for trisomy 21. A total of 8.6% of the pregnancies were 21 weeks or beyond; there were no aneuploid fetuses in this group. All 15 of the common sex chromosome aneuploidies in this population were identified, although there were 11 false-positive results for 45,X. Taken together, the positive predictive value for the sex chromosome aneuploidies was 48.4% and the negative predictive value was 100%.

CONCLUSION: Our prospective study demonstrates that noninvasive prenatal analysis of cell-free deoxyribonucleic acid from maternal plasma is an accurate advanced screening test with extremely high sensitivity and specificity for trisomy 21 (>99%) but with less sensitivity for trisomies 18 and 13. Despite high sensitivity, there was modest positive predictive value for the small number of common sex chromosome aneuploidies because of their very low prevalence rate.

Key words: cell-free deoxyribonucleic acid, massively parallel genomic sequencing, noninvasive prenatal screening

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Retrieving diagnostic information about the fetus through noninvasive technology has been an elusive goal for clinicians interested in prenatal diagnosis. Although fetal cell traffic in the maternal circulation has been known since the 1950s, success at retrieving such cells including the nucleated fetal erythrocyte has met with limited success.[1]

The discovery of cell-free fetal deoxyribonucleic acid in the maternal plasma in 1997 opened up a new frontier in the quest for a noninvasive prenatal screening strategy.[2] In the last decade, a number of investigators have reported successful diagnoses of fetal sex,[3] Rhesus D status,[4] monogenic disorders,[5,6] and fetal aneuploidy using a variety of laboratory

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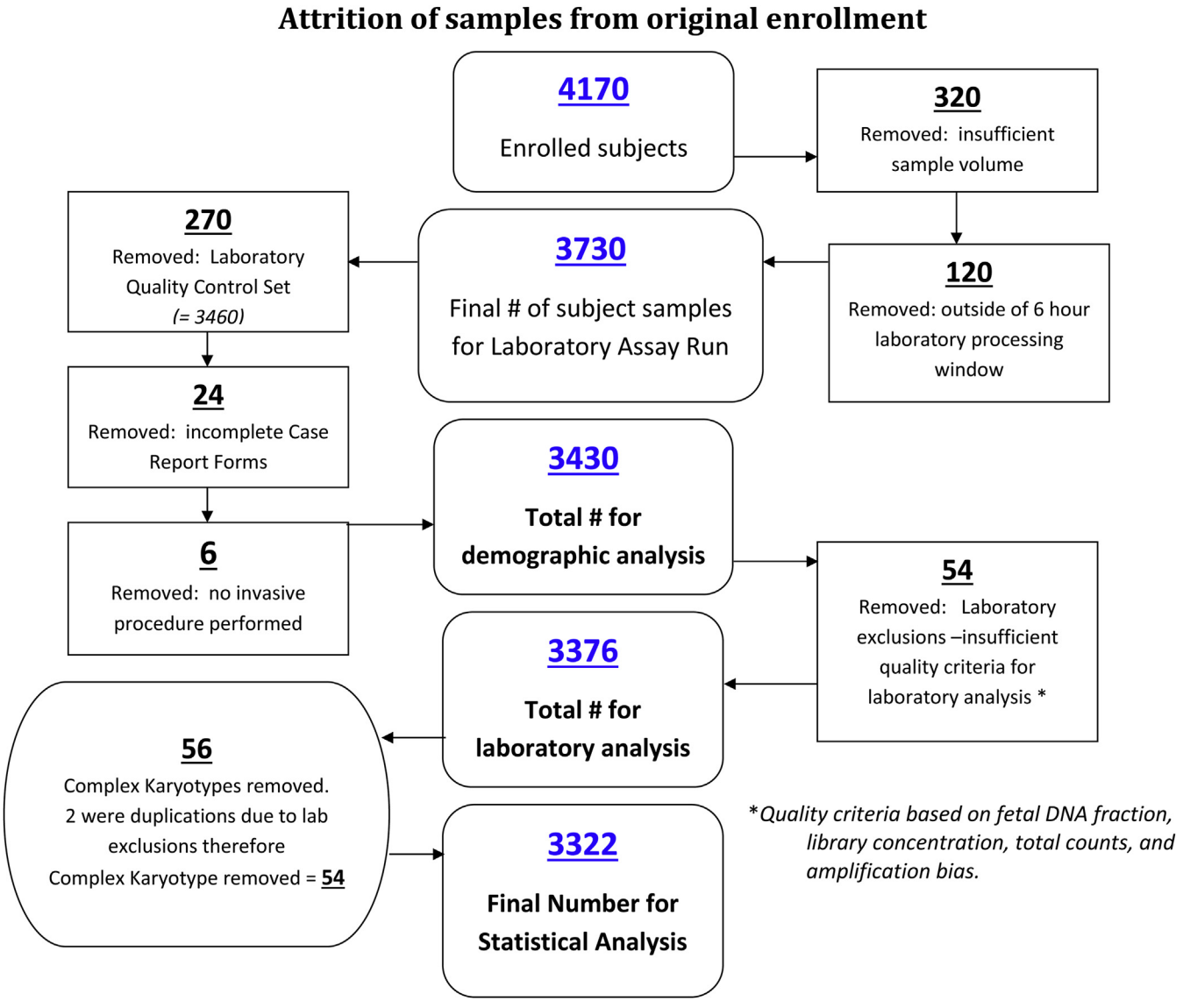
FIGURE 1
Flow diagram showing the process by which patient samples were included for analysis of the common autosomal trisomies (21, 18, 13)



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techniques to retrieve and analyze nucleic acid sequences found in the maternal circulation.[7-9] Currently the DNA sequencing-based approach fulfills the promise of reliably bringing noninvasive screening to the patient.[10]

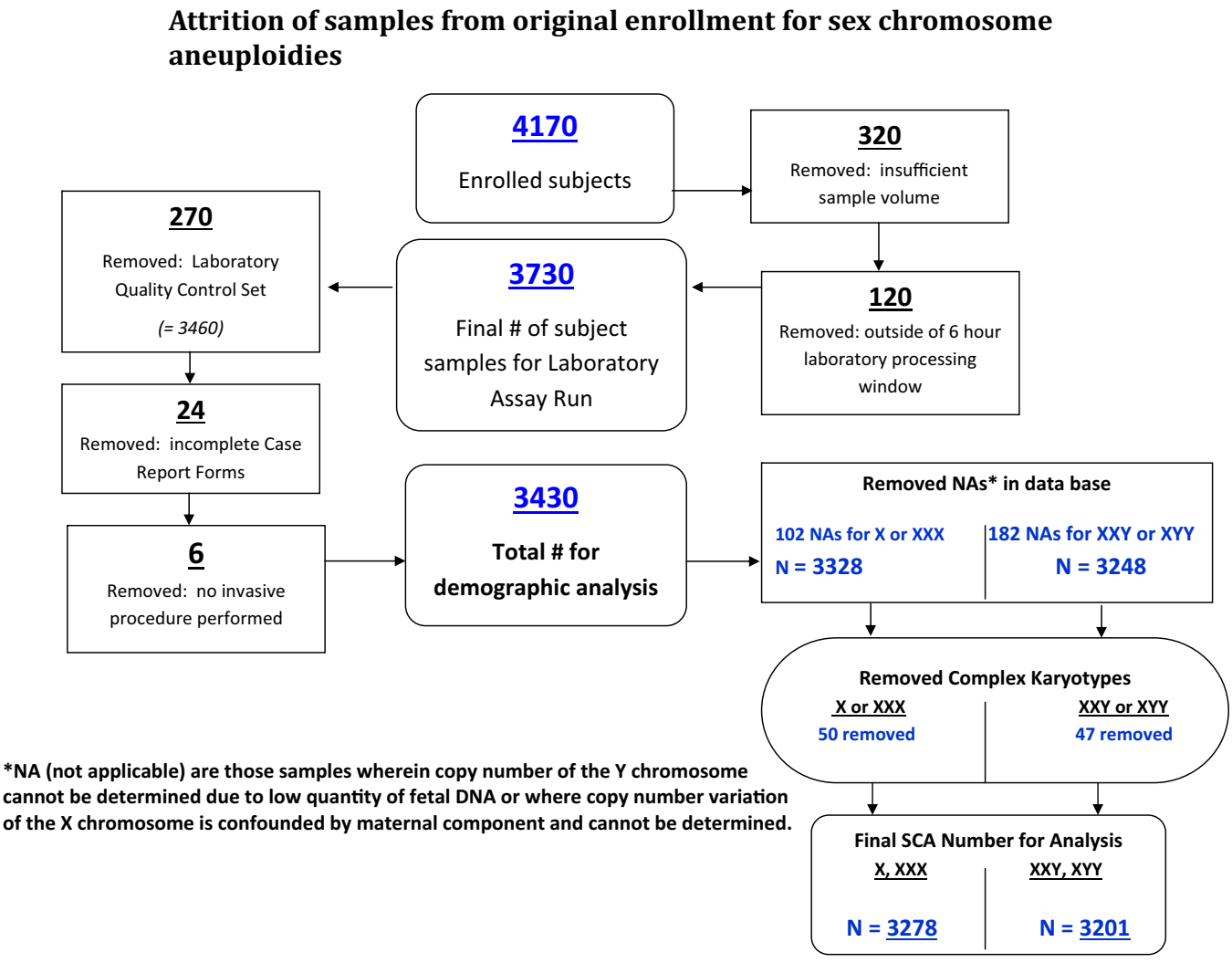
The objective of this study was to evaluate the contemporary use of noninvasive screening as it is currently offered to patients at high risk for fetal aneuploidy. We collected blood samples from women who were pursuing prenatal diagnosis by chorionic villus sampling (CVS) or amniocentesis (AC). These samples were used to validate the clinical performance of massively parallel genomic sequencing (MPS) of cell-free deoxyribonucleic acid (cfDNA) contained in the specimens for the testing of fetuses for trisomies 21, 18, or 13. All samples meeting laboratory analytical criteria also were analyzed for fetal sex and the common sex chromosome aneuploidies (45, X; 47, XXX; 47, XXY; 47, XYY).

Materials and Methods

Clinical methodology

This is a prospective multicenter observational study of the application of a laboratory developed test. Women at 31 clinical sites judged by their attending physicians to be at high risk for fetal aneuploidy, who met the inclusion criteria of the study protocol and who had made the decision to pursue CVS or AC were offered enrollment into the study. Blood samples (20-30 mL drawn into

FIGURE 2
Flow diagram showing the process by which patient samples were included for analysis of the common sex chromosome aneuplodies



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2 or 3 ethylenediaminetetraacetic acid purple/lavender—top tubes) were drawn before the procedure in all instances.

The study participant was considered enrolled and assigned a bar-coded participant identification number after the informed consent was signed but before the sample was collected. The samples were immediately placed on wet ice and transferred to a standard refrigerator at the collection site where they were stored at a temperature between 0°C and 8°C. Within 6 hours all samples were transferred in a chill pack (at approximately 4°C) to a locally contracted laboratory for further processing to plasma. Samples were maintained at a central storage facility at −70°C or colder until transferred to the study sponsor for testing by MPS.

The results of the samples analyzed by the study sponsor were stored separately from the original karyotype results and both secured in password-protected databases. Karyotypes from the AC or CVS specimens were completed at independent commercial laboratories and the results reported to the patients and their providers.

Inclusion criteria were singleton pregnancy in a patient 18 years of age or older who had provided written informed consent and who had made the decision to pursue invasive prenatal diagnosis by CVS or AC. The participant was further judged to be at increased risk for fetal aneuploidy for 1 or more of the following conditions: maternal age 35 years or older at the estimated date of delivery, screen positive on first- or second-trimester serum biochemical screening tests, the presence of a fetal abnormality on ultrasound, or a personal or family history of a chromosomal abnormality.

Exclusion criteria were the inability to give written informed consent, multiple gestation, or fetal demise of an additional

TABLE 1
Baseline characteristics and medical history

Characteristic	AC (n = 2590)	CVS (n = 840)	Total (n = 3430)
Maternal age (y), n (%)			
Mean (SD)	34.7 (5.8)	36.4 (4.9)	35.1 (5.6)
Median	36.0	38.0	36.0
Minimum, maxmum	18.0, 50.0	19.0, 47.0	18.0, 50.0
Maternal age category (y), n (%)			
<35	982 (37.9)	249 (29.6)	1231 (35.9)
≥35	1608 (62.1)	591 (70.4)	2199 (64.1)
Maternal weight (lbs), n (%)			
Mean (SD)	158.2 (37.7)	153.0 (33.3)	156.9 (36.7)
Median	150.0	147.0	149.0
Minimum, maxmum	95.0, 404.0	90.0, 366.0	90.0, 404.0
Maternal height (in), n (%)			
Mean (SD)	64.5 (2.8)	65.0 (2.7)	64.7 (2.8)
Median	64.0	65.0	64.0
Minimum, maxmum	55.0, 74.0	58.0, 73.0	55.0, 74.0
Maternal body mass index (kg/m^2), n (%)			
Mean (SD)	26.7 (5.9)	25.4 (5.2)	26.4 (5.8)
Median	25.3	24.2	25.0
Minimum, maxmum	15.1, 63.3	16.5, 64.8	15.1, 64.8
Maternal race category, n (%)			
American Indian or Alaska Native	25 (1.0)	2 (0.2)	27 (0.8)
Ashkenazi Jewish	4 (0.2)	0 (0.0)	4 (0.1)
Asian	544 (21.0)	98 (11.7)	642 (18.7)
Black/African American	133 (5.1)	23 (2.7)	156 (4.5)
Hispanic or Latino	280 (10.8)	60 (7.1)	340 (9.9)
Multiple	130 (5.0)	45 (5.4)	175 (5.1)
Native Hawaiian or other Pacific Islander	22 (0.8)	3 (0.4)	25 (0.7)
Not reported	0 (0.0)	1 (0.1)	1 (0.0)
White	1452 (56.1)	608 (72.4)	2060 (60.1)
Parity, n (%)			
0	841 (32.5)	287 (34.2)	1128 (32.9)
1	1061 (41.0)	330 (39.3)	1391 (40.6)
2	440 (17.0)	153 (18.2)	593 (17.3)
3	162 (6.3)	45 (5.4)	207 (6.0)
>3	85 (3.3)	25 (3.0)	110 (3.2)
Gestational age, n (%)			
Mean (SD)	17.8 (2.8)	12.0 (1.1)	16.3 (3.5)
Median	17.0	12.0	16.0
Minimum, maxmum	9.0, 37.0	9.0, 24.0	9.0, 37.0

(continued)

embryo during the current pregnancy at 8 weeks or farther in gestation. Gestational age was determined by a reliable menstrual history unless it differed from composite ultrasound measurement dating by more than 7 days, in which case the latter would be used to assign a gestational age.

Maternal demographic data and karyotype results were entered into password-protected databases and maintained by the Obstetrix Medical Group. All databases including study sponsor MPS test results, maternal demographic data, and karyotype results were stored separately until test performance was assessed by an independent biostatistician.

An oversight committee composed of 3 knowledgeable individuals uninvolved in any aspect of the study reviewed study subject enrollment and the prevalence rate of trisomy 21 at predetermined intervals to recommend the final sample size with the goal of identifying sufficient numbers of trisomy 21 fetuses (approximately 80-160); otherwise, the oversight committee was blinded to all study participant test results. A case prevalence rate of up to 4% was anticipated at the outset based on historical data at some of the participating sites. It was estimated that this would translate to a sample size of approximately 4000 patients.

This study was approved by the Western Institutional Review Board (WIRB protocol 20090261) or local institutional review boards. It was deemed a minimal-risk protocol because simple venipuncture is considered to be noninvasive, and the volume of blood drawn was 30 mL or less (CFR Part 812.3[k]).[11] This study was registered with clinicaltrials.gov (identifier NCT00847990).

Laboratory methodology

All samples were collected and processed under the same protocol. A 10 mL aliquot of maternal whole blood was drawn into an ethylenediaminetetraacetic acid-K2 spray-dried Vacutainer (Becton Dickinson, Franklin Lakes, NJ), stored, and transported to the processing laboratory on wet ice. Within 6 hours of the blood draw, the maternal whole blood was centrifuged (Eppendorf 5810R plus swing-out rotor; Eppendorf AG,

Hamburg, Germany) chilled (4°C) at 2500 × *g* for 10 minutes and the plasma was collected. The plasma was centrifuged a second time (Eppendorf 5810R plus fixed-angle rotor) at 4°C at 15,000 × *g* for 10 minutes. After the second spin, the plasma was removed from the pellet and distributed into 4 mL plasma barcoded aliquots and immediately stored frozen at −70°C until the DNA extraction.[12]

The MPS for aneuploidy detection was completed following a previously published method.[13,14] The fetal fraction of the cfDNA was determined using a method relying on differentially methylated markers.[15] DNA extraction and library preparation also followed a previously published method,[13] and clustering and sequencing were performed using the HiSeq 2000 sequencers (Illumina Inc, San Diego, CA). Thirty-six cycles of single-read multiplexed sequencing (libraries pertaining to 12 samples including controls in each lane of a flow cell) were performed and image analysis and base calling were performed with the manufacturer-provided software (Illumina Inc). Sequences were aligned to the UCSC hg19 human reference genome using Bowtie version 2.

Data analysis was completed using sequence reads unique to the chromosomes of interest (21, 18, 13, X, Y) and then standardizing the fractional representation of each chromosome by comparison with a known euploid control group (z-scores).[13] The standardization of chromosome representation was carried out by using the median chromosome representation as computed over the samples from a given flow cell and using a previously established estimate of variability of this representation.

This calculation does not rely on a group of control euploid samples; this is carried out for each flow cell and accounts, implicitly, for flow cell to flow cell variability (this is the same approach taken in clinical practice). Z-scores at or above 3 were considered indicative of trisomy 21 (z-scores of at or above 3.95 were considered indicative of trisomies 18 and 13). The normalization procedure for the sex chromosome aneuploidies (SCA) as well as the classification algorithm was completed in accordance with a previously published report.[16]

All samples were required to meet quality control criteria. These included a minimum fetal fraction of 4.0% and a maximum fetal fraction of 50%; minimum fetal DNA per sample of 26 copies; minimum library concentration of 7.5 nmol; minimum number of autosomal-aligned reads of 9 million. Sequencing results that exhibited strong guanine-cytosine (GC) bias (as estimated from the shape of the counts per 50 kb bin vs GC content of each 50 kb bin) were rejected and the affected samples were reprocessed.

TABLE 1
Baseline characteristics and medical history *(continued)*

Characteristic	AC (n = 2590)	CVS (n = 840)	Total (n = 3430)
Gestational age category (wks), n (%)			
<12	0 (0.0)	284 (33.8)	284 (8.3)
≥12	2589 (100.0)	557 (66.2)	3146 (91.7)
Maternal bleeding during this pregnancy, n (%)			
No	2265 (87.5)	721 (85.8)	2986 (87.1)
Yes	325 (12.5)	119 (14.2)	444 (12.9)
Diabetes, n (%)			
GDM	54 (2.1)	14 (1.7)	68 (2.0)
Type 1, type 2	43 (1.6)	5 (0.6)	17 (0.5)
None	2491	821	3312
No information	2	0	2
Indications for invasive procedure, n (%)			
Abnormal NT	32 (1.2)	72 (8.6)	104 (3.0)
Abnormal triple/quad	447 (17.3)	45 (5.4)	492 (14.3)
Abnormal U/S	248 (9.6)	41 (4.9)	289 (8.4)
AMA	1044 (40.3)	373 (44.4)	1417 (41.3)
Elective	56 (2.2)	19 (2.3)	75 (2.2)
Multiple indications	691 (26.7)	238 (28.3)	929 (27.1)
Not reported	0 (0.0)	1 (0.1)	1 (0.0)
Previous Hx of Down syndrome	10 (0.4)	6 (0.7)	16 (0.5)
Previous Hx of other chromosomes	35 (1.4)	37 (4.4)	72 (2.1)
Previous or family Hx of heredity	7 (0.3)	3 (0.4)	10 (0.3)
Sequential screening	10 (0.4)	1 (0.1)	11 (0.3)
Other	10 (0.4)	4 (0.5)	14 (0.4)

Data are in raw numbers with percentages shown in parentheses.

AC, amniocentesis; *AMA*, advanced maternal age; *CVS*, chorionic villus sampling; *GDM*, gestation diabetes mellitus; *Hx*, history; *NT*, nuchal translucency; *U/S*, ultrasound.

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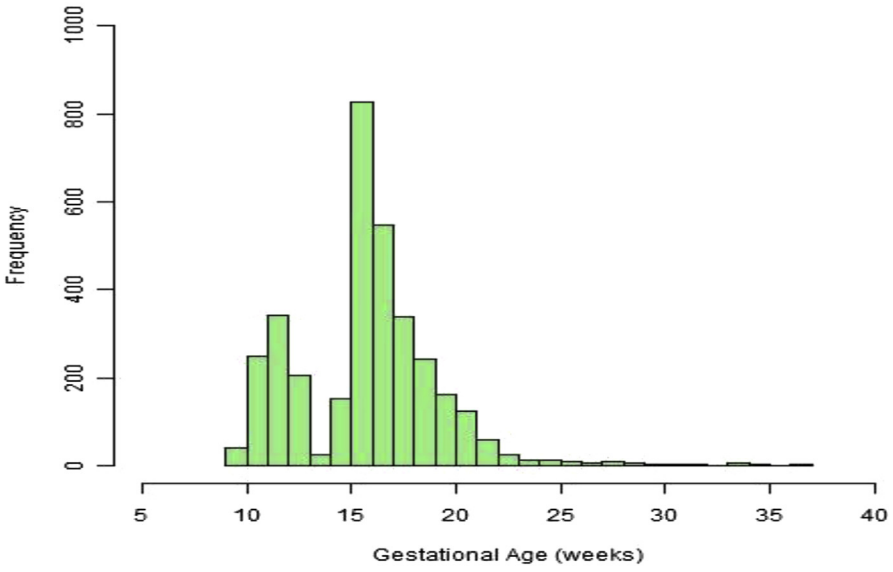
Discordant results resolution

A discordant result resolution plan was developed before the initiation of the sample testing by MPS. This process included investigation for transcription/clerical errors in the case report forms. Twenty-three database entry errors were identified and corrected by the principal investigator. These included 17 errors in fetal sex, 3 errors in complex

FIGURE 3
Histogram showing the stratification of patient samples by gestational age



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karyotypes, and 3 errors in aneuploidy from terminations of pregnancy. All had formal karyotype reports available to assure correct entries. Also included was a review of sample collection procedures, an analysis of secondary aliquots, maternal DNA assessment (in buffy coat), and clinical patient follow-up after the birth.

A set of 52 single-nucleotide polymorphisms was used to identify potential chain of custody events outside the collection sites or the analytical laboratory, which would be considered part of the standard workflow. Affected samples outside this standard workflow were removed from the analysis. There were 3 affected samples whose analyses demonstrated that sample identification was incorrect and they qualified for removal from the analysis. Two samples had a genetic mismatch between the buffy coat and the sequencing library; the third sample had a sequencing library matching a library from a different patient. Karyotypes and sequencing results were concordant once the correct patient samples were identified, but because the chain of custody was compromised, they were excluded from further analysis.

Statistical analysis

The statistics were calculated treating the invasive procedure results as the gold standard. The exact 95% Clopper-Pearson[17] confidence limits are shown alongside the binomial outcomes, which include sensitivity and specificity as well as positive and negative predictive values. Asymptotic 95% confidence limits, computed under a log transform, were also calculated. All statistics were generated using the frequency procedure, SAS/STAT software, version 9.3 of the SAS system (SAS Institute, Cary, NC). All

TABLE 2
Comparison with invasive procedures for trisomy 21 complex karyotypes excluded

Variable	AC (n = 2518)	CVS (n = 804)	Total (n = 3322)
Having T21			
Test positive for T21	100.0 (81/81)	100.0 (56/56)	100.0 (137/137)
Test negative for T21	0.0 (0/81)	0.0 (0/56)	0.0 (0/137)
Not having T21			
Test positive for T21	0.1 (3/2437)	0.0 (0/748)	0.1 (3/3185)
Test negative for	99.9 (2434/2437)	100.0 (748/748)	99.9 (3182/3185)
Sensitivity	100.0 (95.55–100.00)	100.0 (93.62–100.00)	100.0 (97.34–100.00)
Specificity	99.9 (99.64–99.97)	100.0 (99.51–100.00)	99.9 (99.72–99.98)
Positive predictive value	96.4 (89.92–99.26)	100.0 (93.62–100.00)	97.9 (93.87–99.56)
Negative predictive value	100.0 (99.85–100.00)	100.0 (99.51–100.00)	100.0 (99.88–100.00)

Data are in percentages with raw numbers shown in parentheses. Statistical analysis shows 95% confidence intervals in parentheses.

AC, amniocentesis; *CVS*, chorionic villus sampling; *T21*, trisomy 21.

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TABLE 3
Comparison with invasive procedures for trisomy 18 complex karyotypes excluded

Variable	AC (n = 2518)	CVS (n = 804)	Total (n = 3322)
Having T18			
Test positive for T18	92.0 (23/25)	92.9 (13/14)	92.3 (36/39)
Test negative for T18	8.0 (2/25)	7.1 (1/14)	7.7 (3/39)
Not having T18			
Test positive for T18	0.0 (0/2493)	0.0 (0/790)	0.0 (0/3283)
Test negative for T18	100.0 (2493/2493)	100.0 (790/790)	100.0 (3283/3283)
Sensitivity	92.0 (73.97–99.02)	92.9 (66.13–99.82)	92.3 (79.13–98.38)
Specificity	100.0 (99.85–100.00)	100.0 (99.53–100.00)	100.0 (99.89–100.00)
Positive predictive value	100.0 (85.18–100.00)	100.0 (75.29–100.00)	100.0 (90.26–100.00)
Negative predictive value	99.9 (99.71–99.99)	99.9 (99.30–100.00)	99.9 (99.73–99.98)

Data are in percentages with raw numbers shown in parentheses. Statistical analysis shows 95% confidence intervals in parentheses.

AC, amniocentesis; *CVS*, chorionic villus sampling; *T18*, trisomy 18.

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statistical analyses were done by an independent statistician.

Results

Figures 1 and 2 summarize the history of the enrolled patients. Enrollment began in September 2009 and was completed in April 2011. A total of 3430 patients were analyzed for demographic characteristics and medical history. After laboratory exclusions for quality control deviations, 3376 patients were available for autosomal trisomy comparisons. A further 56 samples were excluded as planned from the final analyses because their karyotypes were judged complex. Complex karyotypes were defined prior to the independent analysis and included all mosaic karyotypes, triploidies, and any unbalanced rearrangements with missing or duplicated genetic material (to be reported in a subsequent publication).

All samples were analyzed after enrollment was completed and before the independent statistical analysis. All final results also reflect exclusions of

TABLE 4
Comparison with invasive procedures for trisomy 13 complex karyotypes excluded

Variable	AC (n = 2518)	CVS (n = 804)	Total (n = 3322)
Having T13			
Test positive for T13	77.8 (7/9)	100.0 (7/7)	87.5 (14/16)
Test negative for T13	22.2 (2/9)	0.0 (0/7)	12.5 (2/16)
Not having T13			
Test positive for T13	0.0 (0/2509)	0.0 (0/797)	0.0 (0/3306)
Test negative for T13	100.0 (2509/2509)	100.0 (797/797)	100.0 (3306/3306)
Sensitivity	77.8 (39.99–97.19)	100.0 (59.04–100.00)	87.5 (61.65–98.45)
Specificity	100.0 (99.85–100.00)	100.0 (99.54–100.00)	100.0 (99.89–100.00)
Positive predictive value	100.0 (59.04–100.00)	100.0 (59.04–100.00)	100.0 (76.84–100.00)
Negative predictive value	99.9 (99.71–99.99)	100.0 (99.54–100.00)	99.9 (99.78–99.99)

Data are in percentages with raw numbers shown in parentheses. Statistical analysis shows 95% confidence intervals in parentheses.

AC, amniocentesis; *CVS*, chorionic villus sampling; *T13*, trisomy 13.

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TABLE 5
Summary of autosomal trisomy sequencing results

	Sequencing				
Variable	**Euploid**	**T21**	**T18**	**T13**	**Total**
Karyotype					
Euploid	3127	3	0	0	3130
T18	3	0	36	0	39
T21	0	137	0	0	137
T13	2	0	0	14	16
Total	3132	140	36	14	3322
	Sensitivity	Specificity	PPV	NPV	
T21	100.0%	99.9%	97.9%	100.0%	
T18	92.3%	100.0%	100.0%	99.9%	
T13	87.5%	100.0%	100.0%	99.9%	

NPV, negative predictive value; *PPV*, positive predictive value; *T13*, trisomy 13; *T18*, trisomy 18; *T21*, trisomy 21.

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patients after planned discordancy analyses were completed. Table 1 reviews the demographic characteristics and medical histories of the 3430 patients. We note that 64% of the patients were 35 years of age or older and 60% were white. The patients were generally of low parity, with only 9% para 3 or greater. Forty-one percent of the patients requested their invasive procedure for advanced maternal age alone and another 27% had multiple indications.

Overall, 75% of patients received an AC as the invasive procedure and 25% a CVS. Figure 3 depicts the bimodal gestational age distribution of the samples. A total of 8.6% of the patients were 21 weeks' gestational age or beyond; there were no aneuploid fetuses in this subset.

Tables 2-4 summarize the comparisons of the noninvasive sequencing analysis with the actual karyotypes for the autosomal trisomies, stratified by type of invasive procedure received. Complex karyotypes were excluded in these tables as well as in Tables 5-7. Overall, there were 137 fetuses with trisomy 21, 39 with trisomy 18, and 16 with trisomy 13 for a prevalence rate of the common autosomal trisomies of 5.8% (4.1% for trisomy 21).

The sensitivity for trisomy 21 was 100% (97.34, 100.00). There were 3 false-positive tests (0.1%) for trisomy 21, conferring greater than 99% (99.72, 99.98) specificity and a positive predictive value of 97.9%. There were 3 false-negative tests for trisomy 18 and 2 false-negative tests for trisomy 13; there were no false-positive tests for trisomies 18 or 13 (positive predictive value, 100%). The high negative predictive values for the autosomal trisomies reflect in part the low prevalence rate of the affected fetuses. Table 5 is a summary of the autosomal trisomy MPS results.

Only 1.6% of autosomal trisomy analyses received no MPS report because

TABLE 6
Comparison with invasive procedure for fetal sex complex karyotypes excluded

Variable	**AC (n = 2520)**	**CVS (n = 803)**	**Total (n = 3323)[a]**
Male by invasive procedure			
Male by laboratory assay	99.8 (1221/1223)	99.8 (413/414)	99.8 (1634/1637)
Female by laboratory assay	0.2 (2/1223)	0.2 (1/414)	0.2 (3/1637)
Female by invasive procedure			
Male by laboratory assay	0.2 (3/1297)	0.3 (1/388)	0.2 (4/1685)
Female by laboratory assay	99.8 (1294/1297)	99.7 (387/388)	99.8 (1681/1685)
Sensitivity	99.8 (99.41–99.98)	99.8 (98.66–99.99)	99.8 (99.47–99.96)
Specificity	99.8 (99.33–99.95)	99.7 (98.58–99.99)	99.8 (99.39–99.94)
Positive predictive value	99.8 (99.29–99.95)	99.8 (98.66–99.99)	99.8 (99.38–99.93)
Negative predictive value	99.8 (99.44–99.98)	99.7 (98.58–99.99)	99.8 (99.48–99.96)

Data are in percentages with raw numbers shown in parentheses. Statistical analysis shows 95% confidence intervals in parentheses.

AC, amniocentesis; *CVS*, chorionic villus sampling.

[a] Total reflects 54 excluded complex karyotypes (2 duplications with laboratory quality exclusions) and 53 prior laboratory exclusions for insufficient quality criteria for laboratory analysis. Quality criteria were based on fetal DNA fraction, library concentration, total counts, and amplification bias.

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TABLE 7
Comparison with invasive procedures for sex aneuploidy complex karyotypes excluded

Variable	X (n = 3278)[a]	XXX (n = 3278)[a]	XXY (n = 3201)[b]	XYY (n = 3201)[b]
Having sex aneuploidy				
Test positive for aneuploidy	100.0 (9/9)	100.0 (4/4)	100.0 (1/1)	100.0 (1/1)
Test negative for aneuploidy	0.0 (0/9)	0.0 (0/4)	0.0 (0/1)	0.0 (0/1)
Not having sex aneuploidy				
Test positive for aneuploidy	0.3 (11/3269)	0.1 (3/3274)	0.1 (2/3200)	0.0 (0/3200)
Test negative for aneuploidy	99.7 (3258/3269)	99.9 (3271/3274)	99.9 (3198/3200)	100.0 (3200/3200)
Sensitivity	100.0 (66.37–100.00)	100.0 (39.76–100.00)	100.0 (2.50–100.00)	100.0 (2.50–100.00)
Specificity	99.7 (99.40–99.83)	99.9 (99.73–99.98)	99.9 (99.77–99.99)	100.0 (99.88–100.00)
Positive predictive value	45.0 (23.06–68.47)	57.1 (18.41–90.10)	33.3 (0.84–90.57)	100.0 (2.50–100.00)
Negative predictive value	100.0 (99.89–100.00)	100.0 (99.89–100.00)	100.0 (99.88–100.00)	100.0 (99.88–100.00)

Data are in percentages with raw numbers shown in parentheses. Statistical analysis shows 95% confidence intervals in parentheses.

[a,b] Totals reflect excluded complex karyotypes ([a]50 females and [b]47 males) as well as prior laboratory exclusions for both the female and male samples (102 female and 182 male) because of sequencing aberrations on the X chromosome and the requirement of a higher fetal fraction for Y chromosome sequences resulting in a no-call determination[15] (Figure 2).

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of failure to meet laboratory guidelines. Figure 4 shows the expected relationship between fetal fraction and the Z-scores for chromosome 21.

Fetal sex prediction by the presence or absence of the Y chromosome sequences also showed sensitivity and specificity performance greater than 99% (Table 6). The SCAs are few in absolute number in this prospective data set (Table 7). Sensitivity was 100.0% (78.20, 100.00) (15 of 15) for all of the SCAs taken together. There were 11 false-positive results for 45, X (0.3%), suggesting that ultrasound findings may provide the proper context in interpreting sequencing data for fetuses affected with 45, X. There were 2 false-positive results for 47, XXY, 3 false-positive results for 47, XXX, and none for 47, XYY. The positive predictive value of the SCA taken together was 48.4% (30.15, 66.94) and the negative predictive value was 100% (99.88, 100.00).

Once again, the very high negative predictive values of the test for each of the common SCA reflects the very low prevalence rate of these abnormal karyotypes even among high-risk patients. Taken together (both female and male), laboratory quality guidelines precluded an MPS result in 8.3% of analyses for the SCA.

Comment

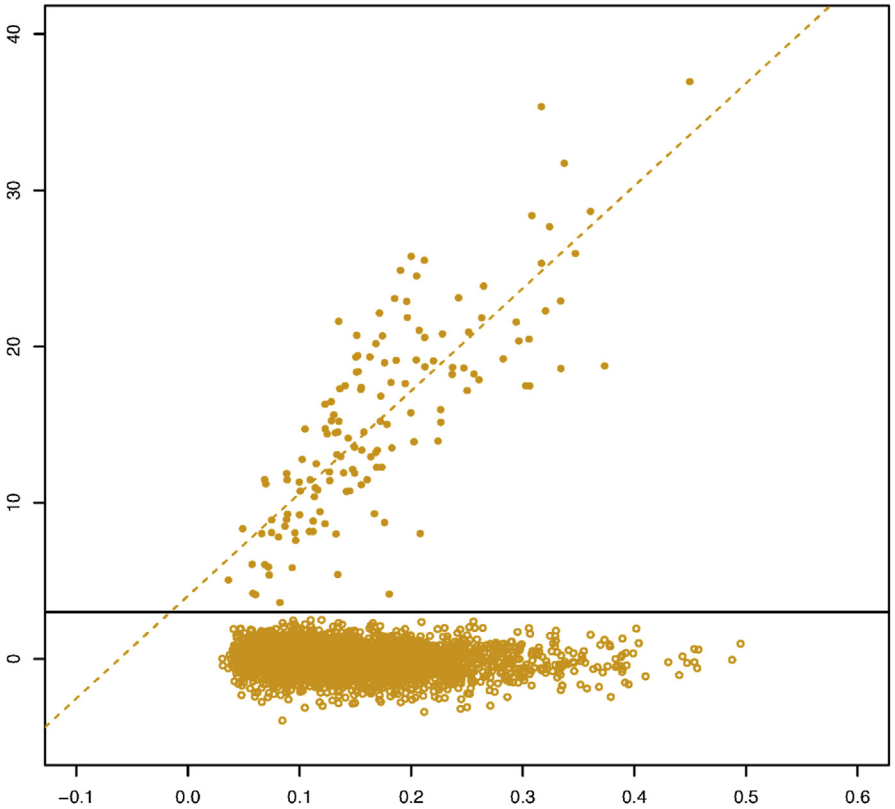
Our study provides the largest prospectively collected sample number concurrently processed and analyzed by this sequencing technology to date. It demonstrates that noninvasive prenatal analysis of cfDNA from maternal plasma is an accurate advanced screening test with extremely high sensitivity and specificity for trisomy 21 (>99%) but with somewhat less sensitivity (although high specificity) for trisomies 18 and 13. Moreover, this study is derived from a clinically based group of patients who elected invasive testing because they were either screen positive by contemporary screening standards or were simply of an advanced maternal age at delivery (the original screening test for fetal aneuploidy) and wanted information from the outset.

MPS was reported independently by 2 groups in 2008 and demonstrated that this technique was capable of identifying aneuploidy in the fetus from cfDNA[8,9] with very high accuracy. A number of follow-up studies using cfDNA sequencing have appeared recently, each adding some unique analytical or methodological nuance to improve the operational throughput of this complex laboratory procedure. Notably, Ehrich et al[12] reported tetra-plexing in 2011 and still achieved 100% negative predictive value and a single false-positive for trisomy 21 among 449 samples, which passed their quality control metrics.

The prevalence rate of trisomy 21 in this case-control study was 8.7%, somewhat higher than one would expect, even in a population of pregnant women at high risk for fetal aneuploidy. Later in 2011, Palomaki et al [18] published a large clinical validation study of trisomy 21 fetuses with excellent test performance, although there were 2 false-negative samples, even after the GC correction analysis was applied. The same investigators early the following year showed similar test performance characteristics for trisomies 18 and 13.[19] By study design, in their nested case-control validation study, the prevalence rate of trisomy 21 was 12.5%.

The prevalence rate of trisomy 21 in our study was 4.1%, in keeping with general clinical expectations in such patients. It is a major strength of this report. The performance of noninvasive prenatal screening in our setting mirrored that of smaller previous studies, especially noting that the false-negative rate remained very low (zero for trisomy 21) and negative predictive values quite high at greater

FIGURE 4
Graph of Z-scores (plotted on y-axis) vs fetal fraction (plotted on x-axis) for chromosome 21



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than 99%, an extremely valuable finding. The small number of false-positive results to achieve this result (≤0.1%) will be of little clinical consequence because all positive results should be confirmed by invasive testing because the test is not currently considered to be diagnostic. Moreover, in this risk cohort, all patients were already candidates for invasive diagnostic testing. No patient, therefore, would be in the position of having to consider an invasive test on the basis of noninvasive screening test results who was not already a traditional candidate for such procedures.

We also report positive predictive values for the various aneuploidies in these patients. Recent editorial opinions regarding the deficits in currently published data in this important area highlight the requirement for a large prospective study with proper attention to the comprehensive reporting of the findings.[20,21] We believe that the current report meets that requirement.

Discrepant or discordant results are onerous and merit some reflection. One of the false-positive test results for trisomy 21 occurred in a pregnancy wherein an early fetal demise of a twin was recognized. It is tempting to speculate that involuting placental tissue was the source of the excess chromosome 21 sequences, which were confirmed in a parallel uniplex sequencing analysis by the laboratory.[22]

The false-negative samples are more difficult to understand and raise the possibility of human error in the chain of custody process between the contracted laboratories to which the specimens were originally sent, the biostorage facility and the analytical laboratory. Two false-negative specimens (1 trisomy 18 and 1 trisomy 13) may have fallen subject to such an error in sample integrity because their extended uniplex sequencing[22] strongly suggested that euploid samples were actually analyzed. Of the 4 second-trimester false-negative test results for these 2 trisomies, all had ultrasound abnormalities that in most settings may have made them a priori candidates for invasive testing. There was no prospective study plan to adjust sample inclusion based on this type of retrospective analysis.

Because the karyotypes are the gold standard in this study, these patient samples are reported as false-positive (trisomy 21) and false-negative (trisomy 18 and trisomy 13) results in their respective tables.

Bianchi et al[23] in 2012 showed similar aneuploidy detection results using a different analytic technique but using massively parallel sequencing. They also reported test performance for 45, X. We showed excellent test performance in predicting sex in these fetuses, although the small number of sex chromosome aneuploidies detected in our population of high-risk patients precluded definitive conclusions. We showed high sensitivity (100%) but variable results in terms of positive predictive value. This was especially true for 45, X, in which maternal mosaicism may be a contributor to discordant noninvasive MPS results.[24]

Maternal chromosomal complement was not investigated in this study because it was not part of the informed consent procedure. The finding of false-positive noninvasive MPS results for monosomy X will require additional investigation to understand the contributing factors to this discordant observation. Other publications have appeared more recently using variations on the sequencing technique but limiting the regions sequenced for detection of trisomies 21 and 18.[25-27] All 3 common autosomal aneuploidies as well as X and Y have been identified using selective sequencing of polymorphic loci.[28]

The American College of Obstetrics and Gynecology and the Society of Maternal-Fetal Medicine issued a combined committee opinion in December 2012 summarizing the available

technology at the time and its possible utility in clinical practice.[29]

Surrogate marker screening among patients at high risk for fetal aneuploidy will have increasingly limited value for aneuploidy detection, although enlarged nuchal translucency in the first trimester may reflect additional embryopathy and requires further scrutiny later in gestation. Moreover, several biochemical markers obtained in the first and second trimester and used primarily for aneuploidy screening have been associated with perinatal complications later in pregnancy.[30,31] It is not known whether their continued use for that purpose alone has sufficient merit to support their place in the routine laboratory evaluation of pregnancy in contemporary practice.

This will be an especially important question once data are available regarding test performance of cfDNA screening among low-risk patients. The fetal fraction of cfDNA stratified by gestational age also may serve the purpose of alerting the clinician to an increased risk of perinatal complications.[32,33] Finally, current noninvasive prenatal screening tests do not assess the risk for open ventral wall or neural tube defects. Maternal serum alpha-fetoprotein testing and/or targeted ultrasound examinations will continue to play a role for all patients requesting comprehensive genetic screening to meet the current standards of care.

Noninvasive prenatal screening for the occurrence of the common autosomal trisomies and the sex chromosome aneuploidies is the first major accomplishment of this sequencing technology. An increasing number of monogenic diseases will undoubtedly be reported in the coming years.

Subchromosomal abnormalities as small as 300 kb already have been detected by massively parallel sequencing.[34] Future investigation will incorporate noninvasive testing by deep sequencing, improved bioinformatics, or some alternative strategy for the detection of deletions and duplications at the subchromosomal level and apply this next-generation technology to large clinical populations.[35,36] Indeed, a proof of concept has suggested that the complete fetal genome can be sequenced from cfDNA in the maternal plasma, raising new ethical implications of information so readily obtained.[37] ■

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The Obstetrix research collaborative network include the following: Richard Porreco, MD, Obstetrix Medical Group of Colorado, Denver, CO; C. Andrew Combs, MD, PhD, Obstetrix Medical Group of California, San Jose, CA; David Luthy, MD, Obstetrix Medical Group of Washington, Seattle, WA; Joseph Kipikasa, MD, Regional Obstetrical Consultants, Chattanooga, TN; Bobby Howard, MD, High Risk Obstetrical Consultants, Knoxville, TN; Brian Iriye, MD, Center for Maternal Fetal Medicine, Las Vegas, NV; George Lu, MD, Obstetrix Medical Group of Kansas City, Kansas City, MO; Mike Nageotte, MD, Magella Medical Group, Long Beach, CA; Melissa Bush, MD, Fetal Diagnostic Center, Laguna Hills, CA; Thomas Strong, MD, Obstetrix Medical Group of Phoenix, Phoenix, AZ; Hugh Miller, MD, Obstetric Perinatal Clinic Tucson, Tucson, AZ; Martin Walker, MD, Obstetrix Medical Group of Washington, Evergreen WA; Patrick Ramsey, MD, Texas Perinatal Associates, Houston, TX; Charles Read, MD, Maternal Fetal Specialists, Atlanta, GA; Mildred Ramirez, MD, The University of Texas at Houston, Houston, TX. A number of the investigative practices had more than 1 clinical site at which patients were enrolled into the study.

REFERENCES

1. Bianchi DW, Simpson JL, Jackson LG, et al. Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY-I data. National Institute of Child Health and Development Fetal Cell Isolation Study. Prenat Diagn 2002;22:609-15.
2. Lo YM, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485-7.
3. Avent ND, Chitty LS. Noninvasive diagnosis of fetal sex; utilization of free fetal DNA in maternal plasma and ultrasound. Prenat Diagn 2006;26:598-603.
4. Bianchi DW, Avent ND, Costa JM, Vanderschoot CE. Noninvasive prenatal diagnosis of fetal Rhesus D: ready for prime (r) time. Obstet Gynecol 2005;106:841-4.
5. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of single gene disorder from maternal plasma. Lancet 2000;356:1170.
6. Amicucci P, Gennarelli M, Novelli G, Dallapiccola B. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained for maternal plasma. Clin Chem 2000;46:301-2.
7. Dhallan R, Guo X, Emche S, et al. A noninvasive test for prenatal diagnosis based on fetal DNA present in maternal blood: a preliminary study. Lancet 2007;369:474-81.
8. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci USA 2008;105:16266-71.
9. Chiu RWK, Chan KCA, Gao Y, et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci USA 2008;105:20458-63.
10. Chiu RW, Akolekar R, Zheng YW, et al. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. BMJ 2011;342:c7401.
11. Title 21— food and drugs. Subchapter H. Medical devices, part 812: investigational device exemptions (2013). Silver Spring, MD: Food and Drug Administration, Department of Health and Human Services.
12. Ehrich M, Deciu C, Zwiefelhofer T, et al. Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: a study in a clinical setting. Am J Obstet Gynecol 2011;204:205.e1-11.
13. Jensen TJ, Zwiefelhofer T, Tim RC, et al. High-throughput massively parallel sequencing for fetal aneuploidy detection from maternal plasma. PLoS One 2013;8:e57381.
14. Chen EZ, Chiu RW, Sun H, et al. Non-invasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma DNA sequencing. PLoS One 2011;6:e21791.
15. Nygren AO, Dean J, Jensen TJ, et al. Quantification of fetal DNA by use of methylation-based DNA discrimination. Clin Chem 2010;56:1627-35.
16. Mazloom AR, Dzakula Z, Wang H, et al. Noninvasive prenatal detection of sex chromosomal aneuploidies by sequencing circulating cell-free DNA from maternal plasma. Prenat Diagn 2013;33:591-7.
17. Pearson ES, Clopper C. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 1934;26:404-13.
18. Palomaki GE, Kloza EM, Lambert-Messerlian GM, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet Med 2011;13:913-20.
19. Palomaki GE, Deciu C, Kloza EM, Lambert-Messerlian GM, et al. DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: an international collaborative study. Genet Med 2012;14:296-305.

20. Morain S, Greene MF, Mello MM. A new era in noninvasive prenatal testing. N Engl J Med 2013;369:499-501.
21. Mennuti MT, Cherry AM, Morrissette JJD, Dugoff L. Is it time to sound an alarm about false-positive cell-free DNA testing for fetal aneuploidy? Am J Obstet Gynecol 2013;209:415-9.
22. Fan HC, Quake SR. Sensitivity of noninvasive prenatal detection of fetal aneuploidy from maternal plasma using shotgun sequencing is limited only by counting statistics. PLoS One 2010;5:e10439.
23. Bianchi DW, Platt LD, Goldberg JD, Abuhamad AZ, Sehnert AJ, Rava RP. MatErnal BLood IS Source to Accurately diagnose fetal aneuploidy (MELISSA) Study Group. Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. Obstet Gynecol 2012;119:890-901.
24. Wang Y, Chen Y, Tian F, et al. Maternal mosaicism is a significant contributor to discordant sex chromosomal aneuploidies associated with noninvasive prenatal testing. Clin Chem 2014;60:251-9.
25. Sparks AB, Struble CA, Wang ET, Song K, Oliphant A. Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012;206:319.e1-9.
26. Ashoor G, Syngelaki A, Wagner M, Birdir C, Nicolaides KH. Chromosome-selective sequencing of maternal plasma cell-free DNA for first-trimester detection of trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012;206:322.e1-5.
27. Norton ME, Brar H, Weiss J, et al. Noninvasive chromosome evaluation (NICE) Study: results of a multicenter prospective cohort study for detection of fetal trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012;207:137.e1-8.
28. Zimmerman B, Hill M, Gemelos G, et al. Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. Prenat Diagn 2012;32:1233-41.
29. The American College of Obstetricians and Gynecologists Committee on Genetics. The Society for Maternal-Fetal Medicine Publications Committee. Noninvasive prenatal testing for fetal aneuploidy. ACOG SMFM Committee Opinion no. 545. Obstet Gynecol 2012;120:1532-4.
30. Dugoff L, Hobbins JC, Malone FD, et al. First-trimester maternal serum PAPP-A and free-beta subunit human chorionic gonadotropin concentrations and nuchal translucency are associated with obstetric complications: a population-based screening study (The FASTER Trial). Am J Obstet Gynecol 2004;191:1446-51.
31. Dugoff L, Hobbins JC, Malone FD, et al. Quad screen as a predictor of adverse pregnancy outcome. Obstet Gynecol 2005;106:260-7.
32. Holzgreve W, Hahn S, Zhong XY, et al. Genetic communication between fetus and mother: short- and long-term consequences. Am J Obstet Gynecol 2007;196:372-81.
33. Sekizawa A, Jimbo M, Saito H, et al. Increased cell-free fetal DNA in plasma of two women with invasive placenta. Clin Chem 2002;48:353-4.
34. Srinivasan A, Bianchi DW, Huang H, Sehnert AJ, Rava RP. Noninvasive detection of fetal subchromosome abnormalities via deep sequencing of maternal plasma. Am J Human Genet 2013;92:167-76.
35. Peters D, Chu T, Yatsenko SA, et al. Noninvasive prenatal diagnosis of a fetal microdeletion syndrome. N Engl J Med 2011;365:1847-8.
36. Jensen TJ, Dzakula Z, Deciu C, van den Boom D, Ehrich M. Detection of microdeletion 22q11.2 in a fetus by next-generation sequencing of maternal plasma. Clin Chem 2012;58:1148-51.
37. Lo YM. Noninvasive prenatal diagnosis in 2020. Prenat Diagn 2010;30:702-3.