OBSTETRICS

Clinical experience and follow-up with large scale single-nucleotide polymorphism—based noninvasive prenatal aneuploidy testing

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OBJECTIVE: We sought to report on laboratory and clinical experience following 6 months of clinical implementation of a single-nucleotide polymorphism—based noninvasive prenatal aneuploidy test in high- and low-risk women.

STUDY DESIGN: All samples received from March through September 2013 and drawn ≥9 weeks' gestation were included. Samples that passed quality control were analyzed for trisomy 21, trisomy 18, trisomy 13, and monosomy X. Results were reported as high or low risk for fetal aneuploidy for each interrogated chromosome. Relationships between fetal fraction and gestational age and maternal weight were analyzed. Follow-up on outcome was sought for a subset of high-risk cases. False-negative results were reported voluntarily by providers. Positive predictive value (PPV) was calculated from cases with an available prenatal or postnatal karyotype or clinical evaluation at birth.

RESULTS: Samples were received from 31,030 patients, 30,705 met study criteria, and 28,739 passed quality-control metrics and received a report detailing aneuploidy risk. Fetal fraction correlated positively with gestational age, and negatively with maternal weight. In all, 507 patients received a high-risk result for any of the 4 tested conditions (324 trisomy 21, 82 trisomy 18, 41 trisomy 13, 61 monosomy X; including 1 double aneuploidy case). Within the 17,885 cases included in follow-up analysis, 356 were high risk, and outcome information revealed 184 (51.7%) true positives, 38 (10.7%) false positives, 19 (5.3%) with ultrasound findings suggestive of aneuploidy, 36 (10.1%) spontaneous abortions without karyotype confirmation, 22 (6.2%) terminations without karyotype confirmation, and 57 (16.0%) lost to follow-up. This yielded an 82.9% PPV for all aneuploidies, and a 90.9% PPV for trisomy 21. The overall PPV for women aged ≥35 years was similar to the PPV for women aged <35 years. Two patients were reported as false negatives.

CONCLUSION: The data from this large-scale report on clinical application of a commercially available noninvasive prenatal test suggest that the clinical performance of this single-nucleotide polymorphism—based noninvasive prenatal test in a mixed high- and low-risk population is consistent with performance in validation studies.

Key words: low-risk, noninvasive prenatal testing, single-nucleotide polymorphism, trisomy 21

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Since becoming clinically available in late 2011, cell-free DNA (cfDNA)-based noninvasive prenatal testing (NIPT) for fetal aneuploidy has seen an unprecedented rapid adoption into clinical care.[1] This followed multiple publications on methodologies, validation, and test performance,[2-14] all demonstrating improved sensitivities

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and lower false-positive (FP) rates than current screening methods. Opinion statements by national and international professional societies support the clinical use of NIPT in pregnant women, with most recommending use restricted to women at high risk for fetal aneuploidy.[15-17]

Two approaches to NIPT have been developed and commercialized. In the first approach, fetal chromosome copy number is determined by comparing the number of sequence reads from the chromosome(s) of interest to those from reference chromosomes.[7,8,11-13,18-22] The second approach entails targeted amplification and sequencing of single-nucleotide polymorphisms (SNPs).[2-5,23,24] This approach requires a sophisticated informatics-based method to compute aneuploidy risk through SNP distribution. Validation of the SNP-based NIPT method at 11-13 weeks' gestation was recently reported, demonstrating high sensitivity and specificity for detection of trisomy 21, trisomy 18, trisomy 13, Turner syndrome (monosomy X), and triploidy.[2,3]

Despite hundreds of thousands of tests already having been performed worldwide, there are few large-scale reports describing performance of NIPT in actual clinical settings,[22,25] with most studies reporting on <1000 total patients.[26-29] Here, laboratory and clinical experience of >31,000 women who received prenatal screening with a SNP-based NIPT is reported.

TABLE 1
Demographics of commercial cases

Demographic	Whole cohort, n = 31,030	Follow-up cohort, n = 17,885
Maternal age, y[a]		
Mean	33.3 ± 6.0	33.7 ± 6.1
Median	35.0	35.0
Range	14.0–60.0	14.0–52.0
Gestational age, wk		
Mean	14.0 ± 4.4	14.5 ± 4.7
Median	12.6	13.0
Range	3.1–40.9	9.0–40.9[b]
Maternal weight, lb[c]		
Mean	158.4 ± 39.2	157.2 ± 37.9
Median	149.0	148.0
Range	83.0–425.0	83.0–385.0
Fetal fraction, %		
Mean	10.2 ± 4.5	10.8 ± 4.4
Median	9.6	10.1
Range	0.6–50.0	3.7–50.0[b]

[a] At estimated date of delivery; [b] As the follow-up cohort does not include any out-of-specification cases, or any cases that failed to receive a noninvasive prenatal testing result, minimum gestational age and fetal fraction are higher than in the whole cohort—however, mean values and SD are equivalent between the 2 cohorts; [c] Analysis of maternal weight was limited to centers and laboratories that provided this information, and samples originating from United States to avoid inconsistent weight units.

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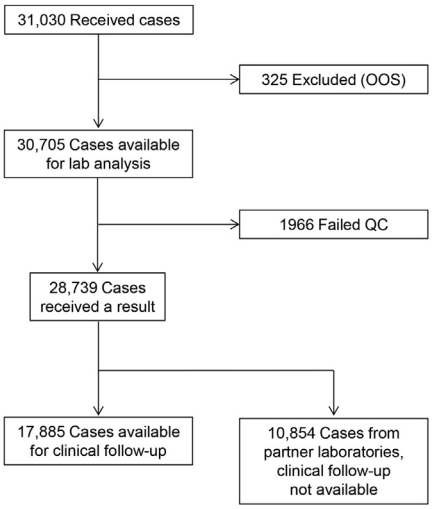
MATERIALS AND METHODS

This is a retrospective analysis of prospectively collected data on 31,030 cases received for commercial testing from March through September 2013. This study received a notification of exempt determination from an institutional review board (Albert Einstein College of Medicine Institutional Review Board: no. 2014-3307). Samples were classified as out of specification and excluded in cases of gestational age <9 weeks, multiple gestation, donor egg pregnancy, surrogate carrier, missing patient information, sample received >6 days after collection, insufficient blood volume (<13 mL), wrong collection tube used, or if the sample was damaged.

Analysis was performed for all samples on chromosomes 13, 18, 21, X, and Y, and included detection of trisomy 21, trisomy 18, trisomy 13, and monosomy X. All samples were processed and analyzed at Natera Inc's Clinical Laboratory Improvement Act (CLIA)-certified and College of American Pathologists (CAP)-accredited laboratory (San Carlos, CA). Laboratory testing was performed as previously described using validated methodologies for cfDNA isolation, polymerase chain reaction amplification targeting 19,488 SNPs, high-throughput sequencing, and analysis with the next-generation aneuploidy test using SNPs (NATUS) algorithm.[2-5] Samples were subject to a stringent set of quality-control metrics. A second blood draw (redraw) was requested if total input cfDNA, fetal cfDNA fraction, or signal-to-noise ratio did not meet quality metrics, or for poor fit of the data to the model. In cases of large regions (>25%) of loss of heterozygosity or suspected maternal or fetal mosaicism, redraw was not requested. Reports included a risk score for the 4 aneuploidies; when requested, reports included fetal sex. Risk scores were calculated by combining the maximum likelihood estimate generated by the NATUS algorithm with maternal and gestational age prior risks. All samples with a risk score ≥1/100 were reported as high risk for fetal aneuploidy and samples with risk scores <1/100 were considered low risk. For the purposes of this study, the high-risk results were further divided into a maximum-risk score of 99/100 or an intermediate-risk score of ≥1/100 and <99/100. The presence of >2 fetal haplotypes (indicative of either triploidy or multiple

FIGURE 1
Study flow chart



OOS: see "Materials and Methods" section.

OOS, out-of-specification; *QC*, quality control.

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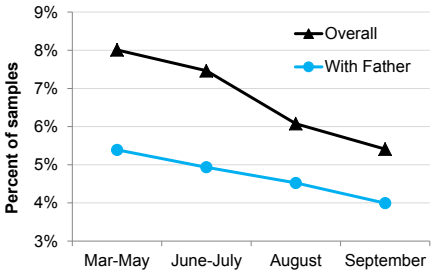
gestation) was reported only when the confidence was >99.9%. Additional sex chromosome aneuploidies (XXX, XXY, and XYY) were reported from June 2013. The following patient characteristics were requested for each sample: maternal date of birth, maternal weight, gestational age, and whether a paternal sample was included.

Patients with available *International Classification of Diseases, Ninth Revision* (*ICD-9*) codes (Appendix; Supplementary Table 1) were categorized into 3 subcohorts: (1) "low risk" if aged <35 years and no aneuploidy-related high-risk codes; (2) "at risk" for fetal aneuploidy based solely on maternal age ≥35 years; or (3) "high risk" for fetal aneuploidy by *ICD-9* code, regardless of maternal age. High-risk indications included positive screening tests, ultrasound anomalies, and relevant family history. Patients without reported *ICD-9* codes were categorized by maternal age as low risk (<35 years) or high risk (≥35 years).

Follow-up information on high-risk results was obtained by telephone and recorded in an internal database. Clinical follow-up was completed on June 14, 2014, at which time all pregnancies were completed. Two partner laboratories accounting for 38.1% of the total 31,030 cases were responsible for their own follow-up efforts and were excluded from outcome calculations. Providers were encouraged to share information about false-negative (FN) results. Samples were categorized as follows: (1) "true positive" (TP) included high-risk samples that were confirmed by prenatal or postnatal diagnostic testing, or based on clinical evaluation at birth; (2) "FP" included high-risk samples that were shown to be euploid by follow-up testing or based on clinical evaluation at birth; (3) "suggestive" included samples where prenatal ultrasound detected at least 1 structural anomaly and 1 soft sonographic marker consistent with NIPT findings, but karyotype confirmation was not obtained; (4) "pregnancy loss" where the patient experienced spontaneous abortion and karyotype confirmation was not obtained; (5) "termination" where the patient elected to end the pregnancy without karyotype confirmation; (6) "no follow-up" included samples where information was unavailable; and (7) "FN" included NIPT low-risk samples that were reported as aneuploid by the provider. When placental and fetal karyotypes were both available and determined to be discordant, NIPT findings were considered TP if they matched the fetal karyotype, and FP if they did not match the fetal karyotype. Pregnancies were considered mosaic when chromosome analysis revealed either placental or fetal mosaicism or there was discordance between placental and fetal karyotypes.

Patient and sample characteristics were expressed as means, SD, medians, and ranges. Linear regression analysis was used to determine the relationship between fetal fraction and gestational age, between fetal fraction and maternal weight, and between fetal/maternal cfDNA and maternal weight; a reciprocal model was used when determining the relationship between fetal fraction and gestational age or maternal weight. For comparison of euploid and aneuploid calls, fetal fractions were expressed as multiples of the median (MoM) relative to low-risk calls weighted by week of gestation, and significance

FIGURE 2
Father sample and clinical laboratory experience reduces redraw rate



Decrease in redraw rates overall and for patients including a paternal sample during the reporting period (March through September 2013) for samples ≥10 weeks of gestation.

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determined using a Mann-Whitney rank sum test. The 2 FN results were included in the appropriate aneuploid category, and FP calls were excluded from aneuploidy fetal fraction analyses. The benefit of a paternal sample on redraw rates and differences in aneuploidy incidence between the a priori risk groups were determined using a χ^2 test. The Kruskal-Wallis 1-way analysis of variance on ranks test was used to evaluate maternal age and gestational age differences for the different risk groups. Positive predictive value (PPV) ([TP]/[TP + FP]) was calculated for cases with known cytogenetic analyses. SigmaPlot 12.5 (Systat Software, San Jose, CA) was used for all statistical analyses. $P < .05$ was considered statistically significant.

RESULTS

Patients and samples

Patient and sample characteristics for the 31,030 cases received during the study period are detailed in Table 1. Mean maternal age was 33.3 years, with 51.4% (15,952) aged ≥35 years at the estimated date of delivery. Mean gestational age was 14.0 weeks, with 64.5% (20,001) of samples drawn in first trimester and 33.8% (10,479) in the second trimester.

Figure 1 depicts the study flow chart. Samples from 325 (1.0%) patients were excluded as being outside of the

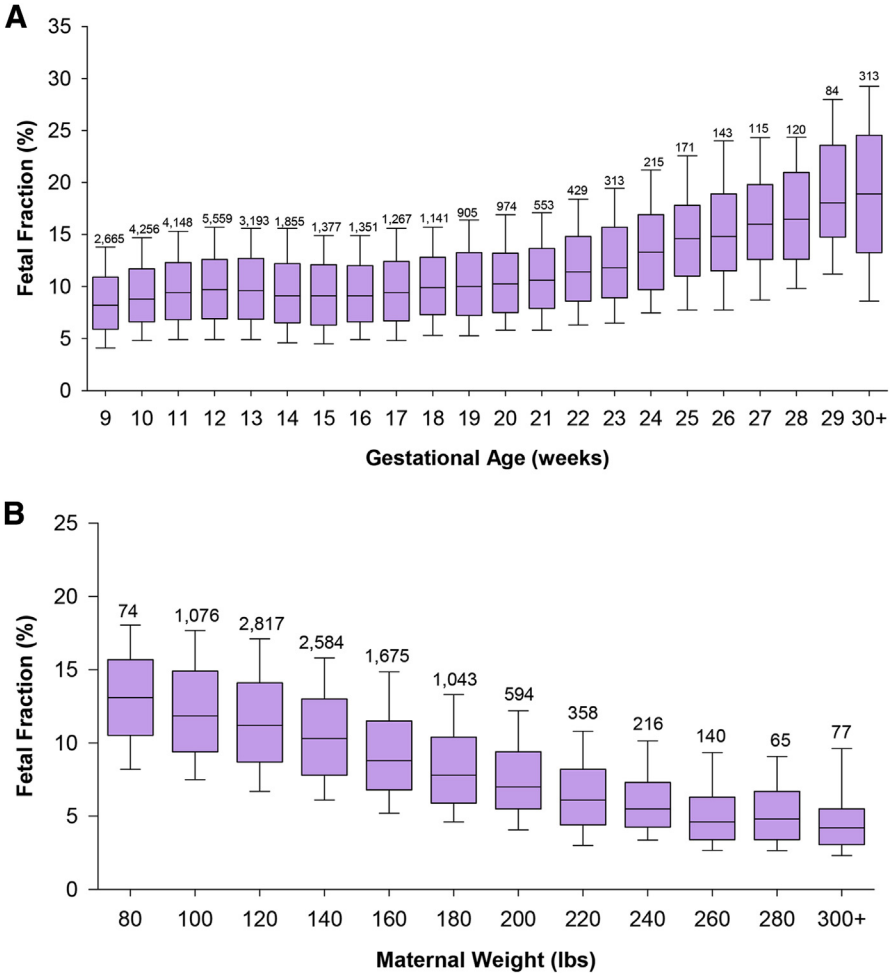
specifications for testing (Supplementary Table 2) and 1966 samples failed quality-control metrics (Supplementary Table 3), mostly due to low fetal fraction, leaving 28,739 cases with NIPT results.

In 21,678 cases from clinics linking patient samples to a single case identification, 386 first draws did not meet requirements, thereby allowing analysis of redraw rates in 21,292 cases. A redraw was requested from 95.4% (1572/1648) of cases without a first draw result, 56.5% (888/1572) submitted a redraw, and 64.3% (571/888) of redraws were reported; 12 (2.1%) resolved redraws received a high-risk call. Redraw rates declined steadily over the reporting period (Figure 2); the most recent first sample redraw rates were 9.4% at 9 weeks', and 5.4% at ≥10 weeks' gestation. Around 30% of patients given the opportunity to submit a paternal sample chose to do so, and inclusion of a paternal sample was associated with a lower redraw rate, with a similar decline over the study period (Figure 2). This effect was more pronounced in women weighing >200 lb, where inclusion of a paternal sample reduced the redraw rate from 27.5% to 16.1% ($P < .001$). The average turn-around time was 9.2 calendar days (95% confidence interval [CI], 9.16–9.23 calendar days), but significant improvements over the study period led to an average turn-around time in the last month of 6.7 calendar days (95% CI, 6.68–6.76 calendar days).

Fetal fractions

The average fetal fraction was 10.2% (Table 1). Regression analysis, using the reciprocal of the independent variable (gestational age or maternal weight), revealed a positive correlation between fetal fraction and gestational age ($r^2 = 0.05$, $P < .001$) (Figure 3, A), and a negative association between fetal fraction and maternal weight ($r^2 = 0.16$, $P < .001$) (Figure 3, B). Furthermore, with increasing maternal weight, there was an increase in maternal cfDNA ($P < .001$) and a decrease in fetal cfDNA ($P < .001$) (Figure 4). Fetal fractions when stratified by aneuploidy were decreased for trisomy 13 (0.759 MoM, $P < .001$), trisomy 18 (0.919 MoM, $P = .012$), and monosomy X (0.835 MoM, $P < .001$), and increased for trisomy 21 (1.048 MoM, $P = .018$) samples.

FIGURE 3
Effect of gestational age and maternal weight on fetal fraction



Box plots depicting effects of **A**, gestational age and **B**, maternal weight on fetal fraction. Boxes indicate 75th (upper) and 25th (lower) quartiles, *solid black line* within box indicates median, *capped whiskers* indicate 90th (upper) and 10th (lower) percentiles, number in each grouping is indicated above 90th percentile whisker.

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NIPT results

The combined rate of high-risk calls for all 4 indications was 1.77% (508/28,739); including 324 trisomy 21, 82 trisomy 18, 41 trisomy 13, and 61 monosomy X (Table 2). One sample was not assigned a risk score for chromosome 21 due to a maternal chromosome 21 partial duplication but was accurately identified as fetal trisomy 21 by the laboratory. Of 20,384 samples evaluated for additional sex chromosome aneuploidies, other than monosomy X, there were 14 (0.07%) identified: 6 XXX, 6 XXY, and 2 XYY. Fetal sex was reported in 24,522 cases. There were no reports of gender discordance from women receiving low-risk reports. For women receiving high-risk reports, confirmation of fetal sex was available for 109 cases, of which 108 (99.1%) were correct; the single discordant case was reported as high-risk for monosomy X (Supplementary Figure) but cytogenetic testing revealed a 46, XY fetus. Although

cases with known multiple gestations were excluded, the NATUS algorithm identified 127 (0.4%) samples as having >2 fetal haplotypes, indicative of either unreported twins, vanishing twin, or triploidy.

ICD-9 codes were associated with 19.0% (5468/28,739) of women: 16.6% were low-risk, 44.1% were high-risk based only on advanced maternal age (≥35 years), and 39.3% had high-risk codes. As expected, the incidence of aneuploidy calls was smallest in the low-risk group (0.7%), followed by advanced maternal age women (1.6%), and largest in the high-risk group (3.4%) (Table 3). Results for the 23,271 samples without *ICD-9* codes showed a similar difference in aneuploidy calls between women aged <35 years (1.0%, 117/11,629) and those aged ≥35 years (2.4%, 274/11,642).

Follow-up of high-risk calls

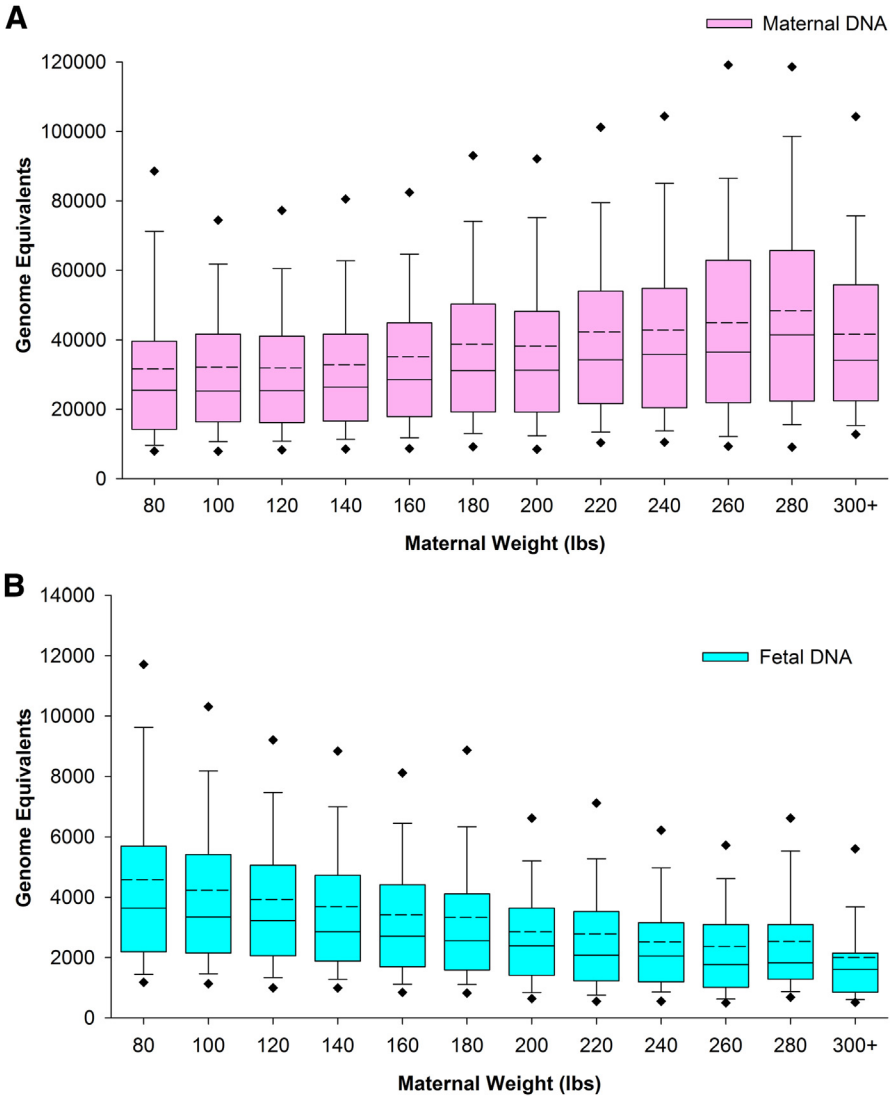
From 17,885 cases in the follow-up cohort, outcome information was sought for the 356 high-risk calls; 152 high-risk calls from the whole cohort described above were not contained within the follow-up cohort.

Information regarding invasive testing uptake was available for 251/356 (70.5%) cases that received a high-risk result: 39.0% (139) elected invasive testing and 31.5% (112) declined invasive tests, and of the remaining 105 (29.5%), 39 had a spontaneous demise or elective termination. Within the 356 high-risk calls, there were in total 58 reported spontaneous abortions, including 16 cases categorized as TP, 2 FP, 4 with ultrasound findings suggestive of aneuploidy, and 36 with unconfirmed outcomes. There were 57 reported elective terminations, including 30 cases categorized as TP, 5 with ultrasound findings suggestive of aneuploidy, and 22 elective terminations with unconfirmed outcomes.

At the conclusion of clinical follow-up, 62.4% (222/356) of high-risk calls had karyotype information or at-birth confirmation: 184 confirmed affected pregnancies (TP) and 38 unaffected pregnancies (FP) (Table 4). Eight cases showed placental or fetal mosaicism: 5 fetal mosaics (TP) were confirmed by amniocentesis (2 trisomy 21, 2 trisomy 18, 1 monosomy X), and 3 cases were considered FP because of confined placental mosaicism (CPM). Two CPM cases were high risk for trisomy 13 and were identified as mosaics by chorionic villus sampling (CVS), one was determined to be euploid by amniocentesis, and the other did not have a follow-up amniocentesis but ultrasound at 20 weeks was read as normal. In the third CPM case, at-birth testing revealed a 100% trisomy 18 placenta and a euploid child. Two FN results (both trisomy 21) were reported to the laboratory following amniocentesis due to other indications.

For the sex chromosome aneuploidies XXX, XXY, and XYY, 7 of the

FIGURE 4
Increasing maternal weight increases maternal cfDNA and decreases fetal cfDNA



Box plots depicting absolute levels of **A**, maternal and **B**, fetal cell-free DNA in maternal circulation as a function of maternal weight. Boxes indicate 75th (upper) and 25th (lower) quartiles, *solid line* within box indicates median, *dashed line* within box indicates mean, *capped whiskers* indicate 90th (upper) and 10th (lower) percentiles, diamonds indicate 95th (upper) and 5th (lower) percentiles.

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14 high-risk calls were within the follow-up cohort. Clinical follow-up revealed 4 cases with known outcomes: 2 TP (1 XXX, 1 XXY) and 2 FP (both XXX).

Based on the cases with cytogenetic confirmation, women with an intermediate-risk score were more likely to have a FP result (19/24, 79.2%) than women with a maximum-risk score (19/198, 9.6%, $P < .001$). For the 36 cases that experienced spontaneous abortion and did not obtain karyotype confirmation, 33 (91.7%) had a maximum-risk score. All 22 patients who elected to terminate the pregnancy without confirmation had a maximal-risk score.

TABLE 2
Number of fetal aneuploidy high-risk calls in reported commercial cases

All cases, N = 28,739[a]	Trisomy 21	Trisomy 18	Trisomy 13	Monosomy X
Risk ≥99/100	298[b]	78[b]	26	53
1/100 ≤ Risk <99/100	25	4	15	8
Total	324[b,c]	82[b]	41	61
Prevalence, 1 in:	88	349	697	467

[a] Total number of cases with reported result at ≥9 wk of gestation; [b] Trisomy 21 and trisomy 18 totals include a single case of double-aneuploidy; [c] Includes 1 case with a detected partial maternal chromosome 21 duplication, the fetus was determined to be high risk for trisomy 21 but the algorithm did not calculate a risk score.

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Positive predictive value

Based only on cases with cytogenetic diagnosis (Table 4), the PPV was 90.9% for trisomy 21 and 82.9% for all 4 cytogenetic abnormalities combined (Table 5). A theoretical PPV was also calculated under the 2 boundary conditions that all unconfirmed high-risk cases were either FP or TP (Table 5). This provided a range for the PPV of 60-94% for trisomy 21 and 52-89% for all abnormalities combined.

Among women without *ICD-9*-coded indications, 63 women aged <35 years received high-risk calls, of which 39 (60.9%) had diagnostic testing and 34 were TP, a PPV of 87.2% (95% CI, 72.6–95.7%). Of 176 women ≥35 years with high-risk calls, 105 (59.7%) had confirmatory karyotyping and 87 were TP, a PPV of 82.9% (95% CI, 74.3–89.5%).

COMMENT

This report of initial clinical experience with this SNP-based NIPT in >31,000 pregnancies demonstrates that performance in clinical settings is consistent with validation studies.[2-5] Using only cases confirmed through chromosome analysis or clinical evaluation at birth, the PPV in this mixed low- and high-risk population is 90.9% for trisomy 21 and 82.9% for all 4 aneuploidies, which is far better than current screening methods. Even under the highly conservative assumption that all unconfirmed high-risk cases are incorrect, this test still offers improved clinical performance over traditional screening.

The main advantage of this study is the robust information it provides on clinical application of NIPT, which can contribute to, and improve, both test performance and counseling of patients. Fetal fraction, the main variable that affects redraw rates, is positively correlated with gestational age and negatively correlated with maternal weight, agreeing with previous studies.[30-33] There are 2 main clinical implications from these findings. First, adequate dating will lower the need for redraw, particularly at early gestational ages. Second, inclusion of a paternal blood sample significantly lowers redraw rates and should be offered to patients, particularly those >200 lb. Importantly, cases with extremely low fetal fraction, which typically do not resolve with redraw, may have an increased risk for fetal aneuploidy.[2] This is likely particularly important for maternal triploidy, which is associated with smaller placentas and lower fetal fractions,[2,5] and trisomy 13 and trisomy 18 pregnancies.

In addition to determining the most likely ploidy state of a fetus, the NATUS algorithm also generates a chromosome-specific risk score, which is a measure of the probability of nonmosaic fetal aneuploidy. As expected, data showed that maximum-risk results are more likely to be TP than intermediate-risk results. Although a high-risk score appears to be more indicative of a TP result, individual numerical values should be interpreted cautiously. Regardless of the risk score, confirmatory studies must be offered to all women with positive results without exception. This is particularly important in light of the finding here that 6.2% of women with high-risk results chose to terminate the pregnancy without invasive test confirmation.

Although referred to as fetal cfDNA, the primary source of cfDNA is placental trophoblast cells.[34] CPM, estimated to be present in 1-2% of 10- to 12-week gestations,[35,36] impacts all NIPTs. Validation studies have typically excluded samples with fetal mosaicism or CPM. Yet, it is clear that when NIPT is performed in a clinical setting, the effect of mosaicism cannot be ignored, and its impact on FP and FN results should be addressed. In this cohort, 8/222 (3.6%) high-risk calls showed evidence of mosaicism. Two cases with CVS results that supported NIPT findings were later categorized as FPs because of CPM. Further, since most FPs in this cohort were determined by amniocentesis or at-birth testing without placental genetic analysis, there may be additional, undetected CPM cases within the FPs. From a retrospective analysis of CVS, Grati et al[37] estimated that the FP rate would be 0.08% for the 4 common aneuploidies. Our findings, combined with the known incidence of CPM-related FPs and FNs, further reinforce the need for adequate pretest counseling, as recommended by American Congress of Obstetrics and Gynecology (ACOG).[17] Patients undergoing CVS following high-risk results with NIPT should be counseled that mosaic conditions can occur and later amniocentesis may be required.

An unexpected finding in this study was that the PPV for women aged <35

TABLE 3
Aneuploidy calls in different a priori risk groups

Variable	Cases with *ICD-9* codes, n = 5468			Cases without codes, n = 23,271	
	Low risk, age <35 y (n = 909)	AMA only, age ≥35 y (n = 2411)	High risk, all ages (n = 2148)	Low risk, age <35 y (n = 11,629)	High risk, age ≥35 y (n = 11,642)
Maternal age, y[a]	28.2 ± 4.4	37.8 ± 2.4	31.3 ± 5.8	28.4 ± 4.5	37.9 ± 2.5
Median (range)	29.0 (15.0–34.0)	37.0 (35.0–48.0)	32.0 (15.0–47.0)	29.0 (14.0–34.0)	37.0 (35.0–52.0)
Gestational age, wk[a]	14.1 ± 4.4	13.3 ± 3.5	15.8 ± 5.0	14.7 ± 4.9	13.4 ± 3.9
Median (range)	12.4 (9.0–33.3)	12.4 (9.0–38.1)	14.4 (9.0–37.0)	13.0 (9.0–38.0)	12.1 (9.0–40.9)
Euploid	903	2368	2073	11,457	11,293
Trisomy 21	2	27[b]	50	57	188
Trisomy 18	1	5[b]	13	21	42
Trisomy 13	1	5	3	11	21
Monosomy X	2	2	6	28	23
Total aneuploids	6	38	72	117	274
Monosomy X prevalence, %	0.22	0.08	0.28	0.24	0.20
Trisomy prevalence, %	0.44	1.49	3.07	0.77	2.16
Overall prevalence, %	0.66[c]	1.58[c]	3.35[c]	1.01[d]	2.35[d]

Women with *ICD-9* codes were sorted into 3 risk populations based on *ICD-9* codes and maternal age: low-risk women aged <35 y, women of AMA (aged ≥35 y) with no other high-risk codes, and high-risk women of any age. Women without *ICD-9* codes were sorted into 2 risk populations based on maternal age: low-risk women aged <35 y and high-risk women of AMA.

AMA, advanced maternal age; *ICD-9*, *International Classification of Diseases, Ninth Revision*.

[a] Mean ± SD, there was a significant difference between risk groups ($P < .001$) for both maternal age and gestational age, as determined by the Kruskal-Wallis 1-way analysis of variance on ranks test; [b] Trisomy 21 and trisomy 18 totals include single case of double-aneuploidy; [c] Significant difference in aneuploidy call rate among 3 groups with *ICD-9* codes ($P < .001$), as determined by χ^2 test; [d] Significant difference in aneuploidy call rate between 2 groups without *ICD-9* codes ($P < .001$), as determined by χ^2 test.

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TABLE 4
Clinical follow-up findings

N = 17,885[a]	Trisomy 21	Trisomy 18	Trisomy 13	Monosomy X	Total
High-risk calls	233[b]	55[b]	30	38	356
Confirmed outcomes					
True positive	140[c]	27	8	9	184
False positive	14[d]	2[e]	13[f,g]	9	38
Unconfirmed outcomes					
Suggestive[h]	8	9	0	2	19
Pregnancy loss[i]	18	6	3	9	36
Termination[j]	14	3	0	5	22
No follow-up[k]	39	8	6[l]	4	57
Low-risk calls					
Confirmed outcomes					
False negative	2	0	0	0	2

[a] Total number of cases with reported result at ≥9 wk of gestation from participating centers; [b] Trisomy 21 and trisomy 18 totals include single double-aneuploidy case; [c] Includes 13 cases reported as trisomy 21 based on at-birth clinical evaluation; [d] Includes 3 cases reported as normal based on at-birth clinical evaluation; [e] Includes 1 confined placental mosaicism case; [f] Includes 2 confined placental mosaicism cases (1 confirmed and 1 unconfirmed); [g] Includes 1 case reported as normal based on at-birth clinical evaluation; [h] Patients declined invasive testing but ultrasound findings were consistent with noninvasive prenatal testing findings (see "Materials and Methods" section); [i] Patients experienced spontaneous abortion and did not obtain karyotype confirmation; [j] Patients chose to terminate pregnancy without diagnostic testing; [k] Follow-up information was not available; [l] One sample tested as high-risk (1/7.6) for fetal aneuploidy, analysis of second sample indicated that patient was at low-risk, follow-up information was not available.

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years (87%) was similar to that of women aged ≥35 years (83%). This does not appear to be attributable to a bias in the referral of cases for karyotyping. Some women aged <35 years may have chosen NIPT because of ultrasound findings or positive results with traditional serum screening. However, the lower aneuploidy call incidence of 1.0% in women aged <35 years, vs 2.4% in women aged ≥35 years (Table 3), supports that these 2 groups of women do differ substantially with respect to aneuploidy incidence. The PPV was expected to be lower in low-risk women because the number of affected pregnancies would be lower but the number of FPs was predicted to be a constant proportion.[38] The similar PPVs determined in both maternal age groups may indicate that FPs, like affected pregnancies, are also proportionately more common in older women; perhaps arising from trisomic conceptions that are rescued but express CPM. More data are needed to confirm this observation.

Based on the current opinion statement from ACOG, NIPT is appropriate for use in high-risk patients.[17] Nevertheless, the ability to detect aneuploidy with cfDNA depends on assay precision and fetal fraction, not on disease prevalence. Reported PPV in studies performed on mixed high- and low-risk populations, as well as the current study, far exceed current screening methodologies. Consistent with this, recent guidelines published by the American College of Medical Genetics and Genomics (ACMG) do not distinguish between high and low risk. Therefore, the transition of NIPT into a universal, first-line, aneuploidy screen should depend on the availability and affordability of NIPT, and not concerns about performance.

In this cohort of women who were thought to have singleton pregnancies at the time of NIPT, 127 cases were identified as having >2 fetal haplotypes suggesting either triploidy or a previously undetected multifetal pregnancy or vanishing twin. The SNP-based NIPT methodology provided the opportunity to identify these cases, pursue further diagnostic avenues, and avoid FPs that can arise using alternative methodologies.[22]

TABLE 5
Positive predictive values

Variable	Trisomy 21	Trisomy 18	Trisomy 13	Monosomy X	Total
Cytogenetically confirmed cases					
TP/(TP + FP) (PPV)	140/154 (90.9%)	27/29 (93.1%)	8/21 (38.1%)	9/18 (50.0%)	184/222 (82.9%)
All unconfirmed cases considered as FPs (lower bound)					
TP/(TP + FP) (PPV)	140/233 (60.1%)	27/55 (49.1%)	8/30 (26.7%)	9/38 (23.7%)	184/356 (51.7%)
All unconfirmed cases considered as TPs (upper bound)					
TP/(TP + FP) (PPV)	219/233 (94.0%)	53/55 (96.4%)	17/30 (56.7%)	29/38 (76.3%)	318/356 (89.3%)

PPV calculated as (TP)/(TP + FP). Data are presented for just those cases where there was cytogenetic or clinical confirmation of result; based on the extreme condition that all unconfirmed cases were FPs (lower bound) and the opposite condition that all unconfirmed results were TP (upper bound).

FP, false positive; *PPV*, positive predictive value; *TP*, true positive.

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The main limitation of this study is the incomplete follow-up data, particularly on low-risk patients, precluding precise calculation of sensitivity and specificity. While follow-up was not conducted on low-risk patients, given the clinical significance of a FN report, and based on our laboratory experience, it is likely that FNs would be voluntarily reported; there were 2 voluntarily reported FNs. However, the lack of comprehensive follow-up on all low-risk patients precluded determination of the negative predictive value. Nevertheless, it is important to note that strong performance characteristics were in keeping with prior validation studies,[2,3,24] even with the inclusion of mosaic samples. Follow-up of normal results remains an issue for all laboratories that wish to track results for quality assurance, and we support the ACMG recommendation for a national registry.[16]

In conclusion, this is a large-scale report of clinical utilization of NIPT. Analysis of >31,000 samples from both low- and high-risk women supported that test performance of this NIPT method in a clinical setting mirrors the robust performance reported in validation studies.

Clinical performance of SNP-based NIPT in a mixed high- and low-risk population is consistent with performance in validation studies. Similar PPVs were found in women aged <35 years and aged ≥35 years. The strength of the study is the robust information it provides on clinical application of NIPT. The primary limitation is the incomplete follow-up data, particularly on low-risk patients, precluding precise calculation of sensitivity and specificity.

This study supports the use of NIPT as a first-line screening test for aneuploidy in all patients. Furthermore, it highlights the importance of, as well as provides data that can improve, counseling of patients. Finally, the results of this study raise the questions of how many FP results may be explained by CPM and how best to manage clinical care and diagnostic confirmation of high-risk NIPT results in light of potential CPM. The extent to which CPM may underlie NIPT FP results requires further investigation. ■

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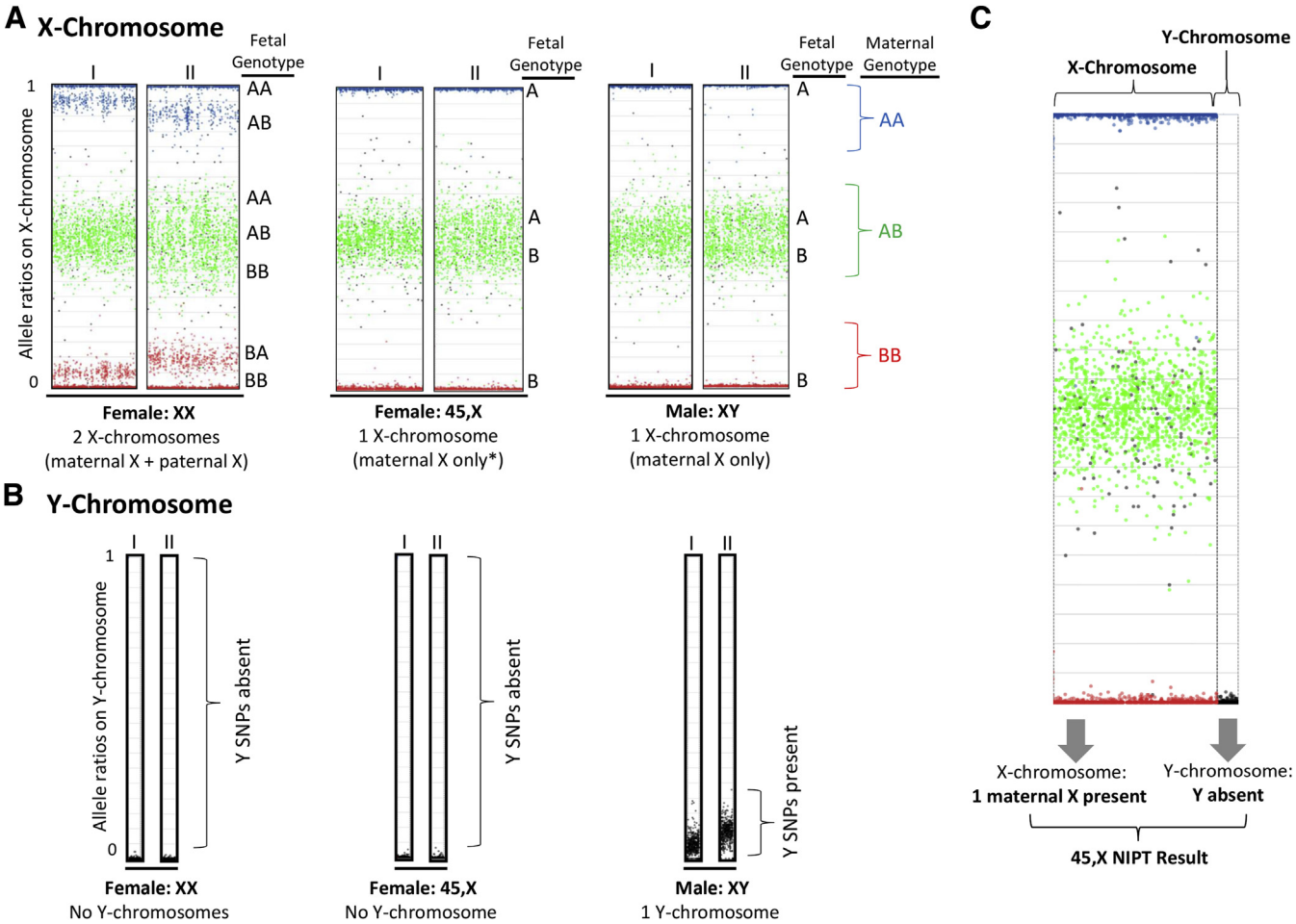
Appendix

SUPPLEMENTARY FIGURE
45,X/46,XY mosaicism may explain the single discordant fetal sex result



Single-nucleotide polymorphism (SNP) data for single discordant fetal sex case are consistent with monosomy X fetus. Representative **A**, X-chromosome and **B**, Y-chromosome SNP plots from female (XX), male (XY), and monosomy X (45,X) fetuses are shown using samples with fetal fractions of around 10% (I) and 20% (II). X-axis of each SNP plot represents the position along the chromosome, and y-axis indicates allele ratio. **A**, Fetal SNP data are colored based on maternal genotype, with alleles arbitrarily labeled as A or B: where AA is *blue*, AB is *green*, and BB is *red*. When the maternal genotype is homozygous at a specific SNP location (*red or blue dots*), the presence of single X-chromosome (45,X fetus or XY fetus) can easily be distinguished from 2 X-chromosomes (XX fetus); 45,X fetus with single paternal X-chromosome has a different SNP profile to that shown, but is easily distinguished by the absence of maternal X-chromosome-derived SNPs in the fetus. **B**, Males are determined by the presence of Y-chromosome SNPs; as fetal fraction increases, Y-chromosome SNPs migrate further away from X-axis, but Y-chromosome SNPs remain detectable down to at least 4% fetal fraction. **C**, For the single discordant fetal sex case that had a fetal fraction of 10%, SNP data clearly indicate the presence of a single maternal X-chromosome, with no paternal X-chromosome or Y-chromosome detected, leading to the monosomy X result. Mosaicism, which is frequently seen in association with a 45,X cell line, is a possible explanation for this discordant result.

NIPT, noninvasive prenatal testing.

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SUPPLEMENTARY TABLE 1

Prevalence of *International Classification of Diseases, Ninth Revision* codes in low-risk, high-risk, and advanced maternal age women

ICD-9 code	Description	LR, n	AMA, n	HR, n	Code type
228.1	Lymphangioma, any site	1	0	2	LR
278	Obesity, unspecified	0	1	1	LR
293.84	Anxiety disorder in conditions classified elsewhere	1	0	0	LR
300	Anxiety, dissociative and somatoform disorders —anxiety state unspecified	0	0	11	LR
305.03	Alcohol abuse, in remission	0	0	1	LR
305.1	Tobacco use disorder (tobacco dependence)	0	0	1	LR
306	Physiological malfunction arising from mental factors —musculoskeletal	0	0	1	LR
313.1	Disturbance of emotions specific to childhood and adolescence—misery and unhappiness disorder	1	0	0	LR
345	Epilepsy and recurrent seizures	0	0	1	LR
622.1	Dysplasia of cervix	6	0	0	LR
648.13	Thyroid dysfunction—antepartum condition or complication—not delivered during current episode of care	0	0	1	LR
649.13	Obesity complicating pregnancy, childbirth, or puerperium—antepartum condition or complication —not delivered during current episode of care	0	1	0	LR
649.43	Epilepsy complicating pregnancy, childbirth, or puerperium (antepartum obstetric condition, not delivered during current episode of care)	0	1	0	LR
655.53	Suspected damage to fetus from drugs (antepartum condition or complication)	1	2	1	LR
655.63	Suspected damage to fetus from radiation	0	1	0	LR
656.13	Other known or suspected fetal and placental problems affecting management of mother—Rhesus isoimmunization	1	0	0	LR
695.3	Rosacea—acne	0	0	1	LR
767.5	Facial nerve injury—facial palsy	0	0	2	LR
780.39	Other convulsions	0	1	0	LR
790.92	Abnormal coagulation profile	0	0	1	LR
795.79	Other and unspecified nonspecific immunological findings (raised antibody titer, raised level of immunoglobulins)	0	0	1	LR
V13.29	Personal history of disease—other genital system and obstetric disorders	0	0	1	LR
V13.63	Personal history of congenital malformations of nervous system	1	0	0	LR
V19.5	Family history of skin condition	1	1	1	LR
V22.0	Supervision of normal first pregnancy	21	7	12	LR
V22.1	Supervision of other normal pregnancy	905	2421	2133	LR
V22.2	Pregnant state, incidental	28	8	6	LR
V23.41	Pregnancy with history of preterm labor	1	0	0	LR

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(continued)

SUPPLEMENTARY TABLE 1

Prevalence of *International Classification of Diseases, Ninth Revision* codes in low-risk, high-risk, and advanced maternal age women *(continued)*

ICD-9 code	Description	LR, n	AMA, n	HR, n	Code type
V23.85	Pregnancy resulting from assisted reproductive technology	0	1	0	LR
V26.31	Testing of female genetic disease carrier status	469	1476	1305	LR
V28.0	Encounter for antenatal screening of mother—screening for chromosomal anomalies by amniocentesis	0	2	1	LR
V28.1	Screening for raised alpha-fetoprotein levels in amniotic fluid	0	0	2	LR
V28.3	Encounter for routine screening for malformation using ultrasonics	2	0	1	LR
V28.6	Encounter for antenatal screening of mother—screening for streptococcus B	1	0	0	LR
V72.40	Pregnancy examination or test—pregnancy unconfirmed	0	1	0	LR
V72.42	Pregnancy examination or test—positive result	0	0	1	LR
V77.2	Special screening for endocrine, nutritional, metabolic, and immunity disorders—malnutrition	0	0	1	LR
V77.6	Special screen for cystic fibrosis	19	19	19	LR
V77.7	Special screen for other inborn errors of metabolism	13	14	14	LR
V78.2	Special screen for sickle-cell disease	13	14	14	LR
V78.3	Special screen for other hemoglobinopathies	13	14	14	LR
V82.9	Unspecified condition	1	0	0	LR
659.53	AMA—first pregnancy	29[a]	556	116	AMA
659.6	Elderly multigravida (unspecified as to episode of care or not applicable)	0	1	1	AMA
659.63	AMA—not first pregnancy	33[a]	1489	343	AMA
V23.82	Supervision of other HR pregnancy, elderly primigravida	0	0	16	AMA
348	Other conditions of brain	0	0	1	HR
429.3	Cardiomegaly (cardiac: dilatation, hypertrophy, Ventricular dilatation)	0	0	1	HR
591	Hydronephrosis	0	0	1	HR
593.89	Other specified disorders of kidney and ureter—other	0	0	1	HR
606.9	Male infertility, unspecified	0	0	1	HR
628	Infertility, female—associated with anovulation	0	0	2	HR
628.8	Infertility, female of unspecified origin	0	0	2	HR
629.9	Unspecified disorder of female genital organs	0	0	1	HR
640	Hemorrhage in early pregnancy, threatened abortion (unspecified as to episode of care or not applicable)	0	0	2	HR
646.03	Other complications of pregnancy, not elsewhere classified—papyraceous fetus (antepartum condition or complication)	0	0	1	HR
646.3	Recurrent pregnancy loss (unspecified as to episode of care or not applicable)	0	0	1	HR

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(continued)

SUPPLEMENTARY TABLE 1

Prevalence of *International Classification of Diseases, Ninth Revision* codes in low-risk, high-risk, and advanced maternal age women *(continued)*

ICD-9 code	Description	LR, n	AMA, n	HR, n	Code type
646.31	Habitual aborter (for 646.3)	0	0	1	HR
646.33	Recurrent pregnancy loss (antepartum condition or complication not delivered during current episode of care)	0	0	4	HR
655.03	Central nervous system malformation in fetus—antepartum condition or complication	0	0	12	HR
655.13	Chromosomal abnormality in fetus (antepartum condition or complication)	0	0	408	HR
655.23	Hereditary disease in family possibly affecting fetus (antepartum condition or complication)	0	0	70	HR
655.8	Other known or suspected fetal and placental problems affecting management of mother	0	0	4	HR
655.83	Other known or suspected fetal abnormality, not elsewhere classified—antepartum condition or complication	0	0	185	HR
655.9	Known or suspected fetal abnormality affecting management of mother—unspecified (unspecified as to episode of care or not applicable)	0	0	1	HR
655.93	Known or suspected fetal abnormality affecting management of mother—unspecified (antepartum condition or complication)	0	0	8	HR
656.43	Intrauterine death (antepartum condition or complication)	0	0	1	HR
656.53	Poor fetal growth—antepartum condition or complication	0	0	2	HR
658.03	Oligohydramnios (antepartum condition or complication)	0	0	2	HR
659.61	Elderly multigravida (antepartum condition or complication)	0	0	1	HR
659.73	Abnormality in fetal heart rate or rhythm (antepartum condition or complication)	0	0	1	HR
663.03	Umbilical cord complication—prolapse of cord—presentation of cord (antepartum condition or complication)	0	0	1	HR
663.83	Other umbilical cord complications—velamentous insertion of umbilical cord	0	0	4	HR
741	Spina bifida with hydrocephalus—unspecified region	0	0	1	HR
742.3	Congenital hydrocephalus	0	0	1	HR
742.4	Other specified anomalies of brain	0	0	3	HR
742.9	Unspecified anomaly of brain, spinal cord, and nervous system	0	0	1	HR
745.1	Congenital anomalies—complete transposition of great vessels	0	0	1	HR
745.4	Ventricular septal defect	0	0	1	HR
746.7	Hypoplastic left heart syndrome	0	0	1	HR
746.9	Unspecified anomaly of heart—congenital	0	0	1	HR

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(continued)

SUPPLEMENTARY TABLE 1

Prevalence of *International Classification of Diseases, Ninth Revision* codes in low-risk, high-risk, and advanced maternal age women *(continued)*

ICD-9 code	Description	LR, n	AMA, n	HR, n	Code type
747.5	Absence or hypoplasia of umbilical artery—single umbilical artery	0	0	3	HR
747.89	Other specified anomalies of circulatory system—other (aneurysm, congenital, specified site not elsewhere classified)	0	0	1	HR
748.1	Other anomalies of nose	0	0	1	HR
753.29	Obstructive defects of renal pelvis and ureter—other	0	0	6	HR
754.7	Other deformities of feet—talipes, unspecified	0	0	1	HR
755.34	Reduction deformities of lower limb—longitudinal deficiency, femoral, complete or partial (congenital absence of femur)	0	0	1	HR
756.17	Anomalies of spine—spina bifida occulta	0	0	1	HR
758	Down syndrome	0	0	18	HR
758.2	Chromosomal anomalies—Edward syndrome	0	0	17	HR
758.5	Other condition due to autosomal anomalies (fetal aneuploidy)	0	0	6	HR
758.9	Condition due to anomaly of unspecified chromosome	0	0	1	HR
759.7	Multiple congenital anomalies, so described	0	0	2	HR
759.9	Congenital anomaly, unspecified	0	0	1	HR
764	"Light for dates" without mention of fetal malnutrition	0	0	1	HR
793.20	Nonspecific (abnormal) findings on radiological and other examination of body structure—other intrathoracic organ	0	0	10	HR
793.60	Nonspecific (abnormal) findings on radiological and other examination of body structure—abdominal area, including retroperitoneum	0	0	1	HR
793.99	Nonspecific (abnormal) findings on radiological and other examination of body structure—other (placental finding by x-ray or ultrasound method, radiological findings in skin and subcutaneous tissue)	0	0	2	HR
796.5	Abnormal/positive serum screening	0	0	548	HR
V13.69	Personal history of other (corrected) congenital malformations	0	0	1	HR
V18.4	Family history of certain other specific conditions —intellectual disabilities	0	0	1	HR
V18.9	Family history of certain other specific conditions —genetic disease carrier	0	0	3	HR
V19.8	Family history of "other condition"	0	0	221	HR
V23.0	Pregnancy with history of infertility	0	0	123	HR
V23.49	Pregnancy with poor reproductive history (prior pregnancy with aneuploidy)	0	0	19	HR
V23.5	Pregnancy with other poor reproductive history	0	0	123	HR
V23.81	Supervision of other HR pregnancy	0	0	15	HR
V23.89	Other HR pregnancy	0	0	5	HR

Dar. Clinical performance of SNP-based NIPT. Am J Obstet Gynecol 2014. *(continued)*

SUPPLEMENTARY TABLE 1

Prevalence of *International Classification of Diseases, Ninth Revision* codes in low-risk, high-risk, and advanced maternal age women *(continued)*

ICD-9 code	Description	LR, n	AMA, n	HR, n	Code type
V23.9	Unspecified HR pregnancy	0	0	6	HR
V26.89	Other specified procreative management	0	0	2	HR
V28.8	Other specified antenatal screening	0	0	17	HR
V28.81	Encounter for fetal anatomic survey	0	0	1	HR
V28.89	Other specified antenatal screening (CVS, genomic screening, nuchal translucency testing, proteomic screening)	0	0	441	HR
V28.9	Unspecified antenatal screening	0	0	337	HR

All *ICD-9* codes recorded in patients in this study were included in table.

AMA, advanced maternal age; *CVS*, chorionic villus sampling; *HR*, high-risk; *ICD-9*, *International Classification of Diseases, Ninth Revision*; *LR*, low-risk.

[a] A small number of women assigned AMA codes but aged <35 y—and therefore not AMA—were included in low-risk cohort (n = 60).

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SUPPLEMENTARY TABLE 2

Exclusion categories for out-of-specification samples

Exclusion category	Count
Redraws accepted	
Insufficient serum/plasma	127
<9 wk of gestation[a]	70
Test cancelled	45
Sample collection date too old	28
Missing information	11
Sample damaged	4
Wrong tube	4
Other[b]	26
Redraws not requested	
Multiple gestation	8
Egg donor	1
Surrogate	1

[a] Redraws are accepted once patient reaches 9 wks of gestation; [b] Includes uncommon exclusion reasons, such as hemolyzed blood samples and missing state-required waivers.

Dar. Clinical performance of SNP-based NIPT. Am J Obstet Gynecol 2014.

SUPPLEMENTARY TABLE 3

Details of samples with failed quality metrics

Exclusion category	Count
Redraws accepted	
Low fetal fraction	1667
Labchip QC failed	48
Contamination	42
Laboratory error	34
Unexplained bad model fit	24
Insufficient DNA	17
Uninformative single-nucleotide polymorphism pattern of unknown origin[a]	13
Multiple sequencing failures	9
Redraws not requested	
Suspected egg donor/surrogate	60
Maternal loss of heterozygosity	38
Fetal loss of heterozygosity	12
Suspected maternal mosaicism	1
Suspected fetal mosaicism	1

QC, quality control.

[a] Unclear whether the uninformative single-nucleotide polymorphism pattern is maternal or fetal in origin.

Dar. Clinical performance of SNP-based NIPT. Am J Obstet Gynecol 2014.