Title: Clinical experience and follow-up with large scale single-nucleotide polymorphism-based non-invasive prenatal aneuploidy testing

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Condensation: Clinical performance of SNP-based NIPT in a mixed high- and low-risk population is consistent with performance in validation studies.

Short Title: Clinical performance of SNP-based NIPT

Abstract

Objective: To report on laboratory and clinical experience following six months of clinical implementation of a single-nucleotide polymorphism (SNP)-based non-invasive prenatal aneuploidy test in high- and low-risk women.

Study Design: All samples received between March and September 2013 and drawn after 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 sub-set of high-risk cases. False negative results were reported voluntarily by providers. Positive predictive value (PPV) was calculated from cases with an available pre- 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. Five-hundred and seven patients received a high-risk result for any of the four tested conditions (324 trisomy 21, 82 trisomy 18, 41 trisomy 13, 61 monosomy X; including one 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%) were 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 over 35 was similar to the PPV for women under 35 years. Two patients were reported as false negatives.

Conclusions: The data from this large-scale report on clinical application of a commercially available NIPT suggests that the clinical performance of this SNP-based NIPT in a mixed high and low risk population is consistent with performance in validation studies.

Keywords: low-risk, non-invasive prenatal testing, single-nucleotide polymorphism, trisomy 21

Introduction

Since becoming clinically available in late 2011, cell-free DNA (cfDNA)-based non-invasive 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 and lower false positive 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 is 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 reference chromosomes.[7, 8, 11-13, 18-22] The second approach entails specific amplification and sequencing of single-nucleotide polymorphisms (SNP's).[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 fewer than 1,000 total patients.[26-29] Here, laboratory and clinical experience of over 31,000 women that received prenatal screening with a SNP-based NIPT is reported.

Materials and Methods

This is a retrospective analysis of prospectively collected data on 31,030 cases received for commercial testing between March and September 2013. This study received a Notification of Exempt Determination from an Institutional Review Board (Albert Einstein College of Medicine Institutional Review Board, IRB#: 2014-3307). Samples were classified as out-of-specification (OOS) 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 (<13ml), 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's CLIA- and CAP-certified laboratory (San Carlos, California). Laboratory testing was performed as previously described using validated methodologies for cfDNA isolation, PCR amplification targeting 19,488 SNPs, high-throughput sequencing, and analysis using 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 four 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 more than two fetal haplotypes (indicative of either triploidy or multiple 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 9 (ICD9) codes (Suppl. Table 1) were categorized into three sub-cohorts: low-risk, if <35 years and no aneuploidy-related high-risk codes, at-risk for fetal aneuploidy based solely on being ≥35 years, or high-risk for fetal aneuploidy by ICD9 code, regardless of maternal age. High-risk indications included positive screening tests, ultrasound anomalies, and relevant family history. Patients without reported ICD9 codes were categorized as low-risk (<35 years) or high-risk (≥35 years) by maternal age.

Follow-up information on high-risk results was obtained by phone 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 results. Samples were categorized as follows: (1) “true positive” (TP) included high-risk samples that were confirmed by pre- or postnatal diagnostic

testing, or based on clinical evaluation at birth; (2) “false positive” (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 one structural anomaly and one 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) “false negative” (FN) included 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 discordancy between placental and fetal karyotypes.

Patient and sample characteristics were expressed as means, standard deviations, 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 determined using a Mann-Whitney Rank Sum Test. The two 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 Chi-square test. The Kruskal-Wallis One 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 was used for all statistical analyses. $P<0.05$ 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) over 35 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 (Suppl. Table 2), 1966 samples failed quality control metrics (Suppl. 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 over 200 pounds, where inclusion of a paternal sample reduced the redraw rate from 27.5% to 16.1% (p=<0.001). The average turn-around time (TAT) 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 TAT in the last month of 6.7 calendar days (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 =<0.001; Figure 3A), and a negative association between fetal fraction and maternal weight (r^2=0.16, p = <0.001; Figure 3B). Furthermore, with increasing maternal weight, there was an increase in maternal cfDNA (p=<0.001) and a decrease in fetal cfDNA (p=<0.001) (Figure 4). Fetal fractions when stratified by aneuploidy, were decreased for trisomy 13 (0.759 MoM, p=<0.001), trisomy 18 (0.919 MoM, p=0.012), and monosomy X (0.835 MoM, p=<0.001), and increased for trisomy 21 samples (1.048 MoM, p=0.018).

NIPT results

The combined rate of high-risk calls for all four 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 (Suppl. Figure 1) 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 more than two fetal haplotypes, indicative of either unreported twins, vanishing twin, or triploidy.

ICD9 codes were associated with 19.0% (5,468/28,739) of women: 16.6% were low-risk, 44.1% were high-risk based only on advanced maternal age (AMA, ≥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 AMA women (1.6%), and largest in the high-risk group (3.4%) (Table 3). Results for the 23,271 samples without ICD9 codes showed a similar difference in aneuploidy calls between women <35 (1.0%, 117/11,629) and those ≥35 (2.4%, 274/11,642) years.

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 abortion. 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 CPM. Two CPM cases were high-risk for trisomy 13 and were identified as mosaics by 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 14 high-risk calls were within the follow-up cohort. Clinical follow-up revealed four cases with known outcomes: two TP (1 XXX, 1 XXY) and two 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=<0.001). 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 that elected to terminate the pregnancy without confirmation had a maximal risk score.

PPV

Based only on cases with cytogenetic diagnosis (Table 4), the PPV was 90.9% for trisomy 21 and 82.9% for all four cytogenetic abnormalities combined (Table 5). A theoretical PPV was also calculated under the two 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 ICD9-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% (CI 72.6%-95.7%). Of 176 older women with high-risk calls, 105 (59.7%) had confirmatory karyotyping and 87 were TP, a PPV of 82.9% (CI 74.3%-89.5%).

Discussion

This report of initial clinical experience with this SNP-based NIPT in over 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 four 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 two 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 over 200lb. 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 non-mosaic 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 true positive 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] Confined placental mosaicism (CPM), estimated to be present in 1-2% of 10-12 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 samples, Grati *et al.* estimated that the FP rate would be 0.08% for the four common aneuploidies.[37] Our findings, combined with the known incidence of CPM-related FPs and FNs, further reinforce the need for adequate pre-test counseling, as recommended by American College 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 under 35 years (87%) was similar to that of women over 35 (83%). This does not appear to be attributable to a bias in the referral of cases for karyotyping. Some women under 35 years of age 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 less than 35 years of age, versus 2.4% in women over 35 years (Table 3), supports that these two 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 FP's, like affected pregnancies, are also proportionately more common in older women; perhaps arising from trisomic conceptions that are rescued but express CPM. More data is 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 that were thought to have singleton pregnancies at the time of NIPT, 127 cases were identified as having more than two fetal haplotypes suggesting either triploidy or a previously undetected or vanishing twin. The SNP-based NIPT methodology provided the opportunity to identify these cases, pursue further diagnostic avenues, and avoid false positives that can arise using alternative methodologies.[22]

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 false negative report, and based on our laboratory experience, it is likely that false negatives would be voluntarily reported; there were two voluntarily reported false negatives. 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 for ACMG's recommendation for a national registry.[16]

In conclusion, this is a large-scale report of clinical utilization of NIPT. Analysis of over 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.

Comment

Clinical performance of SNP-based NIPT in a mixed high- and low-risk population is consistent with performance in validation studies. Similar positive predictive values were found in women under and over 35 years of age. 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 false positive 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 false positive results requires further investigation.

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Table 1. Demographics of commercial cases.

	Whole Cohort (N = 31,030)	Follow-Up Cohort (N = 17,885)
Maternal Age[1] (year)		
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 (weeks)		
Mean	14.0 ± 4.4	14.5 ± 4.7
Median	12.6	13.0
Range	3.1-40.9	9.0-40.9[2]
Maternal weight[2] (pounds)		
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[2]

[1]Maternal age at the estimated date of delivery. [2]As the follow-up cohort does not include any out-of-specification cases, or any cases that failed to receive a NIPT result,

the minimum gestational age and fetal fraction are higher than in the whole cohort. However, mean values and standard deviation are equivalent between the two cohorts.

[3]Analysis of maternal weight was limited to centers and laboratories that provided this information, and samples originating from the United States to avoid inconsistent weight units.

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

All Cases (N = 28,739[1])	Trisomy 21	Trisomy 18	Trisomy 13	Monosomy X
Risk ≥ 99/100	298[2]	78[2]	26	53
1/100 ≤ Risk < 99/100	25	4	15	8
TOTAL	324[2,3]	82[2]	41	61
Prevalence, 1 in:	88	349	697	467

[1]Total number of cases with a reported result at ≥9 weeks of gestation. [2]Trisomy 21 and trisomy 18 totals include the single case of double-aneuploidy.[3]Includes one case with a detected maternal chromosome 21 duplication, as such the fetus was determined to be high risk for trisomy 21 but the algorithm did not calculate a risk score.

Table 3. Aneuploidy calls in different *a priori* risk groups. Women with ICD-9 codes were sorted into three risk populations based on ICD-9 codes and maternal age; low-risk women under 35 years of age, women of advanced maternal age (AMA, ≥35 years of age) with no other high-risk codes, and high-risk women of any age. Women without ICD-9 codes were sorted into two risk populations based on maternal age; low-risk women under 35 years of age, and high-risk women of AMA.

[1] Mean ± SD. [2]Trisomy 21 and trisomy 18 totals include the single case of double-aneuploidy. Different superscripts indicate a significant difference between the groups (P=<0.001), as determined by a Kruskal-Wallis One Way Analysis of Variance on Ranks test.* Indicates a significant difference in the aneuploidy call rate between the three groups with ICD-9 codes (P=<0.001), as determined by a Chi-square test. ‡ Indicates a significant difference in the aneuploidy call rate between the two groups without ICD-9 codes (P=<0.001), as determined by a Chi-square test.

	Cases with ICD9 codes (N = 5,468)			Cases without codes (N = 23,271)	
	Low-Risk, <35 years (N = 909)	AMA only ≥35 years (N = 2411)	High-Risk All ages (N = 2148)	Low Risk, <35 years (N = 11,629)	High Risk, ≥35 years (N = 11,642)
Maternal Age, years[1]	28.2 ± 4.4	37.8 ± 2.4	31.3 ± 5.8	28.4 ± 4.5	37.9 ± 2.5
Median	29.0[a]	37.0[b]	32.0[c]	29.0[A]	37.0[B]
(Range)	(15.0-34.0)	(35.0-48.0)	(15.0-47.0)	(14.0-34.0)	(35.0-52.0)
Gestational Age, weeks[1]	14.1 ± 4.4	13.3 ± 3.5	15.8 ± 5.0	14.7 ± 4.9	13.4 ± 3.9
Median	12.4[a]	12.4[b]	14.4[c]	13.0[A]	12.1[B]
(Range)	(9.0-33.3)	(9.0-38.1)	(9.0-37.0)	(9.0-38.0)	(9.0-40.9)
Euploid	903	2,368	2,073	11,457	11,293
Trisomy 21	2	27[2]	50	57	188
Trisomy 18	1	5[2]	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%*	1.58%*	3.35%*	1.01%[‡]	2.35%[‡]

Table 4: Clinical follow-up findings.

(N = 17,885[1])	Trisomy 21	Trisomy 18	Trisomy 13	Monosomy X	Total
High-Risk Calls	233[a]	55[a]	30	38	356
Confirmed Outcomes					
True Positive	140[b]	27	8	9	184
False Positive	14[c]	2[d]	13[e,f]	9	38
Unconfirmed Outcomes					
Suggestive[2]	8	9	0	2	19
Pregnancy Loss[3]	18	6	3	9	36
Termination[4]	14	3	0	5	22
No Follow-Up[5]	39	8	6[g]	4	57
Low-Risk Calls					
Confirmed Outcomes:					
False Negatives	2	0	0	0	2

[1]Total number of cases with a reported result at ≥9 weeks of gestation from participating centers. [2]Patients declined invasive testing but ultrasound findings were consistent with NIPT findings (see Materials and Methods). [3]Patients experienced spontaneous abortion and did not obtain karyotype confirmation. [4]Patients chose to terminate the pregnancy without diagnostic testing. [5]Follow-up information was not available. [a]Trisomy 21 and trisomy 18 totals include a single double-aneuploidy case.[b]Includes 13 cases reported as trisomy 21 based on at-birth clinical evaluation. [c]Includes three cases reported as normal based on at-birth clinical evaluation. [d]Includes one CPM case.

[e]Includes two CPM cases (one confirmed and one unconfirmed). [f]Includes one case reported as normal based on at-birth clinical evaluation. [g]One sample tested as high-risk (1/7.6) for fetal aneuploidy; analysis of a second sample indicated that the patient was at low-risk; follow-up information was not available.

Table 5: Positive predictive values. Positive predictive values (PPV) calculated as (true positive)/(true positive + false positive).

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

Data is presented for just those cases where there was cytogenetic or clinical confirmation of the result; based on the extreme condition that all unconfirmed cases were false positives (lower bound); and the opposite condition that all unconfirmed results were true positive (upper bound). True positive (TP); false positive (FP).

Supplementary Table 1. Prevalence of ICD9 codes in low-risk, high-risk, and advanced maternal age (AMA) women. All ICD9 codes recorded in patients in this study were included in the table.

ICD-9 Code	Description	Low Risk (N)	AMA (N)	High Risk (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 the current episode of care	0	0	1	LR
649.13	Obesity complicating pregnancy, childbirth or the puerperium - antepartum condition or complication - not delivered during the current episode of care	0	1	0	LR
649.43	Epilepsy complicating pregnancy, childbirth, or the puerperium (antepartum obstetric condition, not delivered during the 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	0	0	1	LR

	immunological findings (Raised antibody titer, Raised level of immunoglobulins)				
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 pre-term labor	1	0	0	LR
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	Advanced maternal age - 1st pregnancy	29[1]	556	116	AMA
659.6	Elderly multigravida (unspecified as to episode of care or not applicable)	0	1	1	AMA
659.63	Advanced maternal age - not 1st pregnancy	33[1]	1489	343	AMA
V23.82	Supervision of other high-risk 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
646.31	Habitual aborter (for 646.3)	0	0	1	HR
646.33	Recurrent pregnancy loss (Antepartum condition or complication not delivered during the 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 the 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 the 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
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 the spine - Spina bifida occulta	0	0	1	HR
758	Down's syndrome	0	0	18	HR
758.2	Chromosomal anomalies - Edward's 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 an aneuploidy)	0	0	19	HR
V23.5	Pregnancy with other poor reproductive history	0	0	123	HR
V23.81	Supervision of other high-risk pregnancy	0	0	15	HR
V23.89	Other high-risk pregnancy	0	0	5	HR
V23.9	Unspecified high-risk 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

[1]A small number of women that were assigned AMA codes but were under 35 years of age – and therefore not AMA – were included in the low-risk cohort (N = 60).

Supplementary Table 2. Exclusion categories for out-of-specification samples.

Exclusion Category	Count
Redraws Accepted:	
Insufficient serum/plasma	127
<9 weeks of gestation[1]	70
Test cancelled	45
Sample collection date too old	28
Missing information	11
Sample damaged	4
Wrong tube	4
Other[2]	26
Redraws Not Requested:	
Multiple gestation	8
Egg donor	1
Surrogate	1

[1]Redraws are accepted once the patient reaches 9 weeks of gestation. [2]Includes uncommon exclusion reasons, such as hemolyzed blood samples and missing state-required waivers.

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
Lab error	34
Unexplained bad model fit	24
Insufficient DNA	17
Stats no-call other	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

Figure Legends

Figure 1: Study flow chart. OOS, out-of-specification samples (see Materials and Methods).

Figure 2: Decrease in redraw rates overall **(A)** and for patients including a father sample **(B)** over the reporting period (March 1, 2013 through September 20, 2013) for samples ≥10 weeks of gestation.

Figure 3: 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 the box indicates median, capped whiskers indicate 90th (upper) and 10th (lower) percentiles, the number in each grouping is indicated above the 90th percentile whisker.

Figure 4: Box plots depicting absolute levels of **(A)** maternal and **(B)** fetal cfDNA in maternal circulation as a function of maternal weight. Boxes indicate 75th (upper) and 25th (lower) quartiles, solid line within the box indicates median, dashed line within the box indicates mean, capped whiskers indicate 90th (upper) and 10th (lower) percentiles, diamonds indicate 95th (upper) and 5th (lower) percentiles.

Supplementary Figure 1: SNP-data for the single discordant fetal sex case is consistent with a monosomy X fetus. Representative X-chromosome (**A**) and Y-chromosome (**B**) 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). The x-axis of each SNP plot represents the position along the chromosome, and the y-axis indicates the allele ratio. **A**. The fetal SNP data are colored based on the maternal genotype, with alleles arbitrarily labelled as A or B: where AA is colored blue, AB colored green, and BB colored red. When the maternal genotype is homozygous at specific SNP location (red or blue dots), the presence of a single X-chromosome (45,X fetus or XY fetus) can easily be distinguished from two X-chromosomes (XX fetus); a 45,X fetus with a 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 the 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 indicates 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.