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Noninvasive Prenatal Testing for Trisomy 21, 18 and 13 - Clinical Experience from 146,958 Pregnancies

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Hongyun Zhang, Ya Gao, Fuman Jiang, Meili Fu, Yuying Yuan, Yulai Guo, Zhongyi Zhu, Mengmeng Lin, Qiufang Liu, Zhongming Tian, Haiquan Zhang, Lijian Zhao, Xin Yi, Ye Yin, and Wei Wang are employees of BGI Diagnostics. Fang Chen is employee of BGI-Shenzhen. Tze Kin Lau has no financial relationship with BGI Diagnostics or BGI-Shenzhen.

Abstract

Objectives: The aim of this study was to report the clinical performance of massively parallel sequencing-based noninvasive prenatal testing (NIPT) in detecting T21, T18, and T13 in over 140,000 clinical samples. NIPT performance in low-risk pregnancies and high-risk pregnancies was also compared.

Methods: from January 1, 2012 to August 31, 2013, 147,314 NIPT requests were received for screening of fetal trisomy 21, 18, and 13 using low-coverage whole-genome sequencing of plasma cell free DNA. NIPT results were validated by karyotyping confirmation or follow-up of clinical outcomes.

Results: NIPT was performed on 146,958 samples, of which outcome data were available in 112,669 (76.7%). 3,213 cases required repeat blood sampling, 145 had no report. Aneuploidy was confirmed in

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720 of 781 T21-positive cases, 167 of 218 T18-positive cases, and 22 of 67 T13-positive cases. There were 9 false negative identified, including 6 T21 and 3 T18 cases. The overall sensitivity of NIPT was 99.17% for T21, 98.24% for T18, and 100% for T13, and the specificity was 99.95% for T21, 99.95% for T18, and 99.96% for T13. There was no significant difference in test performance between 72,382 high-risk and 40,287 low-risk subjects (sensitivity 99.21% vs. 98.97%, p=0.82; specificity 99.95% vs. 99.95%, p=0.98). The major factors contributing to NIPT false positive and false negative results were maternal copy number variant (CNV) and fetal/placental mosaicism, but not fetal fraction.

Conclusions: With stringent protocol, the high performance of NIPT showed by early validation studies could be maintained in large clinical services. NIPT can provide equally high sensitivity and specificity in screening T21 in low-risk population.

Introduction

Conventional prenatal screening for chromosomal abnormalities, mostly T21, relied exclusively on biochemical and sonographic measurements in the first and second trimester. With 5% false positive rate (FPR), conventional screenings achieve a detection rate of about 60-95% for T21 (1-3). In 2008, two studies showed the noninvasive prenatal testing (NIPT) for T21 by sequencing cell-free DNA (cfDNA) in maternal plasma with very low false positive rates (4, 5). Both studies implied that the test could reduce unnecessary invasive procedures and iatrogenic fetal losses. Since then, NIPT has been developed to provide early and safe detection of fetal T21 and other common aneuploidies using shotgun sequencing, targeted sequencing, and single nucleotide polymorphism (SNP)-based sequencing of cfDNA (6-14). With solid evidence of NIPT

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performance in small-scale of predominantly high-risk populations, different professional societies quickly suggested that NIPT can be considered as a second-tier screening test for women with increased aneuploidy risks (15-17).

Recently, several studies suggested that NIPT can offer a comparable performance in the general population as in the high-risk population (18-20). One study showed the high detection rate of T21 and T18 in 11,105 singleton cases with more than 65% from high-risk pregnancy (20). In another study, NIPT prominently improved the positive predictive value (PPV) comparing to conventional screening in a small general population of 1,914 women (21). However, these studies were conducted in populations with small number of low-risk pregnancy, and NIPT performance evaluation in large clinical data in general population is urgently needed.

NIPT has been provided as a screening test in China since 2011, and this test may soon be considered for routine use by local Chinese health authorities after the recent approval by the China Food and Drug Administration (CFDA). However, clinical experience of large-scale NIPT performance in the general population is still lacking, despite the estimation that over 500,000 NIPTs have been offered to date globally (Bianchi DW, personal communication). This study was based on clinical data of 146,958 NIPT tests from Mainland China, the largest to our knowledge. The main purpose was to report the NIPT performance in detecting T21, T18, and T13 in a large-scale clinical service for quality assurance purpose. Possible cause for NIPT false positive (FP) and false negative (FN) results were investigated. In a subset of samples with known clinical outcomes, NIPT performances in detecting T21 between high-risk

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and low-risk subjects were compared.

Methods

Patients and sample collection

This is a prospective and multicenter observation study with the participants enrolled from 508 medical centers in Mainland China from January 1, 2012 to August 31, 2013, excluding the previously reported cases [22]. NIPT was provided either as a primary or secondary screening to the participants. Since the objective of the study was to evaluate the performance of detecting fetal T21, T18, and T13, other chromosomal abnormalities were performed but not analyzed in this study. To be eligible for the test, participants had to be at least 18 years old, with a singleton or twin pregnancy at 9 weeks of gestation or beyond. All participants underwent pre-test counseling and an informed written consent was obtained before blood sampling. Approvals were obtained from the institutional review board of BGI (BGI-IRB).

Sample collection, sequencing and bioinformatics analysis

Five milliliters of peripheral blood was collected into EDTA tubes from each participant. Blood samples were prepared for cfDNA sequencing as described before (20). In general, whole blood was ice-centrifuged twice to extract plasma within eight hours after blood collection. Plasma samples were then frozen and delivered to the Minister of Health (MOH) accredited and ISO/IEC17025-certified clinical laboratories of BGI-Diagnostics where plasma was prepared for library construction, quality control, and pooling. For

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sequencing, 24 libraries were sequenced with 36-cycles single-end multiplex sequencing using Illumina HiSeq2000 platforms. A barcode tracking system was employed during sample preparation. 35 base sequencing reads were trimmed and aligned to a universal unique read set incised from the human reference genome (hg18, NCBI build 36). A binary hypothesis t-test and logarithmic likelihood ratio L-score between the two t-tests were used to classify fetal autosomal aneuploidy of T21, T18, and T13, as described earlier (13, 20). The FCAPS algorithm was implemented into the routine analytic pipeline which can be used to identify chromosome copy number variation (CNV) based on the binary segmentation algorithm (22).

Confirmation of NIPT results and clinical outcome follow-up

Each participant received post-test counseling after NIPT test. Those with a positive NIPT result were advised to have prenatal diagnosis by an invasive test. If refused, the pregnancy was monitored to obtain pregnant outcome. Routine antenatal care was provided to those who had a negative NIPT result. Telephone interviews were performed one month after the expected date of confinement to collect information about fetal outcome, newborn physical examination, or any cytogenetic testing results. To encourage reporting of NIPT FP and FN results, an insurance policy was provided to each participant as a part of the test. The policy would reimburse the cost of invasive tests in case of a positive NIPT result, and would pay CNY 200,000 (approximate five times of GDP per capita in China in 2013) to each case of confirmed NIPT FN result.

Calculating NIPT performance

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Karyotyping results or clinical follow-up results were used as the gold standard to calculate the sensitivity and specificity of NIPT in this population. 95% confidence intervals were calculated on the base of a standard normal distribution.

Comparing NIPT performance in high-risk and low-risk group

Samples with outcome data were further divided into the high-risk group and low-risk group. A subject with any of the following factors was classified as high-risk: advanced maternal age (>35), positive conventional Down screening test (cut-off 1/270 or 1/300 depending on individual hospital's criteria), abnormal sonographic markers, family history of aneuploidy, and previous pregnancy of trisomic fetus. A subject with none of those factors was defined as low-risk. NIPT performance in detection of T21 in these two groups was compared using the karyotyping results or follow-up results as the gold standards. Owning to the small number of positive cases of T18 and T13 in the low-risk group, performance was not compared for these two trisomies.

Fetal cfDNA fraction estimation

To identify the role of fetal cfDNA fraction in NIPT FP and FN results, fetal cfDNA fraction was estimated in samples with FN and FP NIPT results, based on parental-specific homozygous SNP loci, which has been described previously (23). Briefly, parental-specific homozygous loci in the form of ♀AA♂BB were selected. Then the sequence reads from those loci were used to estimate the total fetal cfDNA concentration, using the formula of$f = \frac{2d(B)}{d(A)+d(B)}$, where d meant the depth of the allele A or B.

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Results

Study population

From January 1, 2012 to August 31, 2013, a total of 147,314 maternal blood samples were received for NIPT from 508 hospitals in Mainland China. 211 samples (0.14%) were rejected for further processing due to inadequate sample volume, contamination, <9 gestational weeks, or improper labeling (Figure 1). 3,213 samples (2.18%) required re-sampling because of failing quality control, assay failure, or low fetal fraction. Ultimately, 145 samples (0.098%) failed to provide informative results and were classified as test failure.

The demographic characteristics of the remaining 146,958 cases are shown in Table 1, including 802 cases of twin pregnancies. Maternal age ranged from 17 to 46 with a mean of 30.9 years. Gestational ages at NIPT ranged from 9 to 36 weeks, with a mean of 18.7 weeks. Most samples were collected with maternal age below 35 years old (68.65%). The vast majority of the samples were collected in the second trimester (94.13%).

NIPT positive cases

1,578 in total of the 146,958 samples (1.07%) had positive NIPT results, including 1,107 cases for T21, 352 cases for T18, and 119 cases for T13 (Figure 1). After post-test counseling, 1,055 (66.86%) NIPT positive cases had a prenatal diagnostic test, in which 719 of T21 cases, 167 of T18 cases, and 22 of T13 cases were confirmed by karyotyping

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results, whereas 54 of T21, 50 of T18, and 43 of T13 were unconfirmed (Figure 1).

In the remaining NIPT positive cases without prenatal diagnosis, clinical outcomes were obtained through neonatal physical examination and the records of adverse pregnant outcomes (Figure 1). Seven cases of T21, one case of T18, and two cases of T13 were reported to have apparently normal phenotypes, while one case of T21 was reported to have typical T21 phenotypes at birth. 121 T21, 63 T18, and 21 T13 cases underwent elective abortion due to ultrasound abnormalities (increased NT, cardiac defects, or malformation); 11 T21, 11 T18, and 2 T13 cases ended with stillbirth or spontaneous miscarriage.

283 cases had no pregnant outcomes due to loss of contact or declined follow-up (Figure 1). Nonetheless, 232 cases in these 283 cases (82%) had at least one high-risk indication (advanced maternal age, or previous high risk Down screening results, or abnormal ultrasound findings), which showed good consistence to the NIPT results in identifying high-risk pregnancies (Supplemental Table 1).

NIPT negative cases

A total of 145,380 cases had NIPT negative results, all of which were contacted by telephone interviews one month after the expected date of confinement (Figure 1). Informative follow-up results were available in 111,594 cases, resulting in a successful follow-up rate of 76.76% (111,594/145,380). 106,989 cases had live births with normal neonatal examination results (Figure 1), including 405 cases who also received invasive diagnosis mainly due to anxiety and were confirmed to be normal. Another 4,605 cases

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had live birth with birth defects confirmed to be irrelevant to trisomies, including cleft palate, hearing screening failure, metabolic disease, cardiac defects, and etc. (Supplemental Table 2).

678 cases ended with pregnancy loss, and 33,099 cases had no clinical outcomes because of loss of contact/declined interview. In all the NIPT negative cases, nine FN results were reported through our insurance program, all confirmed by cytogenetic studies (Figure 1 and Table 5). Six live births with typical T21 phenotypes were reported shortly after birth and confirmed by subsequent cytogenetic result. Three cases had termination of pregnancy due to severe developmental malformations by ultrasound examination, and later confirmed as true T18 by cytogenetic examinations of product of conception (Table 5).

Clinical performance of NIPT in testing T21, T18, and T13

Hence in this population of 146,958 pregnancies, cytogenetic or phenotypic confirmation of NIPT results was available in 1,066 NIPT positive cases and 111,603 NIPT negative cases, collectively accounting for 76.67% of the population including twin pregnancies. Further calculation of NIPT sensitivity and specificity were based on this subgroup with outcome data. In this subgroup, for detecting T21, there were 720 true positive cases, 61 FP cases, and six FN cases, resulting in a sensitivity of 99.17% and specificity of 99.95% (Table 2). The false positive rate (FPR) and PPV for T21 were 0.05% and 92.19% respectively. For the detection of T18, there were 167 TP cases, 51 FP cases, and three FN cases, giving a sensitivity of 98.24% and specificity of 99.95%. The FPR

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and PPV for T18 were 0.05% and 76.61% respectively. For NIPT detection of T13, there were 22 TP cases, 45 FP cases, and 0 FN cases, with a sensitivity of 100% and specificity of 99.96%. The FPR and PPV for T13 were 0.04% and 32.84%. The overall sensitivity and specificity for these three chromosomes combined were 99.02% and 99.86%, and the overall FPR and PPV were 0.14% and 85.27%. The disease incidence rate for T21, T18, and T13 were 0.64%, 0.15%, and 0.02% respectively. A theoretical PPV was also calculated under the two boundary conditions that all unproven NIPT positive cases were either assumed TP or FP (Table 2). This provided the range of PPV for T21 as 65-94%, for T18 as 47-85%, and for T13 as 18-62%.

NIPT in twin pregnancies

802 twin pregnancies were included in the population. Seven cases had the NIPT positive result of T21, and 795 cases had NIPT negative results. No T18 or T13 was detected. Karyotyping confirmation showed five TP cases and two FP cases, while follow-up results were available in 397 NIPT negative cases all showing normal phenotypes (Table 2). Hence in twin pregnancies, the NIPT in detection of T21 had the sensitivity of 100% and specificity of 99.50%.

Clinical performance of NIPT in testing T21 in high-risk and low-risk subjects

In the samples with outcome data, 72,382 samples were classified as high-risk group. The remaining 40,287 samples were classified as low-risk group (Table 1). The NIPT performance in detecting T21 in both groups was compared in Table 3. 624 TP cases, 39 FP cases, and 5 FN cases were observed in the high-risk group, resulting in 99.21%

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sensitivity, 99.95% specificity, and 94.12% PPV respectively. In comparison, 96 TP cases, 22 FP cases, and one FN case were observed in the low-risk group, resulting in 98.97% sensitivity, 99.95% specificity, and 81.36% PPV. Statistical analysis by Fisher test showed no significant difference (sensitivity 99.21% vs. 98.97%, p=0.82; specificity 99.95% vs. 99.95%, p=0.98) of the performance between these two groups (Table 3), except the decreased PPV in the low-risk group which was expected due to lower incidences of T21. For T18 and T13 detection, due to insufficient positive cases in the low-risk group, performance was not calculated and compared.

Further investigation of FP and FN results

A total of 157 NIPT FP and nine NIPT FN results were identified in the present study. 41 cases had noticeable explanations, including 39 biological factors (27 of maternal CNV and 12 of mosaicism) causing FP results and 2 confined placental mosaicisms (CPM) causing FN results (Table 4). The remaining cases could not be confirmed by the above reasons mainly because of the lack of samples for confirmation.

Among FP cases, 27 cases had chromosome breakpoints and very large regional T-score identified by the FCAPS analysis, which indicated the existence of maternal background of CNV (Table 4). Confirmation using maternal white blood cells (WBC) sequencing showed that 21 in 27 cases had maternal chromosome duplication ranging from 0.5Mb to 14Mb on the relevant chromosomes, including 13 cases of T21, six cases of T18 and two cases of T13. 12 cases were suspected to have fetal or placental mosaicism based on the finding that the fetal fraction estimated by trisomic chromosome was dramatically

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lower than the fetal fraction estimated by Y chromosome (Table 4). For confirmation, placental tissues were available in four cases for further sequencing or karyotyping, showing confined placental mosaicism (CPM) of T13 in two cases (one with 70% placental mosaicism and one with complete placental T13) and undetected mosaicism in the other two cases (Table 4).

In addition, 10 FP results were based on phenotypic description with no cytogenetic confirmation, and thus cannot rule out the possibility of fetal mosaicism, CPM or maternal CNV. One T13 FP case had the NIPT result of multiple chromosome aneuploidies, which could be caused by sequencing data deviation or other biological factors. Unfortunately consent was not available for testing maternal WBC and hence the maternal genetic background was unknown. Two cases of T21 FP results were from twin pregnancies. Both cases had amniocentesis and karyotyping results showing normal karyotypes in both fetuses.

Among NIPT FN cases, maternal WBC sequencing found no maternal CNV background or mosaicism. Placental samples were obtained in two T18 FN cases, and both showed low level of CPM of T18 (Table 5). In particular, one of the T18 FN cases contained 30% T18 mosaicism and 60% XO mosaicism in multiple placental samples. The NIPT result of this case showed high risk of monosomy X but missed T18.

To verify the role of fetal cfDNA fraction in NIPT FP and FN results, 120 FP cases and eight FN cases with extra sample aliquots were tested with fetal cfDNA fraction. The tested FP cases had fetal fraction ranged from 3.54-21.94% with a mean of 9.74%, all

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above the minimum requirement of NIPT test (3.5%) (Table 4). In the tested FN cases, the fetal cfDNA fraction ranged from 5.18-13.39% with a mean of 10.2%, all higher than the minimum requirement of the test (Table 5).

Discussion

NIPT has been widely used to screen for T21, T18, and T13 in the past few years, yet large clinical data is still absent and concerns have been raised about the performance in large-scale clinical practice (24). To provide the large clinical data as a NIPT audit assessment, this multi-center prospective study collected over 140,000 clinical samples, the largest to our knowledge. The present study was performed in Mainland China where prenatal screening for Down syndrome mainly starts from the second trimester and NIPT is predominantly used as a secondary screening test. Thus most pregnant women received NIPT after 13th gestational week, and more than half of the population was high risk from biochemical screening.

One strength of this study was the estimation of NIPT sensitivity and specificity in a large prospective population with cytogenetic or phenotypic outcomes. Similar estimation has not been reported in several recent studies due to small sample size or insufficient follow-up (19, 21, 25, 26). Our data demonstrated that NIPT in large-scale clinical practice maintained high sensitivity (99.17% for T21, 98.24% for T18, and 100% for T13) and specificity (99.95% for T21, 99.95% for T18, and 99.96% for T13). When compared to studies in high-risk populations (6, 8-13, 27, 28), our data showed

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comparable if not better performance (Supplemental Table 3). Thus with strict protocol and quality management, the clinical efficacy of NIPT did not deteriorate in large scale of practice.

Another strength of this study was the comparison of NIPT performance in 72,382 high-risk pregnancies and 40,287 low-risk pregnancies, which was so far the largest comparison. Previous comparison was conducted with small sample size (21, 29), and conclusive evidence was still required based on large scale of clinical experience. Here, we defined the low-risk group with stringent criteria, containing none of the known high-risk factors. Our data showed comparable sensitivity (p=0.82) and specificity (p=0.98) in detection of T21 in the high-risk and low-risk group, adding to the recent data which only assessed PPV (21). Although NIPT has been only recommended in the high-risk population, similar fetal cfDNA distributions were recently found in the low-risk population, which provides the basis of uniform NIPT performance in the general population (30, 31). Importantly, our comparison data corroborates the effective performance of NIPT in general population. Nonetheless, the reduced PPV in the low-risk group as a consequence of lower disease prevalence reaffirmed the use of NIPT not as a diagnostic test and the necessity of invasive confirmation.

Several conditions have been known to contribute to NIPT FP and FN results: 1) low fetal fraction (32); 2) maternal chromosome abnormality (33); 3) genetic discordance between fetus and placenta i.e. CPM (34, 35); 4) fetal mosaicism (12); 5) vanishing twin (36, 37). In this study, low fetal fraction may not be a major factor in routine practice, since all the tested FP and FN cases in our data had fetal fractions above the NIPT

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requirement. In contrast, biological factors such as maternal background of CNV and CPM had important roles in causing FP and FN results. In addition, fetal pathogenic CNV and fetal mosaicism have also been reported to cause FP and FN results (12, 32, 38, 39). Thus these factors must be taken into account when interpreting NIPT results, and post-test genetic counseling should be provided to the pregnant women following the suggestions such as National Society of Genetic Counselors' statement (15). The NIPT algorithm in this study could not identify the maternal or fetal contribution of abnormality (i.e. CNV or mosaicism). Thus we recommend that each case with identified CNV or mosaicism by NIPT should be confirmed with the source of abnormality using maternal WBC, amniotic fluid, or multiple placental samples.

Most cases with suspected mosaicism or CPM could not be confirmed, mainly because of the difficulty of obtaining confirmatory samples, and the insufficient cells (~20 cells) of examination during routine cytogenetic analysis. We could not verify vanishing twins in this study, due to the lack of sonographic information of the cases. Also, due to the patient's refusal, we could not confirm the reason of one FP case with multiple chromosome aneuploidies, which may relate to maternal malignancy (40). About 2/3 of NIPT FP and FN cases had no noticeable biological factors for explanation. This probably reflected the insufficient resolution of conventional karyotyping in identifying mosaicism and submicroscopic CNV comparing to NIPT (38). Given improved confirmation methods such as microarray, more biological factors may be identified in these NIPT FP and FN cases.

The limitation of this study was the incomplete follow-up of NIPT results, which could

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introduce bias into the performance evaluation. In NIPT positive cases, only 66.8% of women provided the result of confirmatory diagnosis, mainly due to refusal to provide clinical outcomes (17.9%) and elective abortion (13.0%). This raised the importance of reinforcing the genetic counseling in the future clinical utilization of NIPT, and avoiding potential misuse of the test as a diagnostic method. Nonetheless, nearly 77% of the population had outcome data, and the demographic characteristics of this subgroup were similar to the total population. Thus performance based in this subgroup well-represented the total population. Lastly, with a well-operated insurance program to encourage confirmation of positive result and reporting of FN results, the possibility of missing NIPT false results remains low.

Conclusion

In conclusion, our study represented the largest clinical experience of NIPT to date. We showed that the NIPT performance in detecting T21, T18, and T13 was maintained at a high level, comparable to previous small-scale validation studies. Maternal genetic background and fetal/placental mosaicism played important roles in NIPT FP and FN results, whereas low fetal fraction is unlikely to be the significant contributor. Among the low-risk pregnant women, the NIPT performance in detecting T21 showed no statistical difference from the high-risk group. Our findings suggested that it is suitable to offer NIPT as a routine screening test for fetal T21, T18, and T13 in the general population.

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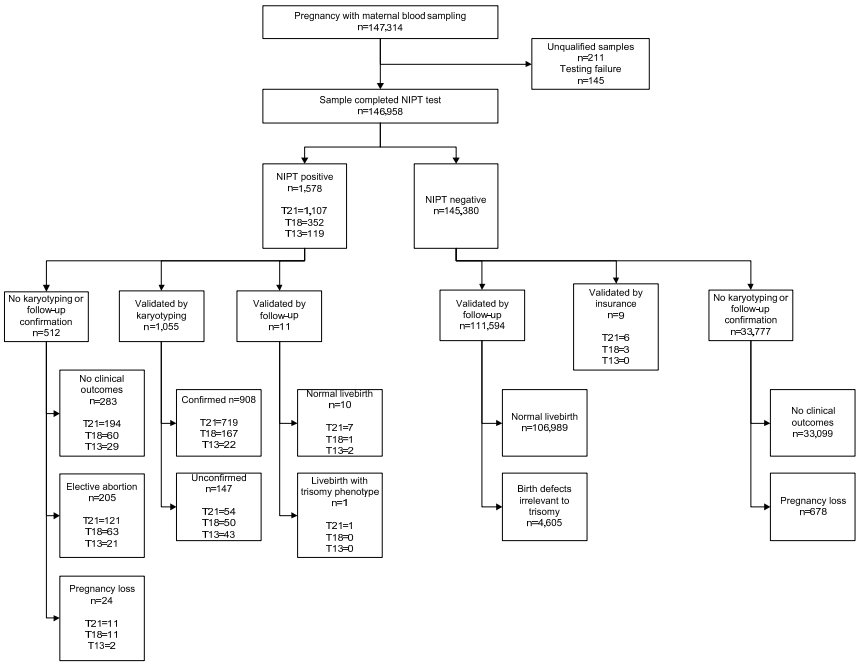

Figure 1: Study population, NIPT results, and clinical outcomes in 147,314 clinical tests. Primary analysis of NIPT performance was carried out in 112,669 cases with outcome data, including 1,055 NIPT positive cases verified by karyotyping results, 11 NIPT positive cases verified by follow-up results, 111,594 NIPT negative cases verified by follow-up results, and 9 NIPT negative cases identified by insurance program.

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