Efficiency of expanded noninvasive prenatal testing in the detection of fetal subchromosomal microdeletion and microduplication in a cohort of 31,256 single pregnancies

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Noninvasive prenatal testing (NIPT) is widely used to screen for common fetal chromosomal aneuploidies. However, the ability of NIPT-Plus to detect copy number variation (CNV) is debatable. Accordingly, we assessed the efficiency of NIPT-Plus to detect clinically significant fetal CNV. We performed a prospective analysis of 31,260 singleton pregnancies, included from June 2017 to December 2020. Cell-free fetal DNA was directly sequenced using the semiconductor sequencing platform for women with high-risk CNV with clinically significant results. Fetal karyotyping and chromosomal microarray analysis (or next-generation sequencing) are recommended for invasive diagnostic procedures. Women at low risk with no other abnormal results continued their pregnancies. We analyzed the expanded NIPT results, diagnostic test results, and follow-up information to evaluate its performance in detecting fetal CNV. Of the 31,260 pregnant women who received NIPT-Plus, 31,256 cases were tested successfully, a high risk of clinically significant CNV was detected in 221 cases (0.71%); 18 women refused further diagnosis; 203 women underwent invasive prenatal diagnosis; and 78 true positive cases and 125 false positive cases, with an overall positive predictive value (PPV) of 38.42% and a false positive rate of 0.40%. For known microdeletion/microduplication syndromes (n = 27), the PPVs were 75% DiGeorge syndrome (DGS), 80% 22q11.22 microduplication, 50% Prader–Willi syndrome, and 50% cri-du-chat. For the remaining clinically significant fetal CNVs (n = 175), the combined PPVs were 46.5% (CNVs > 10 Mb) and 28.57% (CNVs ≤ 10 Mb). NIPT-Plus screening for CNV has certain clinical value. NIPT-Plus yielded relatively high PPVs for 22q11.2 microduplication syndrome and DGS, and low to moderate PPVs for other CNVs.

Abbreviations
NIPT  Noninvasive prenatal testing
CNVs  Copy number variations
CMA  Chromosomal microarray analysis
LOH  Loss of heterozygosity
VOUS  Variants of unknown significance
AMA  Advanced maternal age
MMS  Microduplication/microdeletion syndrome

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NIPT and invasive testing were 17.3 ± 2.0 and 22.7 ± 2.6 weeks, respectively. Prior maternal serological screening included maternal age, gravidity, and prior screening. The pregnant women were 19–48 years old (mean age, 32.2 years). The range for gestational weeks at NIPT was 12–32 weeks. The mean values of gestational week at NIPT, expanded from common aneuploidies to MMSs, will guide pregnancy management.

Many commercially available NIPT cover the detection of specific MMS. The NIPT-Plus showed varying performance in detecting specific MMS, with only low to moderate positive predictive values. It is beneficial to detect clinically significant fetal CNVs in all pregnant women, irrespective of maternal age, including younger pregnant women. Therefore, the application of NIPT in detecting fetal CNVs is promising and feasible to utilize.

At present, karyotyping and chromosomal microarray analysis (CMA) or CNV sequencing (CNV-seq), have been recommended to prenatally identify fetal clinically significant CNVs. In this study, we prospectively investigated 31,256 singleton pregnancies using NIPT-Plus and investigated its efficacy in detecting fetal clinically significant CNVs.

Material and methods

Study subjects and expanded NIPT data sources. This prospective study enrolled 31,260 pregnant women with a singleton pregnancy who underwent NIPT-Plus, in Fujian Maternity and Child Health Hospital, of whom four failed to be detected. The study was approved by the Ethics Committee of the Hospital (2015KYLLD01051). All methods were performed in accordance with the relevant guidelines and regulations. The test method, screening-covered diseases, and limitations and risks were informed.

Blood samples were sent for the NIPT-Plus test, generating 36-bp genomic sequence reads. Reads were assigned to consecutive non-overlapping 100 Kb bins to further filter bins with low coverage and GC content (<30% or >70%). Thus, data regarding data regarding clinically significant CNV cases were obtained from Ion Proton semiconductor sequencing platform (Da An Gene Co., Ltd., Shenzhen, China), the sequencing depth was 0.4×, and the data volume was 8 million reads. The ENET algorithm was applied to calculate the fetal fraction (FF). The mean FF of the samples passing quality control was 11.2% (4.0–48.3%).

Pregnant woman demographics. The demographic characteristics of maternal age, gestational week, gravidity, and prior screening are shown in Table 1. The pregnant women were 19–48 years old (mean age, 32.2 years). The range for gestational weeks at NIPT was 12–32 weeks. The mean values of gestational week at NIPT and invasive testing were 17.3 ± 2.0 and 22.7 ± 2.6 weeks, respectively. Prior maternal serological screening (MSS) tests before NIPT, including abnormal MSS results [high risk, critical risk and abnormal multiple of the median for single marker value (AFP, β-HCG, uE3)] and low risk. Invasive diagnostic procedures include amniocentesis during 16 and 24 gestational weeks, and fetal blood sampling beyond 24 gestational weeks. Autosomal and all chromosome aneuploidies were excluded. Totally, 221 women were suspected to have fetal CNVs, and 203 women voluntarily opted invasive testing by karyotype and CMA/CNV-seq, and 18 women refused invasive testing (Fig. 1).

Invasive prenatal diagnostic testing by karyotyping and CNV analysis. For further validation, women with NIPT-positive clinically significant CNVs results were recommended to undergo invasive diagnostic procedures, and 30 mL of amniotic fluid or 4 mL of cord blood was obtained. In addition, in cases with NIPT-negative CNVs results, fetuses anomaly were examined by either ultrasound prenatally or physical examination postnatally, and were also advised chromosome testing.
Karyotypes were scanned on Leica GSL120. At least 20 metaphases were counted, and five metaphases were analyzed. The naming of abnormal karyotypes were based on ISCN 2020.

CMA was performed using Affymetrix CytoScan 750 K array (Affymetrix Inc., Santa Clara, CA), the experimental processes were performed as previously described30, and data analysis was carried out using Affymetrix Chromosome Analysis Suite Software (version 3.1.0.15). The reporting threshold was set at copy number gains/losses ≥ 500 Kb and loss of heterozygosity (LOH) ≥ 10 Mb.

In regard to CNV-seq, library construction and purification operation was conducted by Biosan chromosomal CNV assay kit (reversible terminal termination sequencing), the concentration of library was quantitatively determined by quantification through KAPA Library KTS. Post-quantitative library pooling was sequenced on Illumina NextSeq 500 sequencing platform at ~ 1 × depth, and software was used for chromosomal CNV above 0.1 Mb finally. The number of reads after sample quality control is more than 8 M Sequence depth is ~ 0.1 ×. Burrows-Wheeler algorithm for calculating CNV was performed according to the previous study29.

CNVs were classified through OMIM, UCSC, International Standard Cytogenomic Array, Database of Genome Variants, and Decipher databases into pathogenic, likely pathogenic, variants of unknown significance (VOUS), likely benign (LB), and benign. Data were analyzed using the human genome hg19 reference sequence. The pathogenicity significance of CNVs was evaluated following the ACMG guidelines31. For fetuses with confirmed abnormal CNVs, parental testing was performed to determine its origin.

NIPT result is true positive (TP) if confirmed by diagnostic testing of the fetus, mother, or placenta. When fetal chromosomal anomalies are detected which are not identified via NIPT, the NIPT results are considered false negative (FN). True negative (TN) refers to cases with negative NIPT results and the diagnostic test is normal.

Follow-up and pregnancy management. All pregnant women received pre- and post-test counseling from a senior genetic counselor. Pregnant couples confirmed to have fetuses with pathogenic/likely pathogenic (P/LP) CNVs go through multi-disciplinary treatment, and they take an informed decision on whether to continue pregnancy. Follow-up began three months after delivery, including ultrasound examination report, diagnostic testing results, final pregnancy outcomes, infant’s sex, and physical examination of newborn results.

Table 1. Clinical characteristic of pregnant women undergoing NIPT-Plus. AMA advanced maternal age, NIPT noninvasive prenatal testing, n number, MoM multiple of the median, MSS maternal serological screening. *Pregnant women with contraindications for invasive diagnostic procedures.
FN clinically significant CNVs results subsequently identified by either ultrasound prenatally or physical examination postnatally were subjected to chromosome analysis. The pregnancy outcomes of the FN samples were recorded via telephone or through a follow-up registry.

Statistics. SPSS software version 19.0 (SPSS, Inc., Chicago, IL) was used for statistical analysis. Measurement data were expressed as mean ± standard deviation, statistical comparisons were performed using $\chi^2$ test, and $p < 0.05$ was considered statistically significant.

Ethics approval and consent to participate. This study was approved by the ethics committee of Fujian Maternity and Child Health Hospital, affiliated to Fujian Medical University (No. 2018KYLLD01051), and informed consent was obtained from all the pregnant women.

Results

Locations of CNVs detected by NIPT-plus. Totally, 31,256 pregnant women who received NIPT-Plus were enrolled finally in this study due to 4 cases failed by NIPT-Plus. The enrollment, and flowchart are presented in Fig. 1. A total of 221 women were suspected to have fetal clinically significant CNVs, thus, the screening positive rate of fetal clinically significant CNVs was 0.71% (221/31,256), including 128 CNVs with microduplications ranging in size from 2.0 to 46 Mb and 98 CNVs with microdeletions ranging in size from 2.2 to 75.29 Mb (and more than one abnormality were detected in 5 cases), CNVs detected by NIPT were distributed in chromosome X and each autosome except chromosome 19, of which, and CNVs on chromosomes 4, 5, 7, 11, 15 and 18 were the most common, as shown in Fig. 2.

Detection efficiency of NIPT-Plus in screening clinically significant fetal CNVs. Of the 221 cases with clinically significant CNVs, 203 (91.86%) cases underwent invasive diagnostic testing via amniocentesis or fetal blood sampling. The remaining 18 pregnant women declined invasive testing. Among the 203 cases with clinically significant CNVs detected by invasive diagnostic testing, 78 cases were TP (of which, 33 were microduplications, 45 were microdeletions), and 125 cases were FP, with an overall PPV of 38.42% and an overall false
positive rate (FPR) of 0.40%. Among 101 fetal positive confirmatory invasive diagnostic testing results, 59 were pathogenic, 11 were likely pathogenic, 25 were VOUS, 6 were LB (Table 2).

Among the 203 cases with validation, P/LP CNVs were identified in 70 cases, including 19 cases with susceptibility loci (SL) for neurodevelopmental disorders (NDD) and 18 cases with abnormal karyotypes. Of the 18 fetuses, 14 had confirmed CNVs ≥ 10 Mb and 4 had CNVs < 10 Mb. In addition, CMA also detected 5 LOH (Tables 2, 3).

There were 27 cases of CNVs associated with classical MMS. This comprised 8 cases at high risk of DGS, 5 cases at high risk of 22q11.2 microduplication syndrome, 6 cases of PWS/AS, 4 cases of CDC, and 4 cases of 1p36 deletion syndromes. Of the eight cases of suspected DGS, there were 6 TPs and 2 FPs yielding a PPV of 75%. Of the five cases of suspected 22q microduplication syndrome, there were four TPs and one FP, yielding a PPV of 80%. For the six suspected PWS/AS cases, there were three TPs and three FPs; for the four suspected CDC cases, there were two TPs and two FPs. Finally, four cases indicated as a 1p36 deletion proved to be FP (Table 4).

The remaining 175 cases of fetal CNVs were segmental CNVs that were classified as other genome-wide CNVs. Of these, there were 34 TPs and 40 FPs for CNVs ≥ 10 Mb (PPV, 45.95%) and 28 TPs and 73 FPs for CNVs < 10 Mb (PPV, 27.72%) (Tables 4, 5).

Pregnancy outcomes. All pregnant women with TP NIPT results underwent genetic counseling to discuss pregnancy intention. While the majority of women diagnosed with fetally clinically significant CNVs elected termination of pregnancy (TOP), a relatively small proportion of pregnant women chose to continue their pregnancies. The TOP rates were much higher in pregnancies diagnosed with known MMS, including DGS (100%), PWS/AS (100%), and CDC (100%). In contrast, elective TOP rates were much lower in women carrying a fetus with 22q11.2 microduplication syndrome (50%) (Table 2).

Seven pregnancies with pathogenic CNVs were missed by NIPT-Plus (negative predictive value 99.98%) (Table 7). The FN cases with clinically significant CNVs included one of eight cases with confirmed DGS, one of five cases with confirmed 22q11.2 microduplication syndrome, one of four cases with confirmed 1p36 deletion syndrome, as well as a Wolf-Hirschhorn syndrome (WHS) case, a 8q24.22q24.3 duplication case, a 16p11.2 deletion case, and a 15q11.2 deletion case. In one of the seven (14.29%) FN cases, prenatal ultrasound detected fetal abnormalities, and pregnancies were terminated upon confirmation via invasive diagnostic testing. The remaining six FN cases were identified only at birth and subsequently confirmed by postnatal chromosome analysis. In the 3–12 months follow-up period after birth, no other FN cases were identified.

The underlying causes of these seven FNs were further investigated. In all seven pregnancies, low FF was unlikely because FF values ranged from 5 to 13.8%. The other five women refused further placenta studies; thus, each one case of WHS and 1p36 deletion syndrome was further investigated via placental tissue chromosome analysis. From four placental biopsy samples, no evidence of 4p16.3 deletion and 1p36 deletion was identifiable, suggesting possible confined placental mosaicism (CPM) as a cause of the two FN results.

The follow-up of 18 pregnant women with high-risk CNVs detected by NIPT-Plus is shown in Table 6. Interestingly, there were three FP cases with normal fetal and placental anatomies but complicated with multiple 5–10.5 cm uterine leiomyomas detected via ultrasound, though their prior obstetrical and gynecologic history was negative. In one case, NIPT indicated a 38 Mb deletion at 7q21.11q31.31 (FF: 6.4%); in another case, NIPT indicated multiple CNVs involving chromosomes 3, 4, 7, 10, and 12 (a 40 Mb deletion at 3q25.2q29, a
| Case ID | MA | GW | NIPT-PLUS results | Fetal karyotype results | Fetal CMA/CNV-seq results/pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome | Chromosome disease syndrome indicated by NIPT-Plus |
|---------|----|----|-------------------|-------------------------|-----------------------------------------------------|--------------------------|------------------|--------------------------------------------------|
| 18      | 24 | 13  | Dup 9q13 (~30 Mb) | 46,XX,add(9)(p13)pat  | arr[GRCh37]9p13.1p24.3 (208,454,38,772,003) × 3 LP | FGR at 22 GW  | TOP | Nonsyndromic |
| 17      | 39 | 14  | Dup 9p24.3p11.2 (~46 Mb) | 46,XX,add(9)(p24) | arr[GRCh37]9p24.3q21.13 (208,454,77,662,508) × 3 P | NT thickening | TOP | Nonsyndromic |
| 27      | 37 | 14  | Dup 13q31.1 (~4.3 Mb) | 46,XN | arr[GRCh37]13q31.1 (78,894,976–81,994,976) × 3 VOUS | None | Born (normal phenotype) | Nonsyndromic |
| 7       | 23 | 15  | Dup 17p12 (~3.34 Mb) | 46,XN | arr[GRCh37]17p12 (13,060,293–15,616,912) × 3 P | None | Born (normal phenotype except high arch) | Charcot-Marie-Tooth 1A type syndrome |
| 26      | 32 | 15  | Dup 21q21.1q22 (~3.5 Mb) | 46,XN | arr[GRCh37]21q21.1q22.11 (29,962,690,32,659,168) × 3 VOUS | None | Born (normal phenotype) | Nonsyndromic |
| 6       | 32 | 16  | Dup 22q3.3q24.2 (~9.93 Mb) | 46,XN | arr[GRCh37]22q3.3q24.2 (154,042,539–162,258,705) × 3 LP | None | TOP | Non-syndromic |
| 9       | 31 | 16  | Dup 15q21.2 (~5.8 Mb) | 46,XN | arr[GRCh37]15q11.2q13.1 (22,770,421,270,804) × 3 da P | None | TOP | 15q11-q13 duplication syndrome |
| 25      | 26 | 17  | Dup 4p13p12 (15 Mb) | 47,XN, +mar | arr[GRCh37]4p13.1q12 (41,896,801–57,724,715) × 3 mat LP | None | TOP | Nonsyndromic |
| 16      | 28 | 17  | Dup 11q23.3q24 (~17 Mb) | 46,XN | arr[GRCh37]11q23.3q25 (117,097,362–134,937,416) × 3 P | Dandy–Walker malformation | TOP | Nonsyndromic |
| 5       | 29 | 17  | Dup 22a1.121q12.1 (~2.0 Mb) | 46,XN | arr[GRCh37]22a1.121q12.13 (22,997,928–25,043,045) × 3 mat P | None | Born (normal phenotype) | 22q11 duplication syndrome |
| 22      | 28 | 17  | Dup 11q23.3q24 (~17 Mb) | 46,XN | arr[GRCh37]11q23.3q25 (117,097,362–134,937,416) × 3 P | Dandy–Walker malformation at 23 GW | TOP | Nonsyndromic |
| 21      | 26 | 18  | Dup 15q11.21q13.1 (~6.3 Mb) | 46,XN | arr[GRCh37]15q11.21q13.1 (22,102,621–28,315,618) × 3 pat P | None | Born (normal phenotype) | 15q11q13 duplication syndrome |
| 19      | 38 | 18  | Dup 3p26.3p22.3 (~35 Mb) | 46,XN,add(3)(p22) | arr[GRCh37]3p26.3p22.3 (1,43,682,691) × 3 P | None | TOP | Nonsyndromic |
| 23      | 34 | 18  | Dup 11q24.3q25 (~4.1 Mb) | 46,XN | arr[GRCh37]11q24.3q25 (130,308,334–134,937,464) × 3 P | None | TOP | Nonsyndromic |
| 10      | 36 | 19  | Dup 5q14.2 (~10 Mb) | 46,XN,add(5)(p14) | arr[GRCh37]5q14.2 (90,230,935–91,382,020) × 3 LP | None | TOP | Nonsyndromic |
| 4       | 35 | 19  | Dup 22a1.12 (~2.7 Mb) | 46,XN | arr[GRCh37]22a1.12 (18,648,855–21,454,872) × 3 P | None | TOP | 22q11 duplication syndrome |
| 1       | 25 | 19  | Dup 15q11.2 q13.1 (~4.9 Mb) | 46,XN | arr[GRCh37]15q11.2q13.1 (23,281,885–28,526,905) × 3 pat P | None | TOP | 15q11q13 duplication syndrome |
| 28      | 29 | 19  | Dup 17g21.3q31.32 (~3.0 Mb) | 46,XN | arr[GRCh37]17g21.31 (44,187,491–44,784,639) × 3 mat VOUS | None | Born (normal phenotype) | Nonsyndromic |
| 29      | 31 | 19  | Dup 9q21.11q22.3 (~25 Mb) | 46,XN | arr[GRCh37]9q21.11q22.3 (71,013,799–95,657,711) × 3 da P | None | Born (normal phenotype) | Nonsyndromic |
| 15      | 29 | 19  | Dup 4p16.315.2 (~25 Mb) | 46,XN | arr[GRCh37]4p16.3p15.2 (68,345–25,296,039) × 3 P | None | TOP | Nonsyndromic |
| 24      | 31 | 20  | Dup 20q13.2q13.3 (~12 Mb) | 46,XN | arr[GRCh37]20q13.2q13.3 (51,504,974–62,913,645) × 3 P | None | TOP | Nonsyndromic |
| 32      | 22 | 20  | Dup1q31.21q12.13 (~3.0 Mb) | 46,XN | arr[GRCh37]1q31.21q12.13 (22,073,046–25,230,759) × 3 LP | None | Born (normal phenotype) | Nonsyndromic |
| 33      | 25 | 20  | Dup 5p15.2p15.1 (~2.5 Mb) | 46,XN | arr[GRCh37]5p15.2p15.1 (14,860,000–16,800,000) × 3 LB | None | Born (normal phenotype) | Nonsyndromic |
| 3       | 20 | 20  | Dup 22q1.121q11.2 (~2.88 Mb) | 46,XN | arr[GRCh37]22q1.121q11.2 (18,648,855–21,461,017) × 3 P | None | TOP | 22q11 duplication syndrome |
| 2       | 24 | 20  | Dup 22q1.12 (~4.0 Mb) | 46,XN | arr[GRCh37]22q1.12 (18,919,477–21,915,207) × 3 P | None | TOP | 22q11 duplication syndrome |
| 14      | 33 | 21  | Dup 5p15.33p15.2 (~31 Mb) | 46,XN,add(21)(p11.2) | arr[GRCh37]5p15.33p15.2 (113,584–32,448,235) × 3 P | None | TOP | Nonsyndromic |

Continued
| Case ID | MA | GW | NIPT-PLUS results | Fetal karyotype results | Fetal CMA/CNV-seq results/pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome | Chromosome disease syndrome indicated by NIPT-Plus |
|---------|----|----|-------------------|-------------------------|----------------------------------------------------------|--------------------------|-----------------|-----------------------------------------------|
| 30      | 29 | 21 | Dup 2p22.3 (~3.1 Mb) | 46,XN | arr[GRCh37]2p22.3 (34,049,512–35,045,602) x 3 LB | None | Born (normal phenotype) | Nonsyndromic |
| 11      | 31 | 21 | Dup 5p15.33p15.2 (~15 Mb) | 46,XN,add(21)(p11.2) | arr[GRCh37]5p15.33p15.2 (113,576–32,448,169) x 3 P | None | TOP | Nonsyndromic |
| 8       | 33 | 21 | Dup 2q23.324.2 (~9.9 Mb) | 46,XN,dup(2) (2q23.3q24.2) | arr[GRCh37]2q23.3q24.2 (154,042,539–162,258,705) x 3 P | None | TOP | Nonsyndromic |
| 13      | 31 | 23 | Dup 20q13.32q13.3 (~12 Mb) | 46,XN | arr[GRCh37]20q13.32q13.3 (51,504,974–62,913,645) x 3 | None | Born (normal phenotype) | Nonsyndromic |
| 20      | 40 | 23 | Dup 5p15.33p11 (~41 Mb) | 46,XN | arr[GRCh37]5p15.33p11 (113,576–46,242,541) x 3 | None | TOP | Nonsyndromic |
| 5       | 32 | 24 | Dup 4p16.3 (~3.4 Mb) | 46,XN | arr[GRCh37]4p16.3 (37,044,025–37,233,386) x 3 | None | Born (normal phenotype) | Nonsyndromic |
| 42      | 37 | 16 | Dup 4p16.3 (~3.4 Mb) | 46,XN | arr[GRCh37]4p16.3 (37,044,025–37,233,386) x 3 | None | Born (normal phenotype) | Nonsyndromic |

### Copy number gain FP (≤ 16 GW)

| Case ID | MA | GW | NIPT-PLUS results | Fetal karyotype results | Fetal CMA/CNV-seq results/pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome | Chromosome disease syndrome indicated by NIPT-Plus |
|---------|----|----|-------------------|-------------------------|----------------------------------------------------------|--------------------------|-----------------|-----------------------------------------------|
| 35      | 35 | 14 | Dup 6q23.3p22.2 (~4.2 Mb) | 46,XN | arr[GRCh37]6q23.3p22.2 (68,345–4,277,002) x 1 | None | TOP | Nonsyndromic |
| 37      | 37 | 15 | Dup 8p12 (~2.5 Mb) | 46,XN | arr[GRCh37]8p12 (29,351,826–30,190,629) x 1 | Separation of renal pelvis | Born (normal phenotype) | Nonsyndromic |

### Copy number loss TP (≤ 16 GW)

| Case ID | MA | GW | NIPT-PLUS results | Fetal karyotype results | Fetal CMA/CNV-seq results/pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome | Chromosome disease syndrome indicated by NIPT-Plus |
|---------|----|----|-------------------|-------------------------|----------------------------------------------------------|--------------------------|-----------------|-----------------------------------------------|
| 78      | 36 | 14 | Del 3q31.1 (~3.7 Mb) | 46,XN | arr[GRCh37]3q31.1 (83,494,767–86,543,280) x 1 | None | Born (normal phenotype) | Nonsyndromic |
| 69      | 38 | 14 | Del 18p11.3p11.21 (~13 Mb) | 46,XN | arr[GRCh37]18p11.3p11.21 (136,227–15,099,116) x 1 | FGR, HPE | TOP | Nonsyndromic |
| 50      | 32 | 15 | Del 1q11q13 (~5.0 Mb) | 46,XN | arr[GRCh37]1q11q13 (23,290,787–28,560,664) x 1 | Intracardiac echogenic focus | TOP | Nonsyndromic |
| 67      | 23 | 15 | Del Xq22.3 (~45 Mb) | 46,XN | arr[GRCh37]Xq22.3 (107,912,179–155,233,983) x 1 | None | TOP | Nonsyndromic |
| 45      | 25 | 15 | Del 22q11 (~3.0 Mb) | 46,XN | arr[hg19]22q11.21 (18,648,855–21,800,471) x 1 | None | TOP | DGS |
| 74      | 34 | 15 | Del 4q11.3q13.2 (~11 Mb) | 46,XN | seq[hg19]4q11.3q13.2 (155,800,001–164,960,000) x 1 | None | TOP | Nonsyndromic |
| 72      | 23 | 15 | Del 6q25 (~17 Mb) | 46,XN | arr[GRCh37]6q25.1q27 (152,176,966–170,914,297) x 1 | None | TOP | Nonsyndromic |

### TP (> 16 GW)

| Case ID | MA | GW | NIPT-PLUS results | Fetal karyotype results | Fetal CMA/CNV-seq results/pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome | Chromosome disease syndrome indicated by NIPT-Plus |
|---------|----|----|-------------------|-------------------------|----------------------------------------------------------|--------------------------|-----------------|-----------------------------------------------|
| 70      | 30 | 16 | Del 7q32q36 (~2.3 Mb) | 46,XN | arr[GRCh7]7q32.1 (149,828,703–152,102,066) x 1 | None | TOP | Nonsyndromic |
| 53      | 23 | 16 | Del Xq23q28 (~3.68 Mb) | 46,XX | arr[GRCh37]Xq23q28 (1475,0751–1552,330,998) x 1 (female) | Intestinal dilatation | Born (normal phenotype) | Nonsyndromic |
| 68      | 30 | 16 | Del 7q34q36 (~17 Mb) | 46,XN | arr[GRCh37]7q34q36 (142,044,268–159,19,707) x 1 | None | TOP | Nonsyndromic |

Continued
| Case ID | MA | GW | NIPT-PLUS results | Fetal karyotype results | Fetal CMA/CNV-seq results/ pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome | Chromosome disease syndrome indicated by NIPT-Plus |
|---------|----|----|-------------------|------------------------|-----------------------------------------------------|------------------------|------------------|-----------------------------------------------|
| 58      | 27 | 16 | Del 18p11.32 (~3.0 Mb) | 46,XN                  | arr[GRCh37]18p11.32p11.31 (2,186,353–5,675,587) × 1 mat P | None                  | TOP              | Nonsyndromic                                  |
| 84      | 25 | 17 | Del 11q22.3 (~5.18 Mb) | 46,XN                  | arr[GRCh37]11q22.3 (104,181,493–106,629,690) × 1 mat LB | None                  | Born (normal phenotype) | Nonsyndromic                                  |
| 85*     | 33 | 17 | Del 2q22,12q22.3 (~11 Mb) | 46,XN                  | arr[GRCh37]21q22.12q22.3 ([36,746,514–48,093,361] × 1 P 8q24.22q24.3 (134,400,222–146,295,771) × 3 LP | None                  | TOP              | Nonsyndromic                                  |
| 44      | 27 | 17 | Del 22q11.21 (~2.8 Mb) | 46,XN                  | arr[hg]19 22q11.21 (18,916,842–21,800,471) × 1 P | VSD                  | TOP              | DGS                                           |
| 66      | 25 | 17 | Del 6q25 (~17 Mb) | 46,XN                  | arr[GRCh37]6q25.1q27 (152,176,966–170,914,297) × 1 P | Intestinal dilatation | Lost follow-up | Nonsyndromic                                  |
| 60      | 28 | 17 | Del 8q12.1q21.2 (~6.69 Mb) | 46,XN                  | arr[GRCh37]8q12.3q13.2 (63,249,655–69,695,857) × 1 P | None                  | TOP              | Nonsyndromic                                  |
| 63*     | 28 | 17 | Del 18q21.31q23 (~35 Mb) | 46,XN,-18, + mar       | arr[GRCh37]18p11.32p11.31 (136,227–3,348,254) × 1 P 18p11.31p11.21 (3,356,536–13,083,388) × 3, P 18p11.21 (13,090,666–15,170,636) × 1 P 18p11.21q21.31 (15,181,207–54,008,143) × 3, P 18q21.31q23 (54,020,488–78,013,728) × 1 P | Fetal aorta coarctation, pulmonary artery stenosis, VSD | TOP              | Nonsyndromic                                  |
| 75      | 31 | 17 | Del 1p31 (~4.5 Mb) | 46,XN                  | arr[GRCh37]1p31.1 (78,282,099–84,553,373) × 1 VOUS | None                  | Born (normal phenotype) | Nonsyndromic                                  |
| 76      | 28 | 17 | Del 18q22.3 (~2.2 Mb) | 46,XN                  | arr[GRCh37]18q22.3 (69,288,801–71,535,501) × 1 VOUS | None                  | Born (normal phenotype) | Nonsyndromic                                  |
| 71      | 28 | 17 | Del 9p24.3p24.2 (~2.5 Mb) | 46,XN                  | arr[GRCh37]9p24.3p24.2 (208,454–2,900,085) × 1 P (152,093,040–159,118,443) × 2 hizn VOUS | Dandy-Walker malformation | TOP              | Nonsyndromic                                  |
| 81      | 20 | 17 | Del 15q25.2q26.3 (~18 Mb) | 46,XN                  | arr[GRCh37]15q25.2q26.3 (83,739,214–102,397,317) × 1 VOUS | None                  | Born (normal phenotype) | Nonsyndromic                                  |
| 49      | 37 | 18 | Del 15q11.2q13.1 (~5.0 Mb) | 46,XN                  | arr[GRCh37]15q11.2q13.1 (23,290,787–28,659,911) × 1 P | None                  | TOP              | PWS/AS                                        |
| 46      | 33 | 18 | Del 22q11 (~2.5 Mb) | 46,XN                  | arr[hg]19 22q11.21 (18,636,749–21,136,749) × 1 P | None                  | TOP              | DGS                                           |
| 52      | 33 | 18 | Del 5p15.32p15.2 (~5.3 Mb) | 46,XN                  | arr[GRCh37]5p15.33p15.2 (113,576–10,477,490) × 1 P | Single umbilical artery | TOP              | Cri-Du-Chat                                    |
| 77      | 30 | 18 | Del 15p13.1p14 (~2.7 Mb) | 46,XN                  | arr[GRCh37]15q13.1q3.3 (30,241,910–32,991,173) × 1 VOUS | Subependymal cyst | Born (normal phenotype) | Nonsyndromic                                  |
| 73      | 28 | 19 | Del 17p13.3–13.2 (4.2 Mb) | 46,XN                  | arr[GRCh37]17p13.3p13.2 (525–4,669,796) × 1 P | None                  | Miller–Dicker syndrome |                                |
| 79      | 31 | 19 | Del 13p15.1p13 (~11.18 Mb) | 46,XN, del(11) (p13p13) | arr[GRCh37]11p15.1p13 (19,973,767–31,001,449) × 1 VOUS | None                  | Born (normal phenotype) | Nonsyndromic                                  |
| 59      | 26 | 19 | Del 10q26.13q26.3 (~7.0 Mb) | 46,XN, del(10)(q26.1) | arr[GRCh37]10q26.13q26.3 (125,262,198–135,426,386) × 1 P | None                  | TOP              | Nonsyndromic                                  |
| 57      | 28 | 19 | Del 17p13.213.3 (~4.2 Mb) | 46,XN                  | arr[GRCh37]17p13.3p13.2 (525–4,669,796) × 1 P | None                  | TOP              | DGS                                           |
| 80      | 31 | 19 | Del 4q26 (~10 Mb) | 46,XN                  | arr[GRCh37]4q26 (114,340,000–119,800,000) × 1 VOUS | Enhanced liver echo | Born (normal phenotype) | Nonsyndromic                                  |
| 43      | 25 | 19 | Del 22q11.21 (~3.72 Mb) | 46,XN                  | arr[GRCh37]22q11.21 (1863,136–2180,0471) × 1 P | None                  | TOP              | DGS                                           |
| 65      | 27 | 19 | Del 5q23.1 (~12 Mb) | 46,XN                  | arr[GRCh37]5q23.1 (107,915,007–120,847,610) × 1 P | None                  | TOP              | Nonsyndromic                                  |
| 47      | 32 | 19 | Del 22q11.21 (~3.0 Mb) | 46,XN                  | arr[hg]19 22q11.21 (18,916,842–21,800,471) × 1 P | None                  | TOP              | DGS                                           |

Continued
| Case ID | MA | GW  | NIPT-PLUS results | Fetal karyotype results | Fetal CMA/CNV-seq results/pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome | Chromosome disease syndrome indicated by NIPT-Plus |
|---------|----|-----|--------------------|------------------------|-------------------------------------------------|-------------------------|------------------|-----------------------------------------------|
| 82      | 31 | 19  | Del 9p23p13.1 (~24 Mb) Del 9q21.11q22.3 (~25 Mb) | arr[GRCh37]9p23p13.1 (13,107,600–38,771,831) x 1 dn VOUS | None | Born (normal phenotype) | Nonsyndromic |
| 83      | 31 | 19  | Del 9p23p13.1 (~25.6 Mb) | arr[GRCh37]9p23p13.1 (13,107,600–38,771,831) x 1 dn VOUS | Subependymal cyst | Born (normal phenotype) | Nonsyndromic |
| 55      | 35 | 19  | Del Xp22.31 (~2.6 Mb) Del 10q21.1 (~3.8 Mb) | arr[GRCh37]Xp22.31 (6,455,152–8,141,076) x 1 mat P | Fetal mild tricuspid regurgitation | Born (mild ichthyosis phenotype) | XLR ichthyosis |
| 54      | 37 | 20  | Del Xp22.31 (~21 Mb) | arr[GRCh37]Xp22.31 (6455152–8135568) x 0 mat P | None | Born (mild ichthyosis phenotype) | XLR ichthyosis |
| 62      | 33 | 21  | Del 18q11.2q22.3 (~18 Mb) | arr[GRCh37]18q11.2q22.3 (59,280,654–78,013,728) x 1 P | None | TOP | Nonsyndromic |
| 48      | 30 | 22  | Del 15q11.1q13.1 (~8.75 Mb) | arr[GRCh37]15q11.1q13.1 (22,770,422–28,928,730) x 1 P | None | TOP | PWS/AS |
| 61      | 34 | 24  | Del 18q11.2q22.3 (~6.0 Mb) | arr[GRCh37]18q11.2q22.3 (19,866,814–27,806,978) x 1 P | None | TOP | Nonsyndromic |
| 51      | 31 | 22  | Del 15q11.3p14.3 (~21 Mb) | arr[GRCh37]15q11.3p14.3 (113,577–21,007,739) x 1 P | None | Top | Cri-Du-Chat |
| 56      | 34 | 26  | Del 4q31.3q32.2 (11 Mb) | arr[GRCh37]4q31.3q32.2 (155,800,001–164,960,000) x 1 P | Abnormal posture of right foot | TOP | Nonsyndromic |
| 64      | 39 | 20  | Del 18p11.3q22.3 (~5 Mb) | arr[GRCh37]18p11.3q22.3 (94,929,201–115,107,733) x 1 P | FGR, absence of a-wave of ductus venosus, HPE, corpus callosum dysplasia | TOP | Nonsyndromic |
| 41      | 30 | 26  | Del 18p11.3q22.3 (~17.8 Mb) | arr[GRCh37]18p11.3q22.3 (94,929,201–115,107,733) x 1 P | Bilateral pleural effusion | Born | Nonsyndromic |
| 98      | 25 | 15  | Del 1p36.32p36.31 (5.7 Mb) | arr[GRCh37]1p36.32p36.31 (107033067–109404131) x 1 P | None | TOP | 1p36 deletion syndrome |
| 95      | 29 | 15  | Del 1p36.32p36.31 (~5.1 Mb) | arr[GRCh37]1p36.32p36.31 (107033067–109404131) x 1 P | None | TOP | 1p36 deletion syndrome |
| 88      | 25 | 15  | Del 1q31.3q31.2 (~3.5 Mb) | arr[GRCh37]1q31.2 (69,344,443–69,565,861) x 1 VOUS | None | TOP | Nonsyndromic |
| 99      | 27 | 16  | Del 1q31.3q31.2 (10 Mb) | arr[GRCh37]1q31.3q31.2 (30,211,776–36,615,043) x 1 P | None | TOP | Nonsyndromic |

Copy number loss FP (≤16 GW)

| 94      | 39 | 15  | Del 18p23.3p23.1 (~5.1 Mb) | arr[GRCh37]18p23.3p23.1 (168,483–6,999,220) x 2 hnm VOUS | Atrial septal defect | Born (normal phenotype) | Nonsyndromic |
| 90      | 34 | 15  | Del 22q11.21 (~3.0 Mb) | arr[GRCh37]22q11.21 (69,344,443–69,565,861) x 1 VOUS | None | Born (normal phenotype) | DGS |
| 98      | 25 | 15  | Del 1p36.32p36.31 (~5.7 Mb) | arr[GRCh37]1p36.32p36.31 (107033067–109404131) x 1 P | None | TOP | 1p36 deletion syndrome |
| 95      | 29 | 15  | Del 1p36.32p36.31 (~75.29 Mb) | arr[GRCh37]1p36.32p36.31 (107033067–109404131) x 1 P | None | TOP | 1p36 deletion syndrome |
| 88      | 25 | 15  | Del 1q31.3q31.2 (~3.5 Mb) | arr[GRCh37]1q31.3q31.2 (69,344,443–69,565,861) x 1 VOUS | None | Born (normal phenotype) | DGS |
| 99      | 27 | 16  | Del 1q31.3q31.2 (10 Mb) | arr[GRCh37]1q31.3q31.2 (30,211,776–36,615,043) x 1 P | None | TOP | Nonsyndromic |

FP (>16 GW)

| 105     | 39 | 17  | Del 15q11.1q11.2 (~4.2 Mb) | arr[GRCh37]15q11.1 (16,727,490–20,433,723) x 1 P | VSD | Born | AS/PWS |
| 89      | 26 | 17  | Del 4q31.3q31.2 (~10 Mb) | arr[GRCh37]4q31.3q31.2 (167230237–190921709) x 2 hnm VOUS | None | Born (normal phenotype) | Nonsyndromic |
| 91      | 34 | 17  | Del 1p31.3p11.2 (~4.9 Mb) | arr[GRCh37]1p31.3p11.2 (46,919,156–51,932,566) x 1 P | None | Born (normal phenotype) | Nonsyndromic |
| 93      | 34 | 17  | Del 1q31.3q32.2 (~4.8 Mb) | arr[GRCh37]1q31.3q32.2 (17,867,202–18,765,914) x 1 P | None | Born (normal phenotype) | Nonsyndromic |
| 86      | 28 | 18  | Del 7q21.11q31.2 (~31 Mb) | arr[GRCh37]7q21.11q31.2 (21,464,764–22,962,962) x 1 P | None | TOP | Nonsyndromic |

Continued
20 Mb deletion at 4q24q28.1, a 70 Mb deletion at 7q11.23q34, a 24 Mb deletion at 10q22.3q24.31, and a 25 Mb deletion at 12q12q14.2 (FF: 10.8%). In the third case (case 86 in Table 2), NIPT indicated a 31 Mb deletion at 7q21.11q31.2; further CMA on amniocytes identified a 1.5 Mb microdeletion at 22q11.21q11.22, which is associated with DGS, and the pregnancy was terminated. Confirmatory CMA on amniocytes did not show any pathogenic CNV in the other two cases. CMA studies of the three placentas after induction or postpartum did not show the existence of abnormal CNVs.

Table 2. A 101 fetal positive confirmatory invasive diagnostic testing results with fetal CNVs indicated by NIPT-Plus. MA maternal age, GW gestational weeks, LFU, TOP terminate of pregnancy, MS-MLPA methylation-specific multiplex ligation-dependent probe amplification, mut maternal, pat paternal, P pathogenic, LP likely pathogenic, VOUS variants of uncertain significance, CMA chromosomal microarray, CNV copy number variation, NIPT noninvasive prenatal testing, TS Turner syndrome, UA ultrasound anomalies, WHS wolf-hirschhorn syndrome, XLR X-linked recessive, SGA small for gestational age, ARSA aberrant right abuclavian artery, VSD ventricular septal defect, FGR fetal growth restriction, HPE holoprosencephaly. *Fetal MS-PLPA: methylation-specific multiplex ligation-dependent probe amplification, paternal duplication. $ When there are multiple CNVs, only the highest pathogenicity classification is calculated.

| Case ID | MA  | GW  | NIPT-PLUS results  | Fetal karyotype results | Fetal CMA/CNV-seq results/pathogenicity classification | Fetal ultrasound finding | Pregnancy outcome |
|---------|-----|-----|---------------------|------------------------|-------------------------------------------------------|--------------------------|------------------|
| 87      | 25  | 18  | Del 18p21.3q23 (~ 22.13 Mb) | 46,XN | seq[hp1] dup (17)(p13.3;p13.3) (1–712,489) × 3 LP | None | Born (normal phenotype except high arch) Nonsyndromic |
| 92      | 29  | 18* | Del 21q22.3 (~ 3.4 Mb) | 46,XN | arr[GRCh37]13q21.2 (596082-66,709,021) × 1 pat VOUS | VSD | Born (normal phenotype) Nonsyndromic |
| 96      | 29  | 20  | Del 13q12 (~ 3.2 Mb) | 46,XN | arr[GRCh37]5q14.1 (76,983,283–77,512,158) × 3 mat LB | VSD | Born Nonsyndromic |
| 97      | 38  | 22  | Del 7p28 (~ 7 Mb) | 46,XN | arr[GRCh37]7p14.1 (155,463,038–162,158,990) × 1 dn P | Complete endocardial cushion defect (unbalanced), coarctation of the aorta | Born Nonsyndromic |
| 100     | 35  | 23  | Del 4p16.3p15.33 (~ 12 Mb) | 46,XN | arr[GRCh37]Xq28 (~ 22.13 Mb) | None | Born (normal phenotype except high arch) Nonsyndromic |

Table 3. The overall PPV and the rate of TP in each of these two cohorts (at ≤ 16 weeks and > 16 weeks). *5 of 18 pregnancies who declined invasive testing with no confirmed test and 23 pregnancies that have been lost follow-up with low-risk results were excluded when making data statistics.

| GW at NIPT | n   | NIPT positive | TP  | FP  | FN  | Refused invasive testing | PPV(%) |
|------------|-----|---------------|-----|-----|-----|--------------------------|--------|
| ≤ 16       | 13,172 | 34             | 13  | 14  | 3   | 7                        | 48.1   |
| > 16       | 18,084 | 187            | 65  | 111 | 4   | 11                       | 36.9   |

Table 4. Performance of NIPT-Plus for detection of clinically significant CNVs in 31,256 pregnancies. CNV copy number variation, TP true positive, FP false positive, FPR false positive rate, PPV positive predictive value, TN true negative, FN false negative, FNR false negative rate, NPV negative predictive value.

| Clinically significant CNVs | TP  | FP  | PPV | TN  | FN  | NPV | Specificity |
|-----------------------------|-----|-----|-----|-----|-----|-----|-------------|
| Classical MMS              | 15  | 1/0.038% | 55.56% | 31,226 | 4/21.1% | 99.99% | 99.96% |
| 22q11.2 deletion syndrome  | 6   | 2/0.006% | 75%  | 31,247 | 1/14.29% | 100% | 99.99% |
| 22q11.2 duplication syndrome | 4  | 1/0.003% | 80%  | 31,249 | 2/33.3% | 99.99% | 100% |
| Cri-du-Chat syndrome       | 2   | 2/0.006% | 50%  | 31,252 | 0/0% | 100% | 99.99% |
| Prader–Willi syndrome/Angelman syndrome | 3  | 3/0.01% | 50%  | 31,250 | 0/0% | 100% | 99.99% |
| 1p36 deletion syndrome     | 0   | 4/0.01% | 0%   | 31,251 | 1/100% | 100% | 99.99% |
| Other genome-wide CNVs     | 63  | 113/0.36% | 35.80% | 31,077 | 3/4.55% | 99.99% | 99.64% |
| ≥ 10 Mb                    | 33  | 38/0.12% | 46.5% | 31,183 | 2/5.71% | 99.99% | 99.88% |
| ≤ 10 Mb                    | 30  | 75/0.24% | 28.57% | 31,150 | 1/3.23% | 100% | 99.76% |
| Total                      | 78  | 125/0.40% | 38.42% | 31,046 | 7/8.24% | 99.98% | 99.60% |
Table 5. The PPVs for all fetal CNVs indicated by NIPT-Plus according to different CNV sizes. TP true positive, FP false positive, PPV positive predictive value.

| CNV size detected by NIPT | NIPT positive | TP | FP | Refused invasive testing | PPV(%) |
|---------------------------|---------------|----|----|--------------------------|--------|
| Within 2–4 Mb             | 97            | 24 | 70 | 3                        | 25.5   |
| Within 4–7 Mb             | 29            | 14 | 13 | 2                        | 51.9   |
| Within 7–10 Mb            | 16            | 6  | 3  | 7                        | 66.7   |
| > 10 Mb                   | 79            | 34 | 39 | 6                        | 46.6   |
| Total                     | 221           | 78 | 125| 18                       | 38.4   |

Table 6. The follow-up of 18 pregnant women with high-risk CNVs detected by NIPT-Plus refused invasive testing prenatally due to fetal ultrasound structural anomalies or contraindications to prenatal diagnosis. VOUS variants of uncertain significance. *Contraindications to prenatal diagnosis.

| Case ID | Prenatal ultrasound finding | Postnatal cord blood CMA results/pathogenicity classification | Cord blood karyotyping | Associated disease with validation | Pregnancy outcome | NIPT-plus result |
|---------|-----------------------------|---------------------------------------------------------------|------------------------|-----------------------------------|-------------------|------------------|
| 1       | Complete endocardial cushion defect, hydramnios | Not done | Not done | No result | TOP | Del 20q11.23q13.31 (18 Mb) |
| 2       | FGR | arr[GRCh37]46, XY | 46, XY | No result | Preterm birth at 35°w, normal phenotype | Del 1p36.32p36.31 (5.5 Mb) |
| 3       | Bilateral pleural effusion | arr[GRCh37]3p26.3 (61,891–2,441,342) × 1 VOUS | Normal | No result | TOP | Del 7q22.1–q31.1 (8 Mb) |
| 4       | FGR, fetal BPD was less than the mean value 3.75D, HC was less than the mean value 4.7SD | Not done | Not done | No result | TOP | Del 4p16.3–p15.33 (13 Mb) |
| 5       | Fetal ventricular septal defect | Not done | Not done | No result | Born (normal phenotype) | Del Xp22.31 (~ 5.8 Mb) |
| 6       | Partial absence of corpus callosum | arr[GRCh37]46,XX | Normal | No result | Born (normal phenotype) | Del 1p36.32-p36.23 (~ 5.1 Mb) |
| 7*      | Fetal pelvic ectopic kidney with multiple cystic changes? | Not done | Not done | No result | Born (normal phenotype) | DupXq28 (~ 7 Mb) |
| 8       | Fetal FL and HL were less than the mean value 2SD | Not done | Not done | No result | Born (normal phenotype) | Del 1q25.31q26.3 (~ 16.8 Mb) |
| 9       | Right hydrocephalus | arr[GRCh37]46,XX | 46, XX | No result | Born (normal phenotype) | Del 16p12.1–p11.2 (4.66 Mb) |
| 10      | Fetal double kidney echo enhanced | arr[GRCh37]46, XY | 46, XY | No result | Born (normal phenotype) | Del 15q11.2–13 (5 Mb) |
| 11      | cerebellar dysplasia, smooth brain/ stenopodia | Not done | Not done | No result | TOP | Del 22q11.2 (5.2 Mb) |
| 12*     | Normal | Not done | Not done | No result | Born (normal phenotype) | Del 15q11.2–13 (4.5 Mb) |
| 13      | Normal | Not done | Not done | No result | Born (normal phenotype) | Del 4p16.3–p15.33 (12 Mb) |
| 14*     | Single umbilical artery | Not done | Not done | No result | Born (normal phenotype) | Del 7q22.1–q31.1 (10 Mb) |
| 15      | Intestinal echo enhancement | Not done | Not done | No result | Born (normal phenotype) | Del 10q25.3q26.3 (16.8 Mb) |
| 16      | Minimal pulmonary regurgitation, skin thickening of head and neck back | Not done | Not done | No result | Born (normal phenotype) | Dup 14q31.1q31.32 (22 Mb) |
| 17*     | Persistent left superior vena cava | Not done | Not done | No result | Born (normal phenotype) | Dup 2p25.3p24.3 (11.5 Mb) |
| 18*     | Fetal nasal bone dysplasia | Not done | Not done | No result | Born (normal phenotype) | Dup 4p16.17.1 (7.1 Mb) |
Case no. | Clinically significant CNVs by CMA | Z-score of CNV | Fetal fraction | Prenatal/postnatal findings
--- | --- | --- | --- | ---
FN-1 | arr[GRCh37] 22q11.21(18631365_21800471) × 1 | - 2.51 | 9.1% | NOT identified by ultrasound prenatally, detected due to ventricular septal defect postnatally
FN-2 | arr[GRCh37]1p36.33p36.32(849,466–4,894,800) × 1 | 1.34 | 11.58% | NOT identified by ultrasound prenatally, detected due to language retardation postnatally
FN-3 | arr[GRCh37] 16p11.2(29,428,531–30,177,916) × 1 | 2.78 | 5% | Fetal ultrasound anomalies: single umbilical artery, left renal parenchyma echogenicity enhancement, upper ureter dilatation on ultrasound
FN-4 | arr[GRCh37] 4p16.3p15.2(68,345–22,489,538) × 3 | - 2.97 | 13.8% | NOT identified by ultrasound prenatally, detected due to VSD postnatally
FN-5 | arr[GRCh37] 8q24.11q24.3(117,830,985_146,295,771) × 3 | 2.56 | 10.8% | NOT identified by ultrasound prenatally, detected due to developmental delay and cleft palate postnatally
FN-6 | arr[GRCh37] 15q11.2(22,770,421–23,625,785) × 1 | 2.83 | 13.2% | NOT identified by ultrasound prenatally, detected due to seizure postnatally
FN-7 | arr[GRCh37] 19p13.2(14,756,611_17,418,160) × 1 | 2.71 | 6.8% | NOT identified by ultrasound prenatally, detected due to mental retardation postnatally

Table 7. Seven cases with false negative NIPT results missed by NIPT-Plus with validation. DGS DiGeorge syndrome, WHS Wolf-Hirschhorn syndrome, FN false negative, VSD ventricular septal defect.

Low-risk NIPT CNV results. Of the 31,035 cases with fetal low-risk P/LP CNVs, 23 were lost to follow-up; thus, 99.93% (31,012/31,035) of these cases were successfully followed. Twenty-five fetuses underwent diagnostic tests because of abnormal ultrasound findings. Of which, 24 cases showed normal karyotype as well as CMA results, and had normal live births. One fetus harbored pathogenic CNVs (FN-3 in Table 7), and the pregnant couple terminated the pregnancy given the test results. Among the 24 cases with normal karyotype and CMA results, one fetus died in utero owing to preeclampsia and multiple malformations, one fetus died in utero owing to oligohydramnios, and three fetuses were born preterm because of fetal growth restriction (FGR), premature rupture of membranes, intrauterine cytomegalovirus infection, respectively, and 15 women terminated the pregnancies due to fetal multiple ultrasound anomalies. No abnormalities were found in the remaining low-risk pregnant women during the 3–12 months postnatal follow-up.

Discussion
Chromosomal abnormality is one of the most important causes of birth defects, and there is no effective method to deal with it. The aim of prenatal screening is to identify fetal chromosomal abnormalities. Currently, in comparison to traditional MSS for Down syndrome, NIPT screening for common trisomy and sex chromosome aneuploidy is more popular among pregnant women; however, it is still controversial whether NIPT should screen for MMS32. In the present study, we investigated the performance of NIPT-Plus for fetal P/LP CNVs in 31,256 pregnant women and assessed its clinical value.

Opponents have argued that the relatively low PPV, high FPR, and uncertain pathogenesis of CNVs cause a dilemma in interpreting reports on high-risk results, putting significant psychological stress on pregnant women, and even increasing unnecessary invasive diagnostic procedures and their associated risks. However, proponents have debated that the purpose of prenatal screening and prenatal diagnosis is to prevent the birth of infants with the burden of fetal chromosomal abnormalities, even for MMS with low PPVs. Most MMSs occur randomly, because the risk of fetal CNVs is not related to the age of pregnant women, which is beneficial for pregnant women of all ages on NIPT screening for CNVs, which are observed in 1.0–2.0% of birth defects without random reasons, because the risk of fetal CNVs is not related to the age of pregnant women, which is beneficial for pregnant women during the 3–12 months postnatal follow-up. Furthermore, the PPV of NIPT-Plus for fetal P/LP CNVs with validation are 75% (DGS), 80% (WHS), and 70% (Nonsyndromic). Of the 31,256 cases screened by NIPT, 78 were chromosome abnormalities, which can provide an important basis for intervention prenatal diagnosis.

The study reported by Liang et al.31 showed that NIPT exhibited high sensitivity and specificity for the detection of clinically significant CNVs. In our study, for classic MMSs (n = 27), the PPV were 75% (DGS), 80%
(22q11.22 microduplication), 50% (PWS/AS), and 50% (CDC). For the remaining clinically significant fetal CNVs (n = 175), combined PPVs were 45.95% (CNVs ≥ 10 Mb) and 27.18% (CNVs < 10 Mb), which is slightly higher than that reported by Liang et al.\textsuperscript{19} The slight difference may be related to the different sample sizes and NIPT sequencing depth.

For the classic MMSs in our study, the PPV for 22q11.2 microduplication syndrome in this study was very high (80%). The overall PPV for the detection of other MMSs varied. For DGS, PWS/AS, CDC, and 1p36 deletion syndrome, the PPVs were 75%, 50%, 50%, and 0%, respectively. The PPVs for PWS and CDC were slightly lower than those reported, with reported PPVs of 75% and 40%, respectively\textsuperscript{15}. Petersen et al. reported that the PPV for CDC, PWS, 1p36 deletion syndrome, and DGS was 0%, 0%, 14%, and 21%, respectively\textsuperscript{35}. Low-level CPM resulted in one FP case of DGS\textsuperscript{30}; Thus, we speculate that CPM may also be the potential etiology in two FP DGS cases, three FP PWS/AS cases, two FP CDC cases, and one four 1p36 deletion cases.

The combined frequency of FN in MMS was 0.022% (7/31,256). These included one fetus identified via ultrasound prenatally and six detected only at birth. Thus, the frequency of FNs can be reduced to 0.019% by prenatal ultrasound examination. Thus, prenatal ultrasound results should be combined to consider the need for further invasive testing, consequently improving the detection rate of fetal MMS\textsuperscript{42}. We speculate that the FNs may be caused by biological factors other than a low FF. In two FN cases of WHS and 1p36 deletion syndrome, placental chromosomal analysis revealed no 4p16.3 and 1p36 deletion, which could explain the two FN NIPT results, suggesting possible CPM is considered as a cause of the two FN results. Although placental chromosome studies of the other four FNs cases are lacking, we speculated that low-level CPM might be the underlying cause of FN.

FP CNVs detected by NIPT can also be attributed to CPM\textsuperscript{30} and the death of a twin in utero. In this study, 125 of the 203 cases were confirmed to be FP. Unfortunately, placental biopsies were obtained after delivery or pregnancy termination for 15 of the 125 fetuses with normal genetic results, and 2 of them ultimately turned out to be CPM with CNVs, which presented with PGR. This supports the fact that CPM involving some P/LP CNVs may be associated with adverse pregnancy outcomes\textsuperscript{39}. In our cohort, there were three FP pregnancies with normal fetal and placental ananoties but complicated with multiple 5–10.5 cm uterine leiomyomas detected via ultrasound. Further diagnostic results revealed that fetal and placental lesions were normal except in case 86. Thus, we speculated that uterine leiomyoma may confound the results of NIPT screening for CNV and lead to FP\textsuperscript{40}. Therefore, when the medical history of the pregnant woman should be further understood when NIPT screening for CNV is positive.

Given the performance of NIPT for detecting MMSs in the present and other reported studies, compared to traditional serological screening, we propose that NIPT could be a candidate for first-line screening of pathogenic CNVs for all pregnancies, irrespective of maternal age\textsuperscript{3}. Currently, there are no other methods available to screen for MMSs, although NIPT has a high FPR.

Factors that influence the performance of CNVs detection include CNV size, sequencing depth, FF, and GC content\textsuperscript{41}. In our study, CNVs detected by NIPT were distributed in chromosome X and each autosome, except for chromosome 19. CNVs were frequently found on chromosomes 2, 4, 15, and 18. We speculated that chromosome 19 is very rarely involved, primarily because of its high GC content.

NIPT has a better detection performance for fetal CNVs ≥ 10 Mb at conventional sequencing depths\textsuperscript{42}. However, their ability to detect smaller CNVs is reduced. In our study, the PPV of nonsyndromic CNVs greater than 10 Mb was slightly higher than that of CNVs less than 10 Mb detected by NIPT (45.95% vs 27.18%, p > 0.05), and our data showed that NIPT demonstrates good performance in detecting fetal CNVs, especially for CNVs ≥ 10 Mb, similar to the results of the study by Yu et al.\textsuperscript{43}

This study had some limitations. First, studies on placentas and maternal CNVs were not routinely conducted to explore the cause of discordance between NIPT results and normal invasive diagnostic results. Second, the sample size was not large enough, and further research is required to accumulate more data. Third, the data are based on a cohort from a single tertiary referral center, and there exists regional bias. Fourth, the genetic information is incomplete due to 23 pregnancies with low-risk results that were lost follow-up and 18 pregnancies who declined invasive testing.

Our data indicate the potential significance of NIPT in screening clinically significant CNVs. NIPT exhibited high performance for the detection of 22q11.2 duplication syndrome and DGS, low to moderate detection performance for other clinically significant CNVs. We believe that NIPT-Plus combined with ultrasound examination and maternal history examination screening for CNVs may be more effective in further multicenter studies with a larger population, increased sequencing depth, and improved bioinformatics analysis algorithms.

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
H.X., M.L. and A.Y. prepared the main manuscript; X.C., L.X., H.H. and Q.G. prepared the figures 1-3 and all the Tables. All authors reviewed the manuscript.

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Competing interests
The authors declare no competing interests.

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