# Supplementary Appendix

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This appendix has been provided by the authors to give readers additional information about the work.

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# Methods

## Subject inclusion

The study was approved by the Region of Southern Denmark Research Ethics Committee (Project-ID: S-20190027). Our project included 42 pregnancies with high-risk criteria for genetic testing. Subjects were recruited via the obstetric departments from the hospitals of the Region of Southern Denmark in 2019-2021 (first 01-05-2019, last 18-10-2021) with informed consent. The selection was done from those pregnant women, who were offered an invasive prenatal test (amniocentesis (AC) or chorionic villus sampling (CVS)) due to a high-risk assessment for diagnostic genetic analysis at the 1st or 2nd trimester screening in the Region of Southern Denmark.

To be included in the study subjects had to belong to one of the following categories: (a) one or more anomalies/malformations identified in the fetus by ultrasound examination; (b) increased NT (nuchal translucency) above 5 mm, cystic hygroma or hydrops fetalis; (c) known genetic disorder (parents are carriers of dominant or recessive pathogenic disease). Additionally, paternal sample availability was mandatory for the inclusion. Out of the 42 pregnancies enrolled, two were excluded due to unavailability of plasma samples and four failed to meet the quality control after processing with no possibility of re-sequencing. Thus, the analysis and results are presented for 36 cases, passing the minimal quality control criteria (Figure S1). In parallel to DES-NIPT, all included cases underwent invasive prenatal analysis, consisting of chromosomal microarray (CMA) and trio WES/WGS on DNA from chorionic villus sampling (CVS) or amniotic fluid sample (AF). The invasive trio analysis results in this study were only utilized as a reference for evaluating the DES-NIPT performance.

At the time of inclusion, gestational age had to be 10-21 weeks in order to obtain the results of analysis before the week 22 of pregnancy, to allow for reproductive choices.

## Project samples

For the clinical invasive trio analysis, the following samples were collected: maternal venous peripheral blood (4 mL EDTA), paternal venous peripheral blood (4 mL EDTA), CVS (30-50 mg) or AF (15-20 ml). No additional CVS or AF from the invasive procedure was needed for the project. For DES-NIPT analysis, additional maternal peripheral blood samples (2 x 8 mL) were collected in Streck Cell-Free DNA BCT® tubes for plasma isolation and cell-free DNA (cfDNA) extraction. Sample processing for clinical analysis was performed as soon as possible by routine protocols of the Department of Clinical Genetics (OUH, Odense University Hospital), where exome or genome sequencing was performed on DNA purified from prenatal invasive samples (CVS or AF) and peripheral blood samples from the parents.

## Cell-free DNA extraction and sequencing library preparation

Plasma isolation from Streck tubes (according to Nonacus Cell3™ Xtract protocol) was performed as soon as possible. Samples with isolated plasma were kept at -20°C until further processing. CfDNA was purified from maternal plasma using the Cell3™ Xtract kit (Nonacus Ltd, Birmingham, UK) according to the manufacturer's instructions. The cfDNA sequencing libraries were preparefead using Cell3™ Target: Exome CG kit (Nonacus Ltd, Birmingham, UK) according to manufacturer's instructions. Sequencing libraries were multiplexed in groups of 8-12 samples and sequenced on the NovaSeq 6000 platform (S4 flow cell) (Illumina, San Diego, CA, U.S.).

## Bioinformatic data analysis

### Alignment and variant calling pipeline

Sequencing data from deep exome sequencing of cfDNA was processed by Illumina DRAGEN Bio-IT Platform v4.03 (Illumina, San Diego, CA, U.S.) using the DNA pipeline in somatic mode. The raw reads were aligned to Illumina DRAGEN Graph Reference Genome GRCh38. Error corrections were performed by collapsing of PCR (Polymerase Chain Reaction) duplicated reads by Unique molecular identifiers (UMI). Only collapsed reads with a UMI family size >= 2 reads were included for variant calling. This strategy increased the median base quality score from Q30 to >Q50. For all analyses, variant calls in Exome CG kit (Nonacus Ltd, Birmingham, UK) in the protein coding regions (+/- 2 bp into introns) of Exome CG kit (Nonacus Ltd, Birmingham, UK) target region were included, excluding regions with low mappability and complexity (stratification files downloaded from: https://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/release/genome-stratifications/v3.1/GRCh38/). Sequencing data from DNA extracted from the CVS, AF samples and parental blood samples were processed by Illumina DRAGEN Bio-IT Platform v4.03 (Illumina, San Diego, CA, U.S.) using the DNA pipeline in germline mode as described previously[1]. Variant annotation, filtering and visualization were carried out using VarSeq v2.2.5 (Golden Helix Inc., Bozeman, MT, USA). In this study, the mean sequencing coverage of our used whole exome target region at 100x was 98.32%, and the mean sequencing coverage of Consensus coding sequence (CCDS) genes at 100x was 97.96% due to difference in exome target regions. For estimation of sensitivity and precision, only autosomal variants were included.

### Fetal de novo SNV/INDEL variant filtering

For fetal de novo SNV/INDEL variants analysis, systematic noise filtering was applied. Site-specific noise was empirically measured in a set of reference samples and then used afterwards for variant filtering to reduce false positive calls. The following strategy was used for inclusion of samples to the noise model: For each of

the 36 samples, the sample in question was left out and the other 36 cfDNA samples in our cohort were used to build the systematic noise model. The systematic noise model was hereafter applied during variant calling of de novo variants. Variants seen more than once in our sample cohort or in >10 individuals in gnomAD database were removed to avoid recurrent artifacts. CfDNA variants were considered fetal de novo variants when meeting the following trio filtering criteria: plasma variant allele frequency (VAF) between 0.4% and 20% with a minimum of 5 supporting reads, additionally the coverage in matched maternal and paternal wbcDNA (DNA from white blood cells) had to be >20X with the VAF<1% for the variant in question. Furthermore, variants were filtered according to expected VAF. Expected VAF were estimated from the fetal fraction: $VAF_{Expected}$ = FF/2. Variants with VAF within the expected range for heterozygous variants: FF*0.2 > VAF > FF*0.8 were considered fetal de novo variants. Variants were considered to be maternal mosaic variants of WBC origin if plasma VAF were between 0.4% and 20% with a minimum of 5 supporting reads and the VAF in matched maternal wbcDNA was >1% (Figure S8). For all patients, the resulting de novo variants were evaluated and classified according to their clinical relevance for the fetus. For all patients, clinical variant interpretations were carried out by two experienced clinical laboratory scientists following the ACMG (American College of Medical Genetics) guidelines[2].

## Paternally inherited SNV/INDEL variant filtering

For detection of paternal sequence variants only positions where the father was found to carry a SNV/INDEL variant in either heterozygous or homozygous with a coverage >20x were included in the analysis. Positions where the mother carried the same SNV/INDEL variant were excluded from the analysis. Analysis of inheritance of paternal variants was carried out with two different purposes. For one purpose, we used paternal variants as a proxy to estimate the sensitivity of the de novo filtering strategy described above. Here, cfDNA variants with VAF between 0.4% and 20% and a minimum of 5 supporting reads in positions with known paternal variants. Sensitivity was estimated by comparing the resulting variants with the variants detected in the matched invasive CVS or AF sample. In addition, inheritance of paternal variants was used to estimate the accuracy/concordance of fetal genotyping of paternal variants. Here, a variant was considered paternally inherited (heterozygous) if it was called with a variant VAF between 0.4% and 20%. No-call positions or variants with VAF <0.4% were considered not inherited (homozygous for the reference allele). Genotyping accuracy/concordance were estimated by comparing the fetal genotype inferred from the cfDNA with the genotype of the matched invasive CVS or AF sample.

## Fetal chromosomal aneuploidy analysis

Detection of fetal chromosomal aneuploidies was carried out using WisecondorX software using default settings (bin size: 100 kb)[3]. Each sample was compared with a pool of normal (PoN) reference samples. The following strategy was used for the selecting of reference samples: For each of the 36 samples, the sample in

question was left out and the remaining cfDNA samples were included in the PoN sample set, except from the 6 samples where a CNV or aneuploidy had been detected in the invasive sample. BAM-files processed using the DRAGEN DNA pipeline as described above, were used as input. WisecondorX estimates the fetal chromosomal copy number of the autosomes (Chr1-22) plus chromosome X.

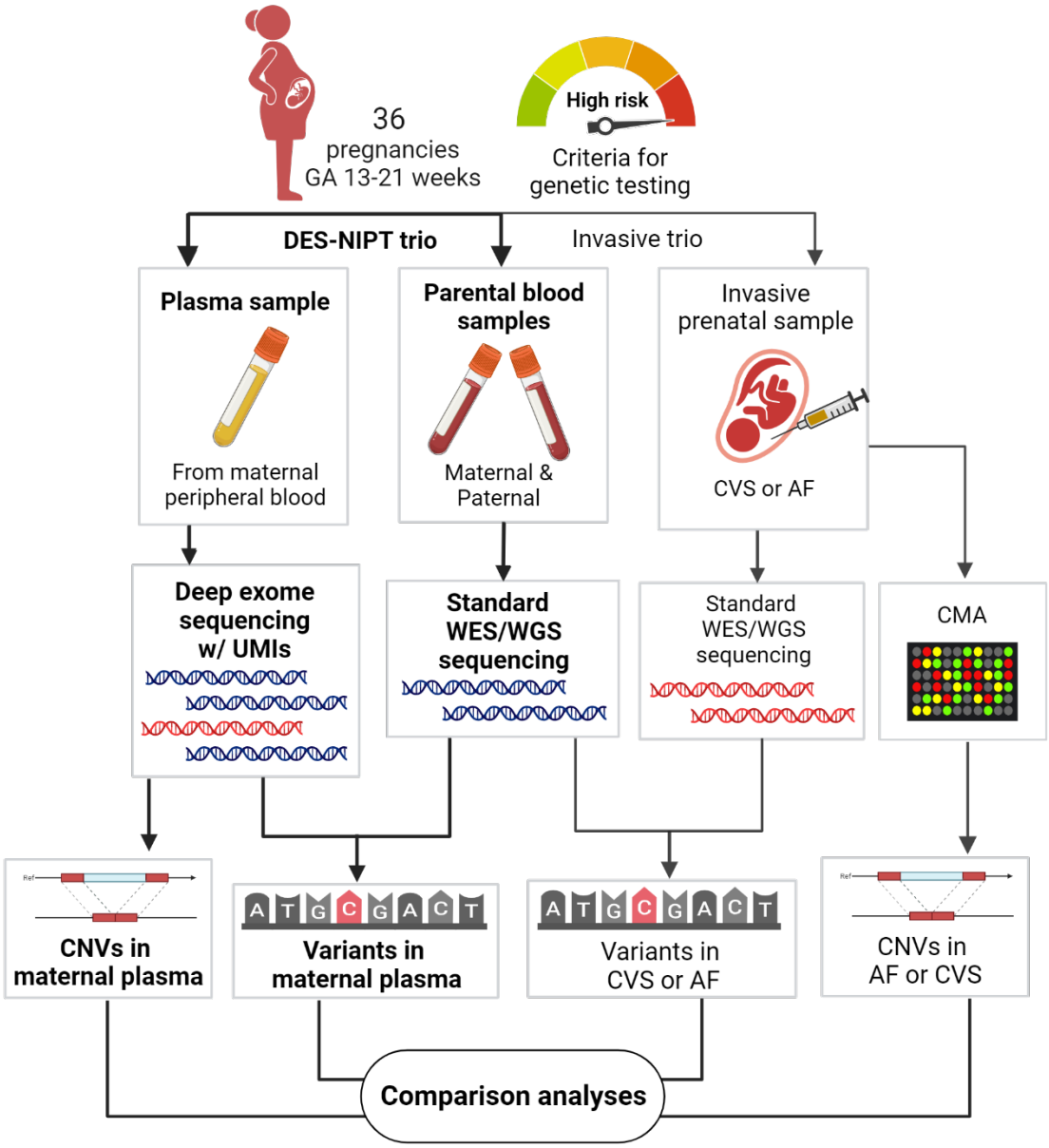
To determine the presence of fetal chromosome Y, the ChrY-ratio was estimated by counting uniquely mapped reads to chromosome Y, divided by the number of reads mapped to chromosome 1. Furthermore, to determine the presence of the paternal X chromosome in the fetus by determining the presence of paternal ChrX SNVs in the plasma sample.

## Fetal copy number analysis

Fetal copy number analysis was carried out utilizing the DRAGEN DNA pipeline sub-workflow for CNV calling in somatic mode. Each sample was compared with a pool of normal (PoN) references samples. The following strategy was used for the selecting of reference samples: For each of the 36 samples, the sample in question was left out and the remaining cfDNA samples were included in the PoN sample set, except from the 6 samples where a CNV or aneuploidy had been detected in the invasive sample. The following filtering for CNV calls were applied: QUAL>100; BC>20; SM<0.98 and SM>1.02. Only CNV calls spanning > 1 Mb regions were considered.

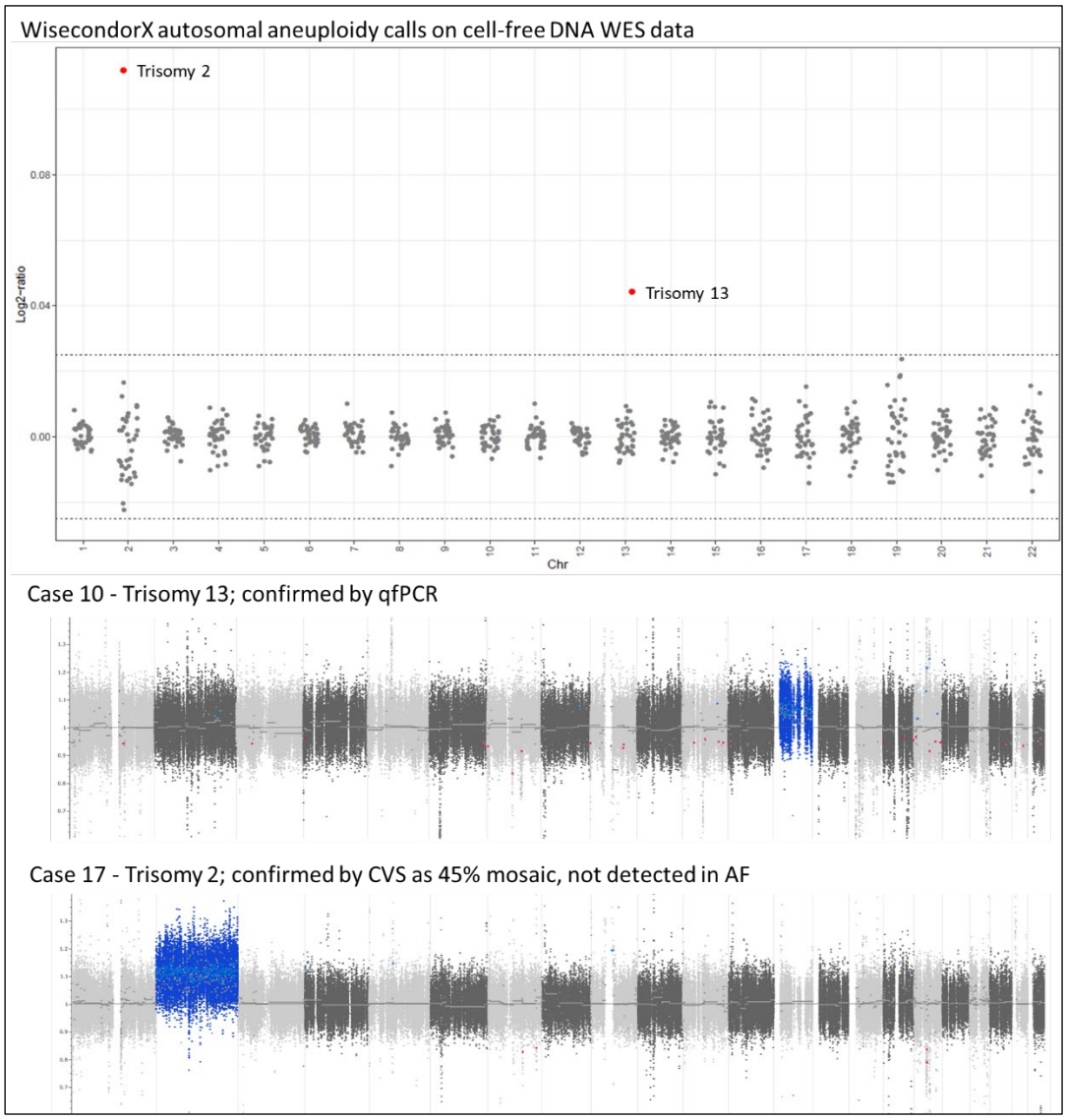
# Supplementary Figures

## Figure S1. Workflow of the DES-NIPT study.




The workflow illustrates the analysis conducted in this study, involving 36 pregnancies evaluated by DES-NIPT (plasma trio analysis). Deep exome sequencing was performed on cell-free DNA extracted from maternal plasma, using unique molecular identifiers (UMIs) for error correction. Simultaneously, invasive trio analysis was conducted on all included cases, using chorionic villus (CVS) or amniotic fluid (AF) samples. The results obtained from the invasive analysis served as the reference for evaluating the performance of DES-NIPT. Maternal plasma sample collection for analysis was performed between 13 to 21 weeks of gestational age (GA). CMA – chromosomal microarray; CNV – copy number variant; WES – whole exome sequencing; WGS – whole genome sequencing. (Created with BioRender.com)

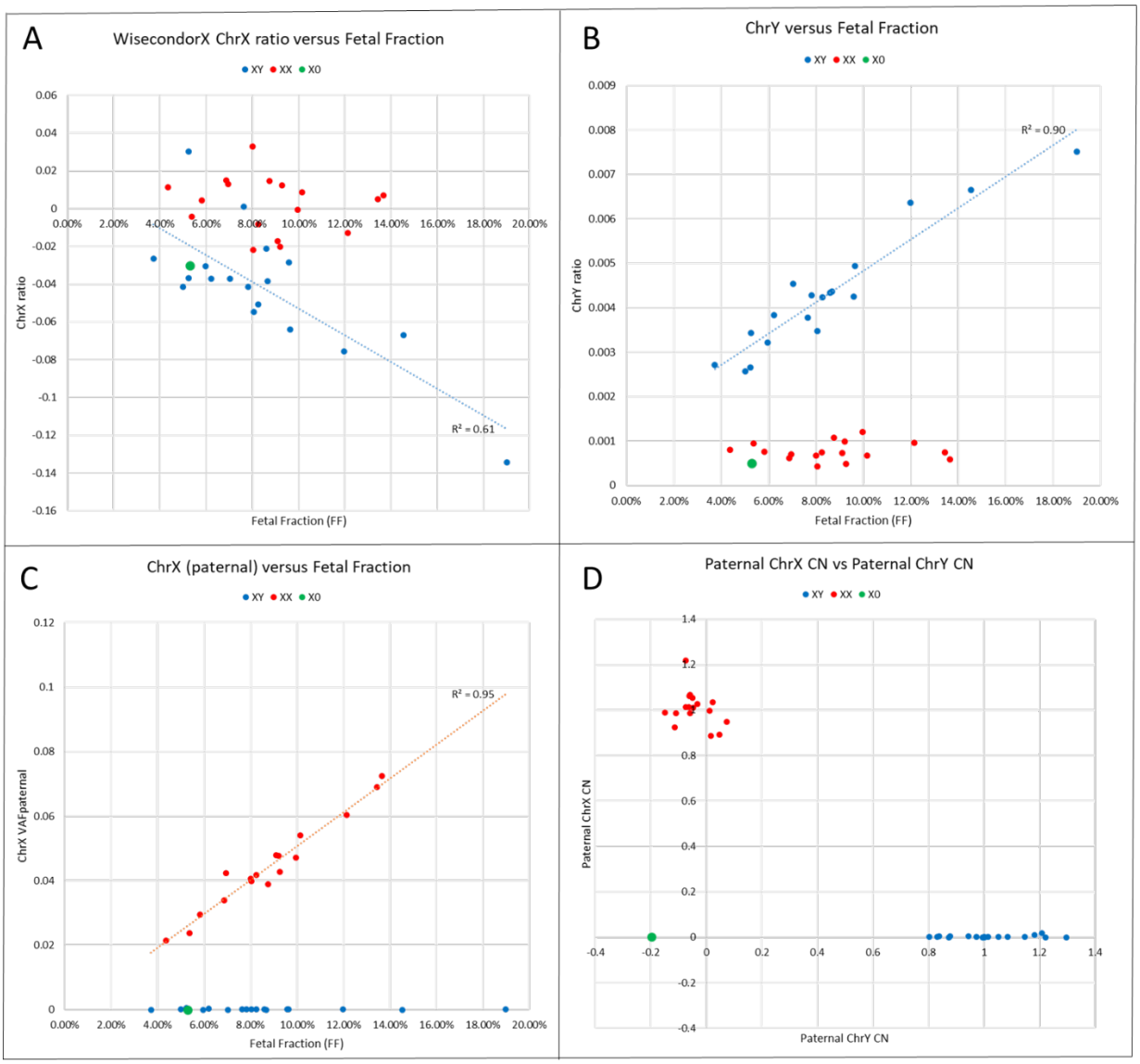
Figure S2. Fetal chromosomal aneuploidy analysis from cell-free DNA in maternal plasma




Fetal autosomal chromosomal copy number estimation of chromosomes (Chr) 1-22 allows detection of chromosomal aneuploidies. Log2 ratios were calculated by WisecondorX software for all autosomal chromosomes in all included cases. Red dots indicate positive trisomy calls. One case was found to have a trisomy 2 and another trisomy 13. No false positive aneuploidy calls were observed. Grey lines at ±0.025 indicate the cut-off. All chromosomal aberrations were confirmed using CMA analysis as part of the invasive analysis.

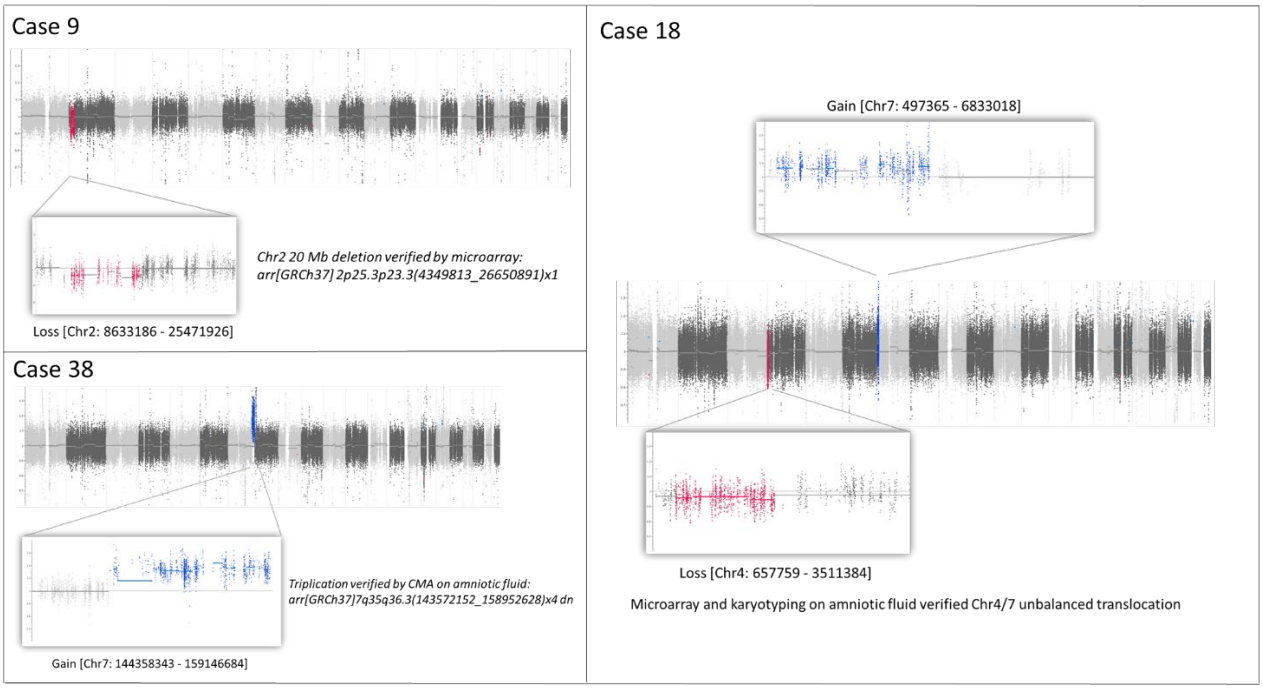
Interestingly, while case 17 exhibited trisomy of chromosome 2 in both chorionic villus sampling (CVS) and plasma samples, it was not observed in the WES analysis of amniotic fluid (AF). Therefore, it was considered to be confined placental mosaicism. The presence of mosaicism does not affect the screening capabilities of DES-NIPT, as it is a known biological characteristic in prenatal samples. Rather it demonstrates that even by analyzing cell-free DNA in maternal plasma, it is possible to detect chromosomal aneuploidies at the mosaic level. In cases of pathogenic findings by DES-NIPT, it is recommended to validate them through invasive sample analysis.

Figure S3 . Fetal sex chromosome analysis from cell-free DNA in maternal plasma



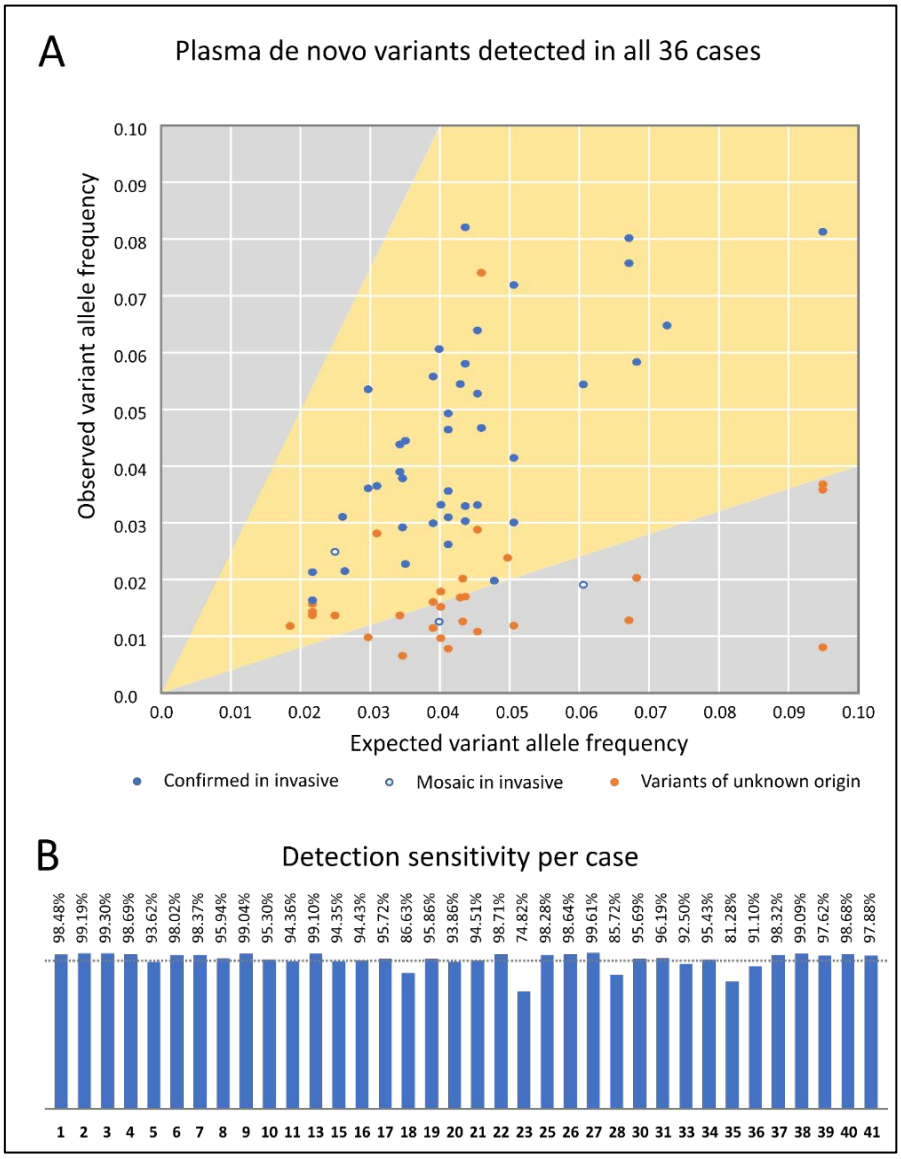

**(A)** Fetal chromosome X (ChrX) copy number estimate versus fetal fraction (FF). ChrX log2 ratios were calculated by WisecondorX software. Samples colored according to the sex of the fetus. WisecondorX ChrX copy number estimate separates female (XX) fetuses from male (XY) and monosomy X (X0) fetuses correctly in 33 out of 36 fetuses**. (B)** ChrY detection in maternal plasma DNA. ChrY ratio versus fetal fraction. Demonstrates the presence of the ChrY in all male (XY) fetuses and absence of ChrY in all female (XX) and monosomy X (X0) fetuses. **(C)** Paternal ChrX detection in maternal plasma DNA. Mean VAF of paternal ChrX SNVs versus fetal fraction. Demonstrates the presence of the paternal ChrX in all female (XX) fetuses and absence of the paternal ChrX all male (XY) and monosomy X (X0) fetuses. **(D)** Paternal ChrX and ChrY copy number (CN) estimate in fetus. The copy number of the paternal ChrX and ChrY were estimated by adjusting the results shown in B and C by the fetal fraction of the sample. The results demonstrate that one copy of ChrX is present in all female (XX) fetuses, one copy of ChrY is present in all male (XY) fetuses, while one fetus (green) was shown to have neither paternal ChrX nor ChrY, indicating that the fetus has monosomy X (X0). The monosomy X was confirmed by invasive testing.

Figure S4. Fetal copy number variant analysis from cell-free DNA in maternal plasma



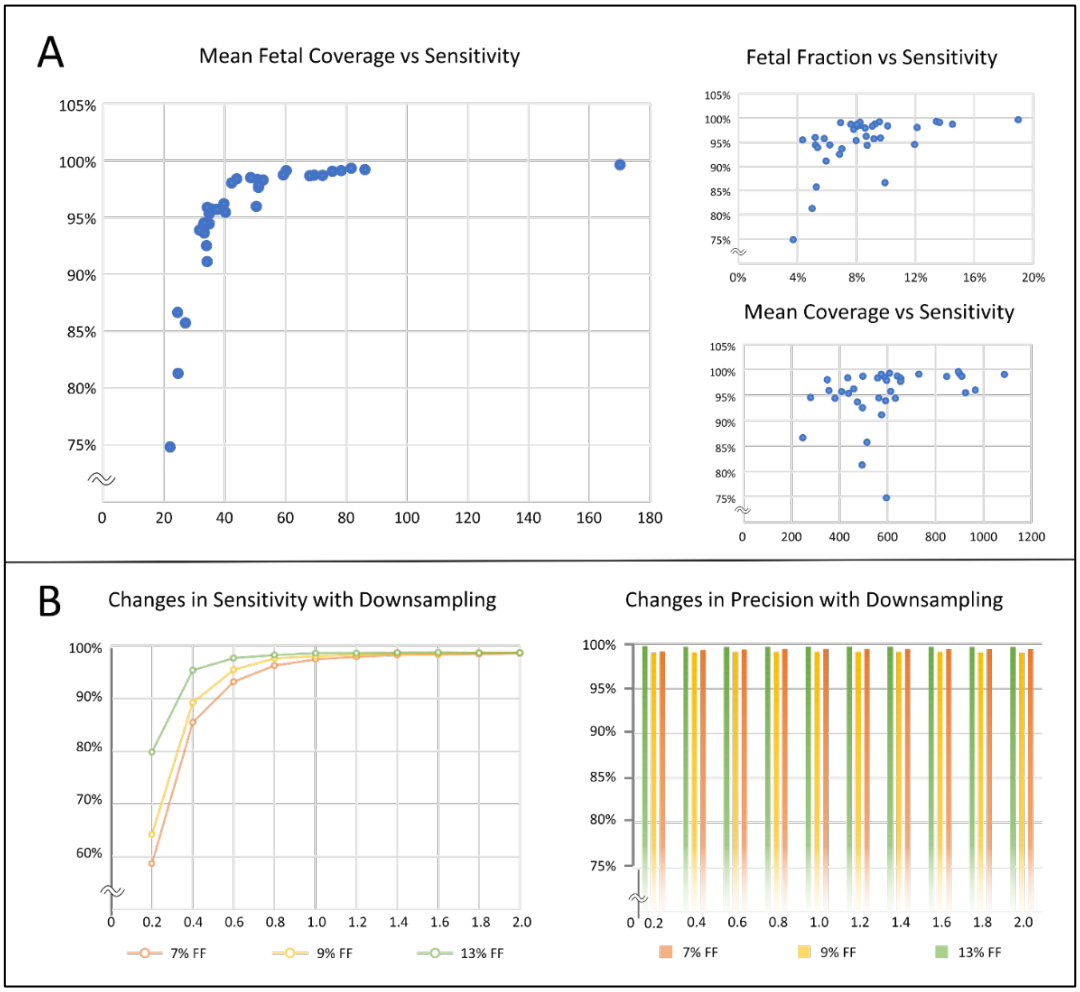

The figure shows copy number variants (CNVs) identified by DES-NIPT in our study cohort. All chromosomal aberrations were confirmed using CMA analysis as part of the invasive analysis. All CNVs were clearly visible and detected in the exome sequencing data of DES-NIPT samples.

Figure S5. De novo sequence variant analysis and sensitivity by DES-NIPT



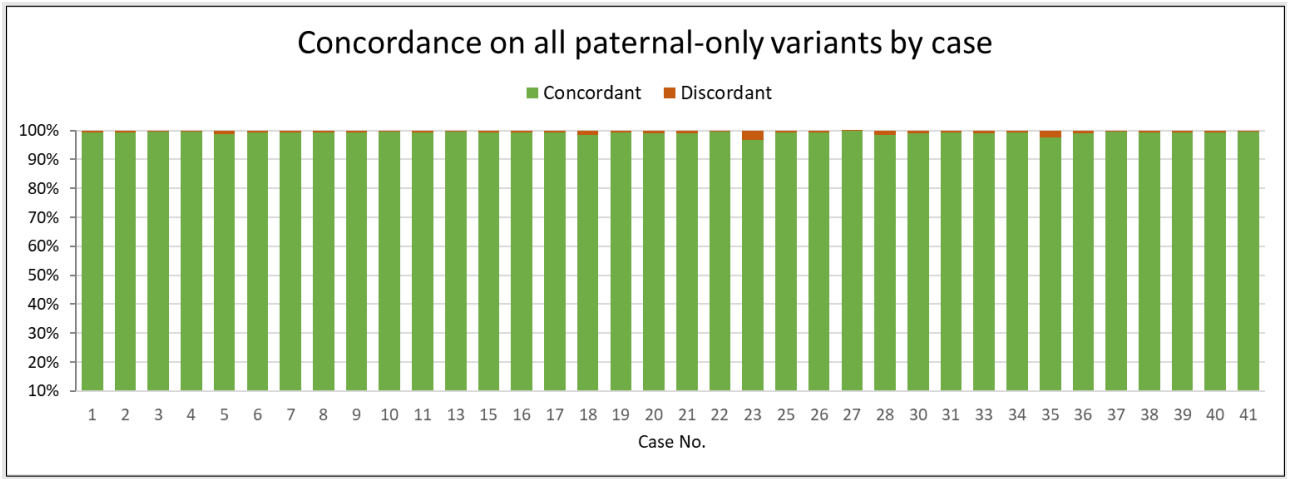

**(A) Plasma de novo sequence variants detected in total in all 36 cases**. De novo variants in cfDNA from maternal plasma were identified by comparing variants in cfDNA with maternal DNA from white blood cells (wbcDNA) and paternal wbcDNA variants to exclude parental germline variants. They were again analyzed to distinguish the SNVs with expected VAF according to the mean VAF of fetal variants. The expected variant allele frequency (VAF) was estimated from the fetal fraction: $VAF_{Expected}$ = FF/2. Variants with VAF in the expected range for heterozygous variants (FF*0.2 > VAF > FF*0.8) are shown in a golden background, while variants with too low or too high VAF are in a grey background and were removed from further analysis. **(B) The detection sensitivity of DES-NIPT for variants across the coding region**. Sensitivity demonstrates the variant recall rate per samples, which had at least five alternative reads in cell-free DNA data. The mean detection sensitivity of 95.12% is marked by a dotted line.

Figure S6. Quality control analysis of DES-NIPT approach




**(A) Correlation between sensitivity and mean fetal coverage**. Mean fetal coverage is a composite measure derived from mean target coverage and fetal fraction (fetal fraction variability across genome analysis depicted in Figure S9), both of which influence sensitivity. Adequate mean fetal coverage, indicative of appropriate sequencing depth and fetal fraction, is crucial for accurate variant detection. The study's mean fetal coverage was 50.08 (range: 22.14-170.13). Higher mean fetal target coverage was associated with increased sensitivity. Mean fetal coverage was utilized as a key criterion to identify high-quality plasma samples, with a minimum threshold of 20x for accurate low-frequency variant calling. Samples falling below this threshold were re-sequenced, and the data were merged to improve coverage.
**(B) Downsampling analysis of three cases with varying fetal fractions (FF).** Each case had two billion reads, which were bioinformatically downsampled to simulate lower sequencing depths. Through downsampling analysis, we observed a strong positive correlation between sensitivity and the total number of reads, reaching a plateau at approximately one billion reads. The analysis revealed that precision remained stable across all downsampling levels, suggesting sample-specific influences related to biological properties, library preparation, and sequencing errors. These findings indicate that a sequencing depth of one billion reads can be considered as the minimum requirement to ensure high sensitivity for the DES-NIPT method.

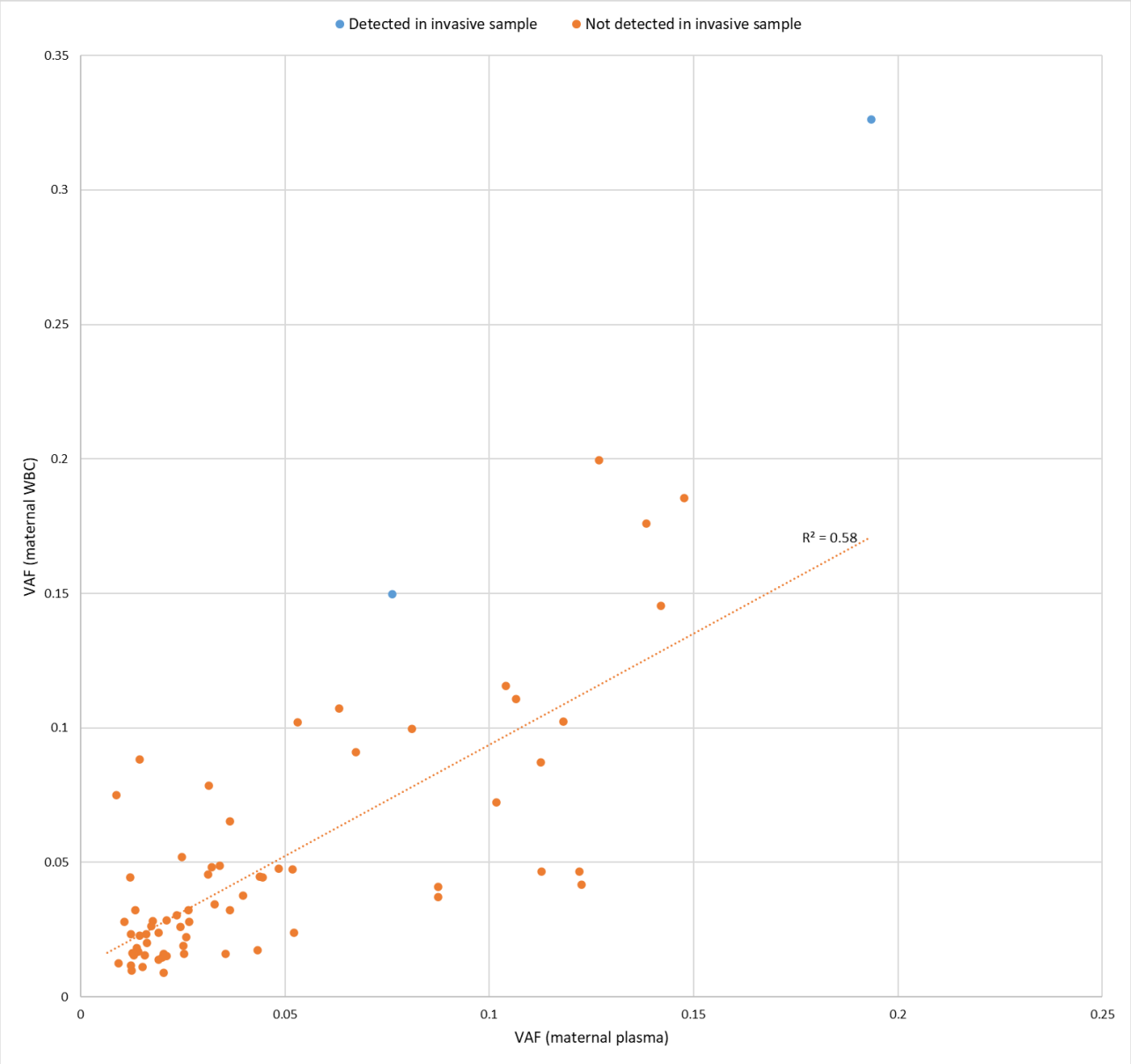
## Figure S7. Performance of DES-NIPT in detecting paternal-only variants




Paternal-only variants are defined as those inherited from the father while not present in the mother. The figure shows the concordance and discordance rates of DES-NIPT compared to invasive trio analysis for detecting these variants. In this study, an average of 3759 paternal-only variants were called per case in invasive analysis, and 3728 were called per case in DES-NIPT analysis across whole coding region. Out of those, there were an average of 3714 overlapping paternal-only inherited variants called per case in both invasive and plasma analyses, showing a high concordance rate for inherited paternal variants between the methods. The average concordance rate for paternal genotyping by DES-NIPT method was 99.12%. These results show that paternal-only variant analysis in DES-NIPT allows exclusion of paternal variants in screening for known monogenic disorders.

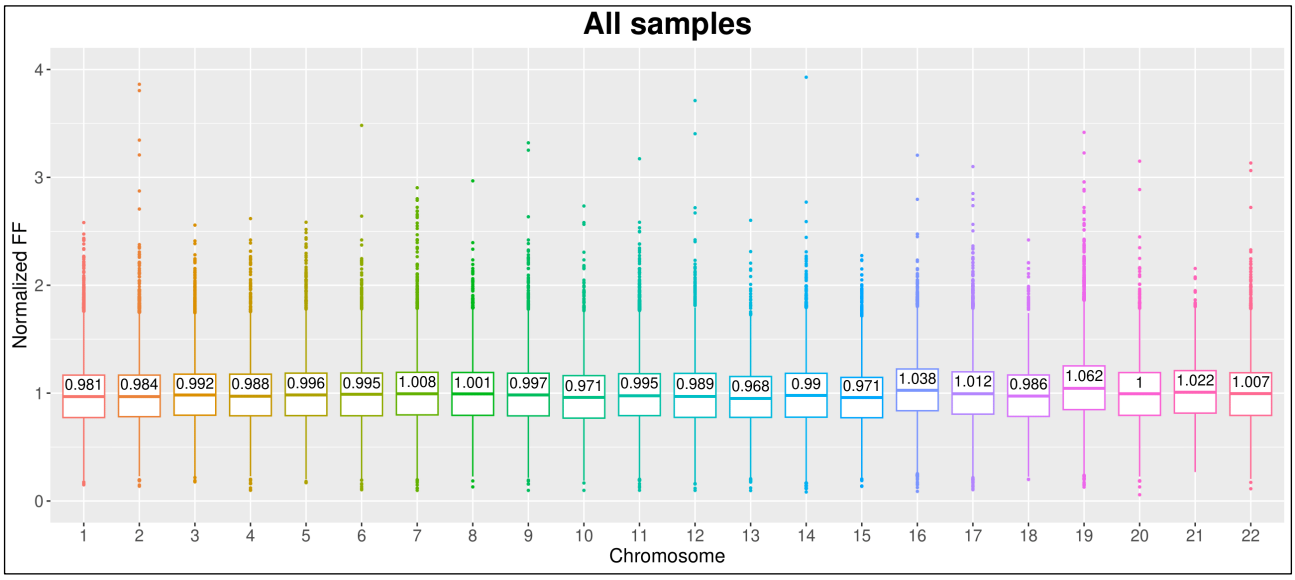
The distribution analysis of discordant and concordant paternal-only variants across different regions is depicted in Figure S10.

## Figure S8. Maternal low-mosaic variants of WBC origin




Correlation of variant allele frequency (VAF) of single nucleotide variants found in maternal plasma cell-free DNA and in maternal wbcDNA (gDNA from white blood cells). In total, 70 plasma variants were seen in both plasma cfDNA and in maternal wbcDNA at low VAF, indicating a low-grade maternal mosaicism within the populations of maternal white blood cells (WBC). In total, 70 variants were detected (1.94 variants were sample). The vast majority of the maternal mosaic variants were not seen in the invasive fetal samples (n=68). However, two variants (blue) were also detected in the invasive fetal sample, indicating that the maternal mosaicism was also present in the maternal germ cells.

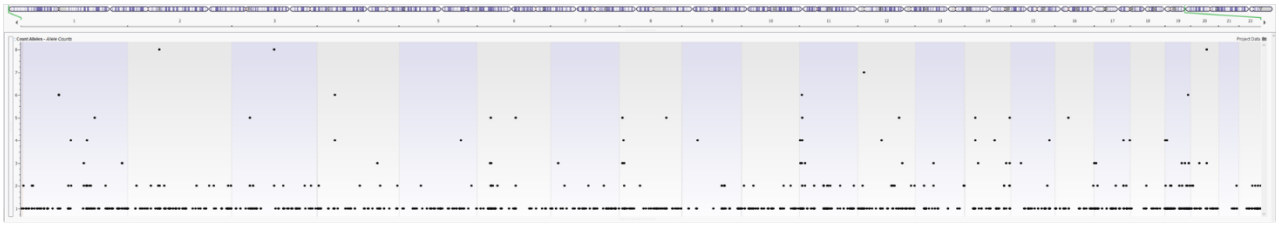
Figure S9. Variability of normalized fetal fraction across different chromosomes for all cases




The fetal DNA are overall uniformly represented across all chromosomes, but we observed a minor increased fetal fraction from chromosome 19 across all cases.

## Figure S10. False negative and true positive paternal-only variant distribution

**Genomic distribution of False Negative (FN) variants**
(paternal-only variants detected in invasive samples but not detected in plasma)



**Chromosomal distribution of False Negative variants**
(paternal-only variants detected in invasive samples but not in plasma)

String Field Counts (1,127 Records)

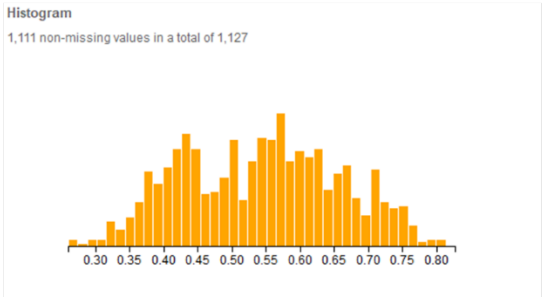
| Category | | Count | Percent |
|---|---|---|---|
| 1 | 9.32% | 105 | 9.32% |
| 2 | 7.28% | 82 | 7.28% |
| 3 | 4.97% | 56 | 4.97% |
| 4 | 4.35% | 49 | 4.35% |
| 5 | 4.26% | 48 | 4.26% |
| 6 | 6.21% | 70 | 6.21% |
| 7 | 4.17% | 47 | 4.17% |
| 8 | 4.53% | 51 | 4.53% |
| 9 | 3.11% | 35 | 3.11% |
| 10 | 4.44% | 50 | 4.44% |
| 11 | 7.99% | 90 | 7.99% |
| 12 | 4.88% | 55 | 4.88% |
| 13 | 2.48% | 28 | 2.48% |
| 14 | 4.70% | 53 | 4.70% |
| 15 | 3.28% | 37 | 3.28% |
| 16 | 2.66% | 30 | 2.66% |
| 17 | 5.50% | 62 | 5.50% |
| 18 | 1.15% | 13 | 1.15% |
| 19 | 8.96% | 101 | 8.96% |
| 20 | 2.66% | 30 | 2.66% |
| 21 | 0.62% | 7 | 0.62% |
| 22 | 2.48% | 28 | 2.48% |
| Total | 0.00% 1.86% 3.73% 5.59% 7.45% 9.32% | 1127 | 100.0% |

**Chromosomal distribution of True Positive (TP) variants**
(paternal-only variants detected in both invasive and plasma samples)

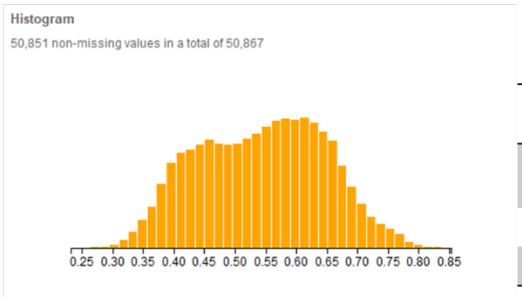
String Field Counts (50,867 Records)

| Category | | Count | Percent |
|---|---|---|---|
| 1 | 9.95% | 5063 | 9.95% |
| 2 | 7.12% | 3623 | 7.12% |
| 3 | 5.33% | 2709 | 5.33% |
| 4 | 4.09% | 2081 | 4.09% |
| 5 | 4.56% | 2320 | 4.56% |
| 6 | 5.59% | 2845 | 5.59% |
| 7 | 4.78% | 2432 | 4.78% |
| 8 | 3.33% | 1692 | 3.33% |
| 9 | 4.06% | 2064 | 4.06% |
| 10 | 4.11% | 2091 | 4.11% |
| 11 | 6.75% | 3435 | 6.75% |
| 12 | 4.91% | 2497 | 4.91% |
| 13 | 1.66% | 843 | 1.66% |
| 14 | 3.27% | 1665 | 3.27% |
| 15 | 3.37% | 1712 | 3.37% |
| 16 | 4.57% | 2323 | 4.57% |
| 17 | 5.82% | 2958 | 5.82% |
| 18 | 1.66% | 843 | 1.66% |
| 19 | 8.42% | 4283 | 8.42% |
| 20 | 2.77% | 1408 | 2.77% |
| 21 | 1.29% | 656 | 1.29% |
| 22 | 2.60% | 1324 | 2.60% |
| Total | 0.00% 1.99% 3.98% 5.97% 7.95% 9.95% | 50867 | 100.0% |

**GC-content distribution of exons with FN variants**




**GC-content distribution of exons with TP variants**




| GC-content | FN | TP |
|---|---|---|
| Median | 0.55 | 0.55 |
| Mean | 0.54 | 0.54 |
| SD | 0.10 | 0.11 |

No obvious regional clustering of the false negative variants across the whole coding region, or across the different chromosomes. There was no significant difference in GC-content observed in the regions with false negative variants compared with exons with true positive inherited paternal variants. (Screenshots from VarSeq).

# Supplementary Tables

## Table S1. Overview of cases included in the DES-NIPT study.

| *Case* | *Plasma sample, GA (weeks)* | *Maternal age (years)* | *Paternal age (years)* | *Indication for DES-NIPT* | *Invasive prenatal sample* | *Invasive NGS type* |
|---|---|---|---|---|---|---|
| *1* | 22+0 | 25 | 24 | Megaureter, slightly short bones and bradycardia, increased nuchal translucency | CVS | WES |
| *2* | 14+5 | 26 | 28 | NT 10.2 mm | CVS | WES |
| *3* | 14+0 | 29 | 41 | NT 5.8 mm, increased risk for T21 1:32 & T18 1:47 | CVS | WES |
| *4* | 17+3 | 38 | 37 | Cystic hygroma first trimester, since short bones | CVS | WES |
| *5* | 18+4 | 39 | 46 | Large dysplastic kidney (NT 3.8 mm) | CVS | WES |
| *6* | 14+5 | 31 | 30 | NT 5.2 mm | CVS | WES |
| *7* | 17+5 | 32 | 30 | Severe heart malformations & suspected anotia | AF | WES |
| *8* | 19+5 | 31 | 33 | Severe IUGR | AF | WES |
| *9* | 18+3 | 33 | 36 | Cyst fossa posterior, cleft unilateral lip-gum, SUA, suspicion heart malformation | AF | WES |
| *10* | 14+4 | 32 | 31 | Cystic hygroma | CVS | WES |
| *11* | 20+1 | 22 | 43 | Short radius and ulna, rudimentary hand (right side) | AF | WES |
| *13* | 19+0 | 38 | 48 | Short bones, bilateral clubfoot, bilateral pyelectasis, suspected skeletal dysplasia | AF | WES |
| *15* | 20+1 | 33 | 35 | Diaphragmatic hernia, suspected heart malformation | CVS | WES |
| *16* | 20+3 | 26 | 31 | Large echogenic kidneys bilat, consanguinity | AF | WES |
| *17* | 14+0 | 36 | 36 | Abdominal bubble, suspected intestinal atresia | CVS | WES |
| *18* | 17+6 | 27 | 27 | NT 5.6 mm on 1. trimester scan, persistent oedema at GA16+0 (5.1 mm), heart and feet were suspicious | AF | WES |
| *19* | 13+1 | 24 | 24 | Omphalocele | CVS | WES |
| *20* | 20+3 | 25 | 30 | Malformation of one hand | AF | WES |
| *21* | 16+3 | 27 | 32 | Bilateral pyelectasis | AF | WES |
| *22* | 20+0 | 26 | 32 | Omphalocele, bilateral clubfoot | CVS | WES |
| *23* | 19+0 | 32 | 41 | Heart malformation suspected | AF | WES |
| *25* | 17+5 | 21 | 25 | NT 6.1 mm | CVS | WES |
| *26* | 20+0 | 37 | 42 | IUGR | CVS | WES |
| *27* | 21+1 | 35 | 34 | Small for date, asymmetric | AF | WES |

| | | | | | | |
|---|---|---|---|---|---|---|
| *28* | 13+1 | 29 | 31 | NT 5.1 mm, edema around heart and lungs | CVS | WGS |
| *30* | 13+2 | 32 | 38 | NT 6.0 mm, Hydrops fetalis | CVS | WGS |
| *31* | 20+4 | 25 | 24 | Malformation of both feet, suspected malformation of hands | AF | WGS |
| *33* | 18+1 | 26 | 52 | Heart malformation (AVSD) | CVS | WGS |
| *34* | 14+5 | 30 | 34 | NT 8.9 mm, Heart malformation and diaphragmatic hernia suspected | CVS | WGS |
| *35* | 20+5 | 31 | 39 | CNS: Enlarged 3. ventricle | AF | WGS |
| *36* | 20+5 | 39 | 44 | Diaphragmatic hernia suspected | AF | WGS |
| *37* | 19+6 | 31 | 35 | Diaphragmatic hernia, hydronephrosis | AF | WGS |
| *38* | 20+0 | 30 | 30 | Large head, abnormal cardiac axis, hyperechogenic kidneys, femur in lower normal range | AF | WGS |
| *39* | 20+2 | 25 | 26 | Short bones, narrow thorax, large head circumference | AF | WGS |
| *40* | 20+4 | 42 | 49 | Bilateral severe hydronephrosis | CVS | WGS |
| *41* | 14+0 | 26 | 31 | Large abdominal bubble | CVS | WGS |

GA - gestational age; NT – nuchal translucency; IUGR – intrauterine growth restriction; CNS – central nervous system; CVS - chorionic villus sample; AF - amniotic fluid; CMA - chromosomal microarray; WES - whole exome sequencing; WGS - whole genome sequencing.

Table S2. Clinically relevant de novo sequence variants identified by DES-NIPT

| Case No. | 02 | 05 | 13 | 34 | 39 |
|---|---|---|---|---|---|
| **Chr:Pos** | 1:155904495 | 12:6601658 | 7:94412095 | 15:96337419 | 4:1801841 |
| **Ref/Alt** | A/C | G/C | G/A | TACGT/- | C/G |
| **Gene Names** | RIT1 | CHD4 | COL1A2 | NR2F2 | FGFR3 |
| **HGVS c., p.** | NM_006912.6:c.245T>G; NP_008843.1:p.Phe82Cys | NM_001273.5:c.547C>G; NP_001264.2:p.Gln183Glu | NM_000089.4:c.1378G>A; NP_000080.2:p.Gly460Ser | NM_021005.4:c.1044_1048delCGTTA; NP_066285.1:p.Tyr348Ter | NM_000142.5:c.746C>G; NP_000133.1:p.Ser249Cys |
| **Sequence Ontology** | missense | missense | missense | frameshift | missense |
| **ACMG classification** | C4: Likely Pathogenic | C4: Likely Pathogenic | C5: Pathogenic | C5: Pathogenic | C5: Pathogenic |
| **FF, %** | 9.58% | 7.01% | 8.27% | 4.51% | 7.83% |
| **VAF in plasma** | 0.0198 | 0.0227 | 0.0464 | 0.0163 | 0.0558 |
| **Site read depth in plasma** | 455 | 352 | 560 | 734 | 538 |
| **Allelic Depths** | 446,9 | 344,8 | 534,26 | 722,12 | 508,30 |
| **Alternative allele depth** | 9 | 8 | 26 | 12 | 30 |
| **SQ** | 51.5 | 11.79 | 11.80 | 10.32 | 51.5 |
| **Confirmed by invasive** | True | True | True | True | True |
| **VAF in invasive** | 0.513 | 0.488 | 0.420 | 0.645 | 0.385 |
| **Allelic Depths in invasive** | 150,158 | 205,195 | 83,60 | 11,20 | 64,40 |
| **Site read depth in invasive** | 308 | 400 | 143 | 31 | 104 |
| **Conditions** | Noonan syndrome 8 | Sifrim-Hitz-Weiss syndrome | Combined Osteogenesis imperfecta and Ehlers-Danlos syndrome 2 | Congenital heart defects/ NR2F2-related disease | Thanatophoric dysplasia type 1, type 2 |
| **OMIM ID** | 615355 | 617159 | 619115 | 615779 | 187600-187601 |

Table S3. Sequencing coverage metrices and performance details for each case

| Case | Fetal fraction, % | SD of FF across exome | Coefficient of variation | Average target coverage after UMI collapsing | % of target coverage at 100x | Mean UMI family size | Mean fetal target coverage after UMI collapsing | Paternal variant in invasive | Paternal variants in plasma | Paternal variants in both | Precision, % | Sensitivity, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **01** | 8.25 | 0.012 | 0.29 | 588.81 | 98.42 | 6.67 | 48.60 | 3353 | 3326 | 3302 | 99.28 | 98.48 |
| **02** | 9.58 | 0.013 | 0.27 | 900.28 | 98.44 | 4.58 | 86.20 | 4176 | 4167 | 4142 | 99.40 | 99.19 |
| **03** | 13.44 | 0.016 | 0.24 | 608.07 | 98.44 | 5.32 | 81.73 | 3723 | 3705 | 3697 | 99.78 | 99.30 |
| **04** | 7.64 | 0.011 | 0.28 | 909.55 | 98.32 | 4.58 | 69.51 | 3830 | 3787 | 3780 | 99.82 | 98.69 |
| **04** | 7.03 | 0.012 | 0.34 | 474.75 | 98.25 | 11.07 | 33.36 | 3749 | 3526 | 3510 | 99.55 | 93.62 |
| **06** | 12.13 | 0.018 | 0.30 | 349.04 | 98.10 | 17.85 | 42.34 | 3884 | 3813 | 3807 | 99.84 | 98.02 |
| **07** | 10.14 | 0.015 | 0.29 | 434.29 | 98.35 | 7.11 | 44.02 | 3429 | 3379 | 3373 | 99.82 | 98.37 |
| **08** | 5.23 | 0.008 | 0.31 | 966.21 | 98.33 | 4.16 | 50.49 | 3599 | 3467 | 3453 | 99.60 | 95.94 |
| **09** | 6.95 | 0.009 | 0.27 | 1086.73 | 98.54 | 7.43 | 75.51 | 3748 | 3729 | 3712 | 99.54 | 99.04 |
| **10** | 8.00 | 0.013 | 0.33 | 438.02 | 98.36 | 6.59 | 35.03 | 3827 | 3656 | 3647 | 99.75 | 95.30 |
| **11** | 5.24 | 0.009 | 0.34 | 632.60 | 98.44 | 12.51 | 33.15 | 4081 | 3858 | 3851 | 99.82 | 94.36 |
| **13** | 8.26 | 0.011 | 0.27 | 730.45 | 98.54 | 7.76 | 60.31 | 3658 | 3634 | 3625 | 99.75 | 99.10 |
| **15** | 8.75 | 0.015 | 0.34 | 381.33 | 98.27 | 10.06 | 33.35 | 3897 | 3679 | 3677 | 99.95 | 94.35 |
| **16** | 6.21 | 0.010 | 0.34 | 563.77 | 98.30 | 9.35 | 35.01 | 3160 | 2989 | 2984 | 99.83 | 94.43 |
| **17** | 5.82 | 0.009 | 0.32 | 613.47 | 98.43 | 8.80 | 35.73 | 2569 | 2461 | 2459 | 99.92 | 95.72 |
| **18** | 9.96 | 0.018 | 0.35 | 247.03 | 97.68 | 29.92 | 24.60 | 3716 | 3278 | 3219 | 98.23 | 86.63 |
| **19** | 9.64 | 0.016 | 0.32 | 356.05 | 98.17 | 12.78 | 34.33 | 3959 | 3812 | 3795 | 99.55 | 95.86 |
| **20** | 5.37 | 0.009 | 0.35 | 591.63 | 98.46 | 12.24 | 31.79 | 3827 | 3601 | 3592 | 99.75 | 93.86 |
| **21** | 11.97 | 0.020 | 0.33 | 278.80 | 97.92 | 12.54 | 33.37 | 3804 | 3616 | 3595 | 99.42 | 94.51 |
| **22** | 9.27 | 0.013 | 0.28 | 640.64 | 98.42 | 4.23 | 59.37 | 3804 | 3777 | 3755 | 99.42 | 98.71 |
| **23** | 3.72 | 0.007 | 0.40 | 595.22 | 98.33 | 9.05 | 22.14 | 3753 | 2812 | 2808 | 99.86 | 74.82 |
| **25** | 8.05 | 0.012 | 0.29 | 654.77 | 98.47 | 5.64 | 52.69 | 4581 | 4522 | 4502 | 99.56 | 98.28 |
| **26** | 8.04 | 0.010 | 0.26 | 846.59 | 98.49 | 4.66 | 68.08 | 3749 | 3708 | 3698 | 99.73 | 98.64 |
| **27** | 19.00 | 0.019 | 0.20 | 895.36 | 98.39 | 4.37 | 170.13 | 3579 | 3565 | 3565 | 100.00 | 99.61 |
| **28** | 5.29 | 0.010 | 0.37 | 514.22 | 98.35 | 6.64 | 27.20 | 3914 | 3364 | 3355 | 99.73 | 85.72 |
| **30** | 9.21 | 0.014 | 0.31 | 408.30 | 98.23 | 15.53 | 37.59 | 3711 | 3561 | 3551 | 99.72 | 95.69 |
| **31** | 8.67 | 0.014 | 0.32 | 459.14 | 98.30 | 6.55 | 39.82 | 4359 | 4203 | 4193 | 99.76 | 96.19 |
| **33** | 6.87 | 0.012 | 0.34 | 495.87 | 98.35 | 6.16 | 34.08 | 3840 | 3556 | 3552 | 99.89 | 92.50 |
| **34** | 4.36 | 0.008 | 0.36 | 925.12 | 98.43 | 6.98 | 40.38 | 4116 | 3945 | 3928 | 99.59 | 95.43 |
| **35** | 5.01 | 0.010 | 0.39 | 494.12 | 98.34 | 6.31 | 24.76 | 3553 | 2904 | 2888 | 99.45 | 81.28 |
| **36** | 5.96 | 0.010 | 0.34 | 575.22 | 98.23 | 5.35 | 34.28 | 3788 | 3458 | 3451 | 99.80 | 91.10 |
| **37** | 9.10 | 0.013 | 0.29 | 558.68 | 98.25 | 7.67 | 50.82 | 3340 | 3290 | 3284 | 99.82 | 98.32 |
| **38** | 13.66 | 0.018 | 0.26 | 573.83 | 98.28 | 7.59 | 78.40 | 3738 | 3727 | 3704 | 99.38 | 99.09 |
| **39** | 7.82 | 0.011 | 0.29 | 653.84 | 98.29 | 6.69 | 51.16 | 4082 | 3996 | 3985 | 99.72 | 97.62 |
| **40** | 14.53 | 0.019 | 0.26 | 497.60 | 98.20 | 5.80 | 72.32 | 3711 | 3667 | 3662 | 99.86 | 98.68 |
| **41** | 8.60 | 0.013 | 0.29 | 596.31 | 98.34 | 6.61 | 51.31 | 4004 | 3925 | 3919 | 99.85 | 97.88 |

Table S4. Representativeness of study participants

| Category | Characteristics |
|---|---|
| Disease, problem, or condition under investigation | Prenatal genetic testing cohort |
| Special considerations related to: | |
| Sex and gender | The study included pregnant individuals (referred to as mothers of the fetus) and their partners (biological fathers of the fetus). The biological sex of the fetus was not a relevant factor for inclusion, as prenatal testing must be applicable for fetuses of all sexes. |
| Age | There was no age restriction for couples, and it was not an inclusion criterion for the study. Maternal age at inclusion ranged from 22 to 42 years, while paternal age ranged from 24 to 49 years.<br>Pregnancies included in the study were between 13$^{th}$ and 22$^{nd}$ weeks gestational age at inclusion. This timeframe aligns with the study's objective to provide results by the 22nd week of pregnancy, allowing for timely decision-making. |
| Race or ethnic group | Race or ethnic group was not a selection criterion, and this information was not collected. Prenatal genetic testing is applicable to individuals of all racial and ethnic backgrounds, and this cohort represents the diversity of races and ethnicities in Denmark. |
| Geography | Region of Southern Denmark |
| Other considerations | Pregnancies were included if they met one or both of the following criteria: increased nuchal translucency detected during 1st-trimester screening and/or the identification of one or more fetal anomalies during 2nd-trimester ultrasound. Informed consent was obtained from all participants. |
| Overall representativeness of this study | The study cohort is deemed representative, as it aligns with the inclusion criteria established in this study, making it applicable to a broader population adhering to these criteria. |

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