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CASE REPORT

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Cell-based noninvasive prenatal testing (cbNIPT) detects pathogenic copy number variations

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Abstract

In two cases, cell-based noninvasive prenatal testing (cbNIPT) detected pathogenic copy number variations (CNVs) in the fetal genome. cbNIPT may potentially be an improved noninvasive alternative for the detection of smaller CNVs.

KEYWORDS

3p deletion, 3p26 deletion, cell-based noninvasive prenatal testing, copy number variation, Noninvasive prenatal testing, Prader-Willi syndrome

1 | INTRODUCTION

In two cases, cell-based noninvasive prenatal testing (cbNIPT) detected copy number variations (CNVs): a 7 Mb deletion of 15q11q13 covering the Prader-Willi region and a 4.6 Mb deletion at 3p26.3p26.1. This may potentially be an improved noninvasive alternative for the detection of smaller CNVs.

Denmark has a tax-financed combined first trimester screening program that consists of a nuchal translucency scan, maternal age, fβ-hCG, and PAPP-A measurements. The program has an uptake of above 93%.[1,2] If a woman has a risk equal to or greater than 1:300 for trisomy 21 or 1:150 for trisomy 18 or 13, she may choose between invasive and noninvasive prenatal testing after appropriate counseling on detection rates and risk.[3,4] In Denmark, the majority of women opt for invasive testing if the risk is high.[2,5]

Cell-free noninvasive prenatal testing (cfNIPT) based on sequencing analysis of cell-free DNA has been widely implemented for screening of common aneuploidies and sex chromosomal aberrations (SCA).[6] However, a substantial number of fetal genetic disorders caused by subchromosomal copy

number variations (CNVs) are often missed by cfNIPT.[1,7-9] There have been several reports on cfNIPT's ability to detect CNVs, but the predictive values for microdeletion and microduplication syndromes are still low, with high false positive and false negative rates.[8,10]

An emerging new technology using intact fetal extravillous trophoblasts (fEVTs) isolated from maternal blood could be an alternative to cfNIPT.[11] These cells encompass an intact fetal genome that can be analyzed without maternal contamination and, hence, can help avoid some of the inherent problems of the cfNIPT analysis. The major obstacle for the implementation of this technology has been the rarity of these fetal cells and the limited knowledge of specific and sensitive markers to allow isolation of fEVTs from every pregnancy.[12] We and others have previously shown that fEVTs can be isolated from gestational weeks 10-14 without maternal contamination and that these cells can be used to detect fetal aneuploidies as well as subchromosomal CNVs.[13-15] As part of our validation of cbNIPT, we have initiated a clinical study in the Central Denmark Region where pregnant women opting for cfNIPT are also offered a cbNIPT for comparison. In the current study, we present two cases; first, where cbNIPT detected a 7 Mb deletion at 15q11q13 covering the Prader-Willi Syndrome (PWS) region and second, where cbNIPT detected a 4.6 Mb deletion at 3p26.3p26.1 covering the ITPR1 gene leading to Spinocerebral Ataxia type 15.

2 | CASE PRESENTATION

Case 1 was a 28-year-old primipara with two early miscarriages within the last 6 months. The pregnancy was spontaneously conceived. The woman attended combined first trimester screening at a Department of Obstetrics and Gynecology at a Regional Hospital in Denmark. The nuchal translucency was 3.0 mm, PAPP-a 0.9, and fβ-hCG 1.2 MOM, leading to a combined risk for trisomy 21 of 1:294 at gestational age (GA) 11 weeks and 4 days (11 + 4). She was counseled on invasive versus noninvasive testing and opted for the latter. She was offered cbNIPT in addition to cfNIPT to which she agreed and signed the consent form.

50 ml of peripheral blood was drawn in Cell-Free DNA BCT tubes (Streck laboratories, USA). The first sample was unfortunately discarded due to a laboratory misunderstanding at the Department of Clinical Genetics in the handling of the cfNIPT sample; therefore, a new sample was obtained at GA 13 + 0.

For the cbNIPT analysis, fEVT cells were isolated from 30 mL of blood as previously described, using a cocktail of antibodies and Magnetic Activated Cell Sorting (Miltenyi Biotech, Germany).[13] Each isolated cell was amplified by whole genome amplification (WGA) using Smart Picoplex WGA (Takara, Japan). Subsequently, to exclude any maternal contamination, isolated cells were validated for their origin by short tandem repeat (STR) analysis using the GlobalFiler kit (Thermo Fischer Scientific, USA), analyzing 24 different STR loci in multiplex. The STR analysis was run on a capillary electrophoresis (ABI3500), and data analysis was performed in GeneMapper ID-X fragment size analyze software (Thermo Fischer Scientific). The cbNIPT genetic analysis was performed by array comparative genomic hybridization (aCGH) on a pool of WGA DNA from three fEVTs using SurePrintG3Human CGH 4x180K arrays from Agilent Technologies. The DNA used as a reference for fEVT cell WGA product was a pool of 5 WGA reactions from lymphoblast genomic DNA (Promega, USA). The copy numbers were determined using the adm-2 algorithm. The filters used for detection of aberrations were minimum size of regions of 5 Mb and minimum absolute average logratio of region of 0.3 for gains and 0.4 for losses. The cbNIPT result showed a 7.4 Mb deletion on chromosome 15 (15q11.2q13.1(22480969-29838300) x1) at a mean logratio of −0.75 in a male fetus (Figure 1).

For the cfNIPT analysis, DNA extraction, genome-wide massive parallel sequencing, and data analysis were carried out as described by the kit manufacturer (Illumina©) applying TG TruSeq® Nano DNA Sample Preparation kit v1.0 + TG NSQ 500/550 High Output Kit v1.3 and VeriSeq NIPT Analysis Software v1. The cell-free fetal DNA (cffDNA) fraction was 10%, and the result showed a male karyotype and 2 copies of each of the chromosomes 13, 18, and 21. The deletion of the 15q11.2q13.1 region was not detected with this method.

CbNIPT and cfNIPT reports were generated at GA 14 + 1.

At the genetic counseling following the cbNIPT result, the woman opted for a CVS analysis. aCGH using SurePrint G3Human CGH 4x180K arrays from Agilent Technologies was performed on DNA from uncultured CVS material. CNVs are called when at least 4 or more neighboring probes are deleted/duplicated resulting in a resolution of approximately 50 kb.

The aCGH analysis on CVS showed an 8 Mb deletion on chromosome 15 (15q11.1q13.1(20481702-28535051)x1) in a male fetus. The deletion from 15q11.1 to 15q11.2 is a known benign CNV that is not clinically reported, so the final clinical conclusion resulted in a 5.8 Mb deletion on chromosome 15 (arr[GRCh37] 15q11.2q13.1(22765628_28535051) x1) covering the PWS region. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) (ME028-C1, MRC-Holland, the Netherlands) revealed the deletion to be of paternal origin and thus causative of Prader-Willi syndrome. The couple were subsequently counseled by IV.

Based on the abnormal cbNIPT result, it was decided to further examine the cbNIPT on a single cell level for research

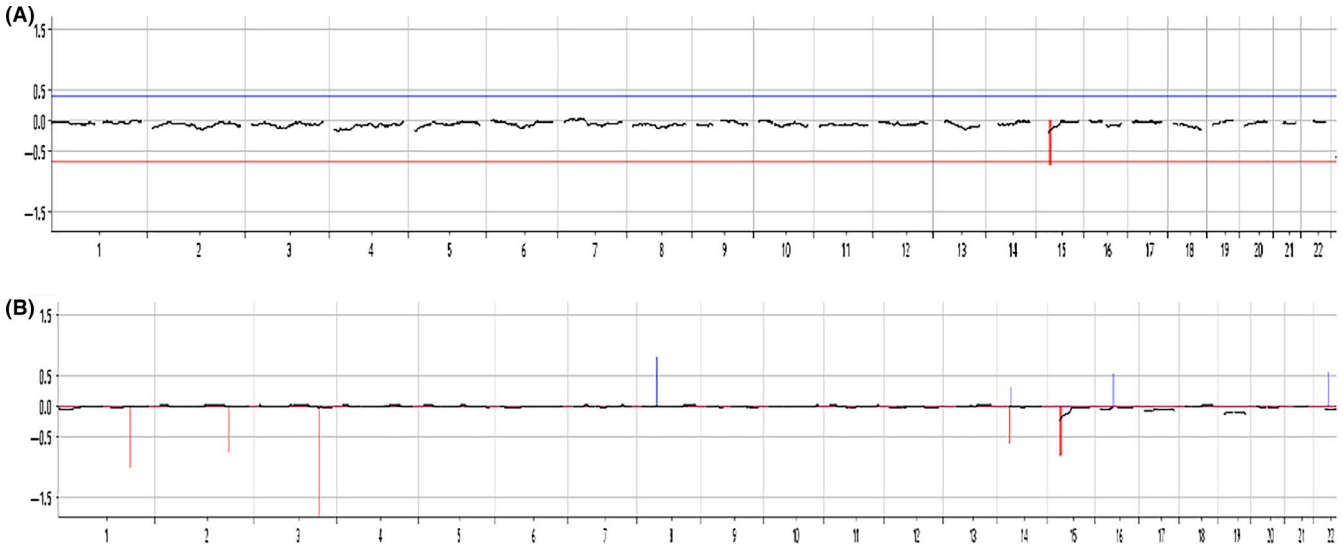

FIGURE 1 Cell based NIPT (cbNIPT) and chorionic villi sampling(CVS) chromosomal microarray results for case 1. A, cbNIPT chromosomal microarray using a pool of three fetal extra villous trophoblast (fEVT) whole genome amplified DNA identifies a 7.4 Mb deletion on chromosome 15 (15q11.2q13.1) covering the Prader-Willi syndrome(PWS) region. B, Validation by CVS chromosomal microarray confirms an 8 MB deletion on chromosome 15 (15q11.1q13.1). The deletion from 15q11.1 to 15q11.2 is however a known benign copy number variation (CNV), so the final clinical conclusion resulted in a 5.8 Mb deletion on chromosome 15 (15q11.2q13.1) covering the PWS region. CNVs were called when at least 4 probes were deleted/duplicated, resulting in the detection of several smaller, benign CNVs

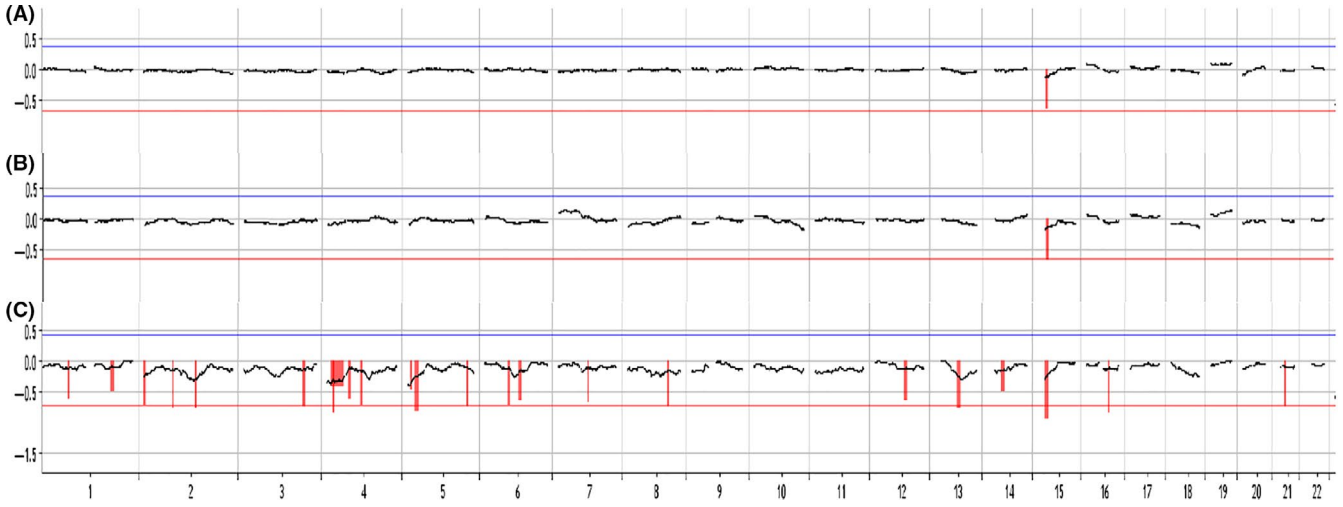

FIGURE 2 Chromosomal microarray for the three individual fetal extra villous trophoblast (fEVT) cells analysed in case 1. A and B, single cells identifies the deletion at chromosome 15 (15q11.2q13.1). C) The microarray result for the third cell gives a noisy uninterpretable result

purposes after the clinical report was made. Hence, the three fEVTs were analyzed individually by additional aCGH. Two out of these three fEVTs corroborated the deletion at chromosome 15 (15q11.2q13.1), but the third cell failed, only giving a noisy uninterpretable result (Figure 2).

Case 2 was a 45-year-old primipara, with one prior early miscarriage within the last 6 months. The pregnancy was achieved by assisted reproduction at one of the regional fertility clinics, and the pregnant woman attended combined first trimester screening at a Department of Obstetrics and Gynecology in the Central Denmark region in Denmark. The nuchal translucency was 1.4 mm, PAPP-a 0.5, and fβ-hCG 1.0 MOM, leading to a combined risk for trisomy 21 of 1:63 at GA 13 + 0. She was counseled on invasive versus noninvasive testing and initially opted for CVS. However, as a placenta biopsy was not obtainable, at GA 13 + 3, the woman opted for noninvasive testing. She was offered cbNIPT in addition to cfNIPT to which she consented and signed the consent form.

Blood was collected, processed, and fEVTs isolated using the same protocol as used in Case 1. The cbNIPT genetic analysis was performed by aCGH on a pool of WGA DNA

from three fEVTs. The result showed a drop at the terminal end of chromosome 3. However, it was not called by the software due to the 5 Mb minimum size of regions threshold set by us, but recognized visually by the analyzing personnel (IV and RC). Lowering the threshold to 3 Mb resulted in the software calling a 4.6 Mb deletion at chromosome 3 (3p26.3p26.1(73914-4722234) x1) at a mean logratio of −0.88. A 3 Mb deletion at chromosome 4 was also called by the software, but this was discarded by IV and RC as noise (false-positive signal) due to a mean logratio of only −0.48 as opposed to the expected mean logratio for a deletion at −1 (Figure 3).

The cfNIPT analysis revealed a cffDNA fraction of 11%, and the result showed a female karyotype and 2 copies of each of the three chromosomes 13, 18 and 21. The deletion of the 3p26.3p26.1 region was not detected. CfNIPT and cbNIPT reports were generated at GA 14 + 4.

At the genetic counseling following the cbNIPT result of the deletion at 3p26, the woman opted for invasive testing, and finally an amniocentesis was performed. Parental blood samples were included in the same run of the aCGH. All samples were analyzed by SurePrint G3 Human CGH 4x180K arrays using the same analysis settings as for CVS analysis. A 4.7 Mb deletion at chromosome 3(3p26.3p26.1(73914-4732996) x1) was verified in the amniocytes as well as in the paternal sample. The parents were subsequently offered genetic counseling.

For research purposes, it was decided to re-analyze the cbNIPT on single cell level. Two out of these three individually run fEVTs demonstrated the deletion at chromosome 3 (3p26.3p26.1), but the third cell failed, only giving a noisy uninterpretable result (Figure 4).

The project was approved by the local Danish Scientific Ethics Committee (S-20 070 045) and the Danish Data Protection Agency (2008-58-0035). The women gave written informed consent for their cases to be published as case reports.

3 | DISCUSSION

We describe that cbNIPT was able to detect two different pathogenic CNVs that were not identified by cfNIPT, designed for the common trisomies.

The 7.4 Mb deletion was on chromosome 15, covering the Prader-Willi syndrome region. PWS is a serious neurodevelopmental imprinting disorder that is rarely detected prenatally, particularly in the first trimester. This is due to few ultrasound abnormalities, and because the combined first trimester algorithm is designed to find trisomies. Further, PWS is characterized by a sporadic occurrence, with an estimated prevalence of 1 in 15 000-30 000 live births[16,17]; thus, a high risk group for PWS has not been identified. The phenotype of PWS varies significantly depending on age. In the neonatal and early childhood, it is dominated by infantile hypotonia, severe feeding difficulties, and developmental delay. In late childhood/preteen ages, this develops into an overeating disorder with possibly morbid obesity.[18] While the fetal phenotype is largely unknown, polyhydramnios, fetal hypomobility, small for

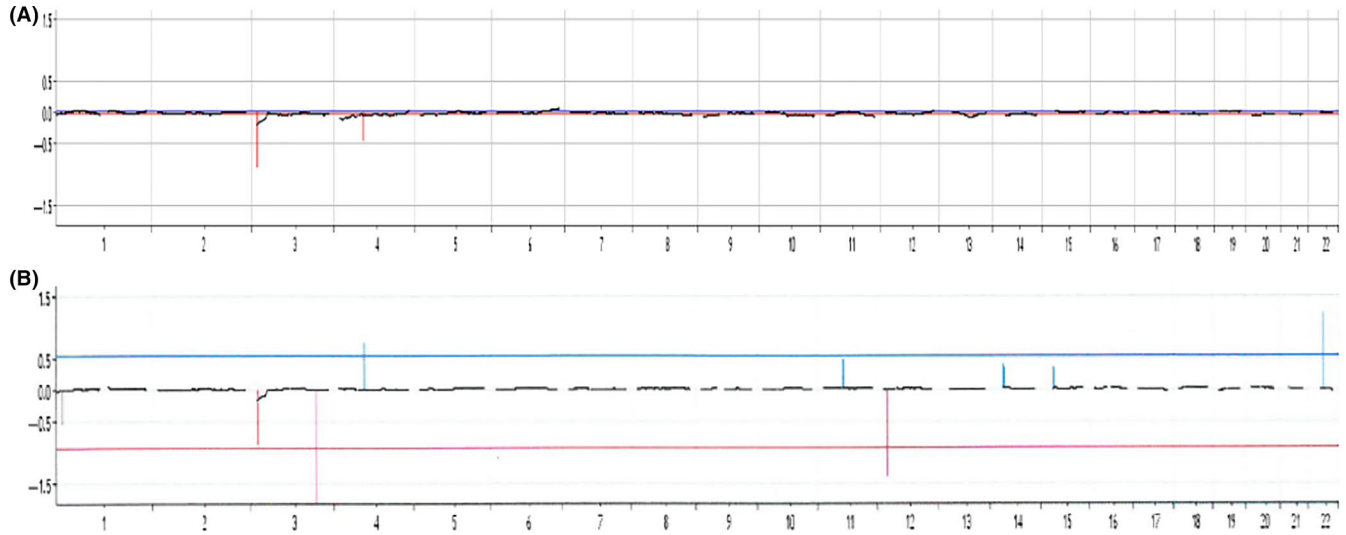

FIGURE 3 Chromosomal microarray results from cell based NIPT (cbNIPT) and amniocentesis for case 2. A, cbNIPT chromosomal microarray using a pool of three fetal extra villous trophoblast (fEVT) whole genome amplified DNA identifies a 4.6 Mb deletion on chromosome 3 (3p26.3p26.1). A 3 Mb deletion at chromosome 4 was also detected. However, due to a low mean logratio, this was interpreted as noise. B, Chromosomal microarray from amniocentesis confirms a 4.7 Mb deletion on chromosome 3 (3p26.3p26.1). Copy number variations (CNVs) were called when at least 4 probes were deleted/duplicated, resulting in the detection of several smaller, benign CNVs

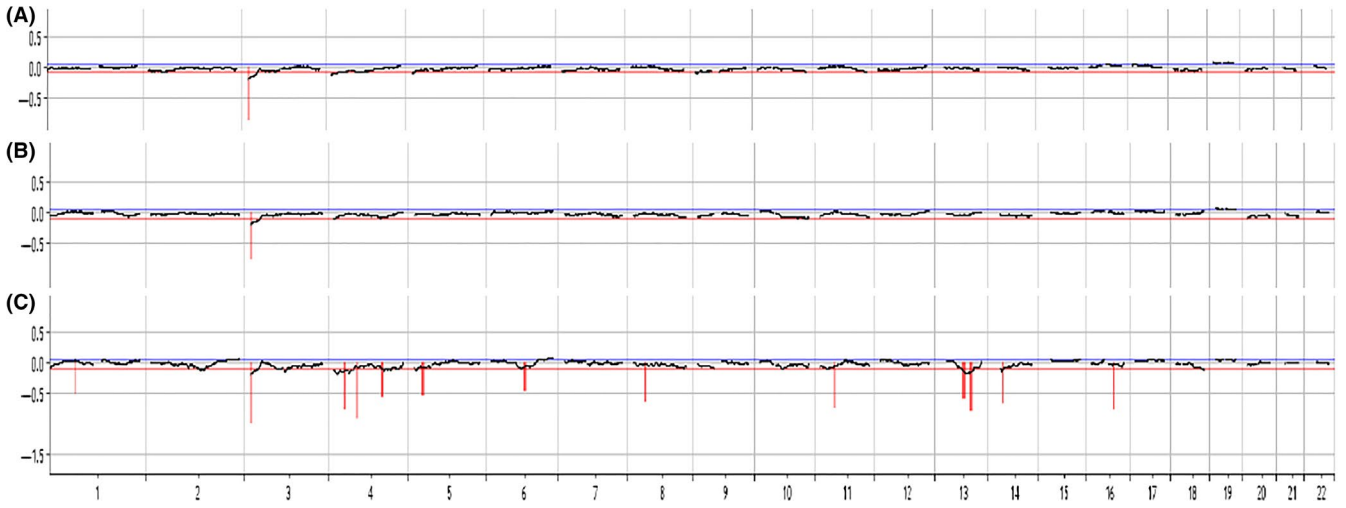

FIGURE 4 Chromosomal microarray for the three single fetal extra villous trophoblast (fEVT) cells analyzed in case 2. A and B, single cells identifies the deletion at chromosome 3(p26.3p26.1). C, The microarray result for the third cell gives a noisy uninterpretable result

gestational age, and abnormal positioning of the hands may be seen in third trimester pregnancy.[19-21]

The 3p26 deletion covers exon 1-31 of the *ITPR1* gene, which leads to the autosomal dominant Spinocerebral Ataxia type 15 (OMIM# 606 658) with a debut between 25-72 years of age of a slowly progressive ataxia.[22,23] The 3p26.3p26.1 region also contains other recessive genes (TRNT1, CRBN and SUMF1) and a gene with unknown penetrance (CHL1). The father is, however, yet unaffected, and we have a similar deletion in our in-house database from an apparently also yet unaffected female. This is not surprising as this is a disorder with a late debut and full penetrance.

It has previously been estimated that up to 23% of atypical CNVs are not detected using current cfNIPT.[1] The detection of PWS by cfNIPT has previously been published [24,25]; however, the low detection and high false-positive rates are remaining challenges.[10,26] To the knowledge of the authors, the 3p deletion has not been detected by NIPT to date. In the current study, we used the Veriseq NIPT analysis software v1 from Illumina, which is accredited for chromosomal aneuploidies 13, 18, 21, X and Y, and can therefore not be expected to detect such subchromosomal CNVs. Illumina has launched an upgraded version, VeriSeq NIPT solution v2 that screens for duplications and deletions ≥7 Mb for all autosomes. The 7.4 Mb deletion at chromosome 15 could possibly be detected by the new version but most likely not the 4.6 Mb deletion at chromosome 3.

Circulating fEVTs used for cbNIPT are estimated to be extremely rare, but several studies have confirmed that cbNIPT based on these cells is achievable.[13,14,27,28] Vossaert et al 2018 demonstrated that it is possible to detect CNVs down to 1 Mb in size.[29] This may be achievable as fEVTs can be isolated with an intact genome and without being contaminated with maternal DNA. Large-scale multicenter clinical validation is needed to determine the full clinical utility of cbNIPT. As cbNIPT is based on the isolation and analysis of fEVTs, which are of placental origin, fetoplacental mosaicism could be a challenge, as it is in prenatal diagnosis and noninvasive prenatal testing today.[14,15] Importantly, whereas cfNIPT has a negative correlation with increasing BMI, cbNIPT does not seem to be sensitive to high maternal BMI.[30]

In the current study, the deleted region in the cbNIPT analysis varied slightly in the breakpoints from the deleted region detected in the CVS and amnio analysis. Further, the size of the deletion also varied marginally between the fEVTs. This is possibly caused by a variation in the coverage during the WGA step. We observe that WGA increased the derivative spread of the microarray analyses thereby decreasing the precision of the estimation of the breakpoints. In spite of this, it is our experience that Picoplex WGA is presently the only amplification kit for reliable CNV calling on fixed cells, as has also been published by others.[29,31]

The current case report combined with previous data indicate that cbNIPT may in time be developed into a real alternative to cfNIPT in the detection of subchromosomal CNVs. In this study, for case 1, the risk from first trimester screening was 1:294. Had the risk been less than 1:300, in the Danish setting, the pregnant woman would not have been given the option of invasive or noninvasive prenatal tests. The fact that CNV syndromes can be particularly difficult to detect in first trimester screening, and that these CNV syndromes are independent of maternal age, opens the possibility that cbNIPT could be used as a prenatal screening test in the future, also for pregnancies at low risk.

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CONFLICT OF INTEREST

LH, RS, KR, BHN, MK, LDJ, IBC, and PS are all employed in ARCEDI Biotech, a Danish company that has developed the fetal cell enrichment and detection procedure described in this manuscript.

AUTHOR CONTRIBUTIONS

LH was co-project leader and wrote the article, RS, KR, BHN, MK, LDJ, IBC and PS designed and performed experiments, analyzed data, and contributed to writing. RF was responsible for human subject approval and collected the clinical material. RC and LA performed aCGH, cfNIPT and MLPA analysis and interpretation. NU was responsible for study management and approval, providing expertise and advice. IV managed the project in Denmark and contributed to the writing.

ETHICAL STATEMENT

The project has been approved by the local Danish Scientific Ethical Committee (S-20 070 045) and the Danish Data Protection Agency (2008-58-0035). The pregnant women gave written informed consent for their cases to be published as a case report.

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