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ORIGINAL ARTICLE

On the road to replacing invasive testing with cell-based NIPT: Five clinical cases with aneuploidies, microduplication, unbalanced structural rearrangement, or mosaicism

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Abstract

Objective: Trophoblastic fetal cells harvested from maternal blood have the capacity to be used for copy number analyses in a cell-based non-invasive prenatal test (cbNIPT). Potentially, this will result in increased resolution for detection of subchromosomal aberrations due to high quality DNA not intermixed with maternal DNA. We present 5 selected clinical cases from first trimester pregnancies where cbNIPT was used to demonstrate a wide range of clinically relevant aberrations.

Method: Blood samples were collected from high risk pregnancies in gestational week 12 + 1 to 12 + 5. Fetal trophoblast cells were enriched and stained using fetal cell specific antibodies. The enriched cell fraction was scanned, and fetal cells were picked using a capillary-based cell picking instrument. Subsequently, whole genome amplification (WGA) was performed on fetal cells, and the DNA was analyzed blindly by array comparative genomic hybridization (aCGH).

Results: We present 5 cases where non-invasive cell-based prenatal test results are compared with aCGH results on chorionic villus samples (CVS), demonstrating aneuploidies including mosaicism, unbalanced translocations, subchromosomal deletions, or duplications.

Conclusion: Aneuploidy and subchromosomal aberrations can be detected using fetal cells harvested from maternal blood. The method has the future potential of being offered as a cell-based NIPT with large high genomic resolution.

1 | INTRODUCTION

A long-sought goal in prenatal care has been the replacement of invasive procedures by analyzing circulating fetal cells or free fetal DNA from maternal blood. The development of a sensitive and accurate cell-based non-invasive prenatal test (cbNIPT) has the potential of diagnosing small copy number variations (CNVs) and point mutations based on a maternal blood sample early in pregnancy. cbNIPT may in theory also be useful for the prenatal diagnosis of diseases and aberrations also carried by the mother, where NIPT based on cell free fetal DNA in maternal plasma (cffNIPT) is challenged by the mixture of maternal and fetal cells which limits the detection to *de novo* and paternally inherited diseases.

We previously reported a method for enriching fetal cells from maternal blood followed by amplification of the fetal genome and detection of chromosomal and subchromosomal variations in the fetal genome.[1]

As a part of a validation study of the cbNIPT method on fetal cells in maternal circulation, we now present 5 cases with a representative selection of clinically significant chromosome abnormalities which were confirmed by cbNIPT. All maternal blood samples were collected for cbNIPT prior to the invasive testing and analysed blindly.

2 | METHODS

2.1 | Clinical material

Participants were recruited among pregnant women that took part in the Danish publicly financed combined first trimester screening

program. Pregnant women who had high risk (>1:300) and opted for invasive testing at any of the 5 Obstetrics and Gynecology departments in the Central Denmark Region were asked to participate in a research program that aims at establishing a clinical cbNIPT method and evaluating the clinical accuracy of this cbNIPT method through the comparison of clinical results obtained from chromosomal microarray (CMA) on CVS with the CMA on fetal cells enriched from maternal blood (cbNIPT). Upon written consent, blood samples were collected prior to the invasive testing, and the samples were given a blinding reference. The project was approved by the local Danish Scientific Ethical Committee (S-20070045) and the Danish Data Protection Agency (2008-58-0035). The cases in the present study are selected from this research program in order to exemplify the diagnostic potential of cbNIPT.

2.2 | Blood processing, fetal cell enrichment, and staining

Thirty milliliters of peripheral blood collected in Cell-Free DNA BCT tubes (Streck, Omaha, USA) was processed and fetal cells enriched and stained using Magnetic Activated Cell Sorting (MACS) from Miltenyi (Miltenyi Biotec, DE) as previously described.[1]

2.3 | Cell smearing and automated scanning using MetaSystems (dry scan)

The final stained cell pellet from case 1 was smeared on 2 glass slides and dried at room temperature overnight. The slides were subsequently submerged in 4% formaldehyde for 10 minutes, washed in PBS for 5 minutes, and finally mounted in Vectashield with 0.6 μg/mL DAPI (Vector Laboratories). The slides were scanned using a MetaSystems scanner (MetaSystems, DE) in MetaCyte mode and with an in-house developed "classifier". For the automated scanning, a 10× objective was used. Green fluorescent cells picked out by the microscope scanner were finally validated manually using a 40× objective based on a set of in-house developed and validated criteria including cell morphology, green fluorescent staining pattern, and intensity.[1,2]

2.4 | Cell smearing and automated scanning using CellCelector (wet scan)

For all other cases, enriched and stained cells from each sample were put on 2 glass slides and scanned with a 10× objective using the CellCelector (ALS Automated Lab Solutions, DE) with an in-house developed "classifier". Green fluorescent cells picked out by the microscope scanner were finally validated manually using a 40× objective based on a set of in-house developed criteria.

2.5 | Picking of fetal cells and whole genome amplification (WGA)

Fetal cells on the scanned slides were picked individually or in groups using the CellCelector following the manufacturer's protocols.

WGA on pools of picked cells (2–7 cells) was performed using the Picoplex WGA Kit (Rubicon Genomics, MI USA) using the manufacturer's protocols.

WHAT'S ALREADY KNOWN ON THIS TOPIC:

- Enriched fetal cells from maternal blood can be used for performing whole genome amplification, followed by array CGH.

WHAT DOES THIS STUDY ADD:

- cbNIPT can detect aneuploidy, microduplication, unbalanced structural rearrangements, and mosaic cases.
- cbNIPT can identify subchromosomal aberrations >10 Mb

2.6 | Array comparative genomic hybridization

Array comparative hybridization (aCGH) was performed on DNA from uncultured CVS and on WGA DNA for cbNIPT using SurePrint G3 Human CGH 4x180K arrays from Agilent Technologies as described by the kit manufacturer. The protocol was modified in the following way: Using a Nanodrop spectrophotometer (Thermo Scientific) for quantification 300 to 700 ng of WGA DNA was labelled using the SureTag Complete DNA Labelling Kit (Agilent). Fetal DNA was labelled with Cy3, and reference DNA was labelled with Cy5 for 2 hours at 37°C. A MultiScreen-PCRμ96 Filter Plate (Millipore) was used to remove unincorporated nucleotides. Hybridizations were carried out at 67°C for 20 to 24 hours, after which they were washed and scanned using an Agilent Microarray Scanner (G2505C). Data were extracted and analysed using Agilent CytoGenomics Edition 3.0.6.6. The DNA used as a reference for each fetal cell WGA product was a pool of WGA DNA from multiple (5–10 single cell) WGA reactions from either male or female lymphoblast reference cell lines. Gender-mismatched references were used. Copy number was 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 log ratio of region of 0.35 for gains and 0.45 for loss. The interpretation of arrayCGH results rests upon the evaluation of quality control metrics including derivative log ratio spread (DLRS) integrated into the arrayCGH analysis software, and these metrics facilitate the visual inspection of overall quality of array results. DLR-spread >1.8 led to failed analyses.

2.7 | cffNIPT

For cases 3 and 4, peripheral blood was collected in Cell-Free DNA BCT tubes (Streck, Omaha, USA). For cases 1, 2, and 5, no blood samples were collected for cffNIPT analysis. DNA extraction, genome-wide massive parallel sequencing, and data analysis (VeriSeq NIPT Analysis Software) were carried out as described by the kit manufacturer (Illumina ©, San Diego, CA, USA).

3 | RESULTS

Fetal cells were isolated from peripheral blood and analyzed by aCGH after WGA from 5 first trimester high risk pregnancies (Table 1 and Figure 2). In the first part of the study, the "dry" method was used, and this method was later replaced by the more efficient "wet" method. On average, 12.8 fetal cells per 30 mL of maternal blood can be isolated,[1] and in the current study we used 2 to 7 cells for analysis.

In case 1, aneuploidy for chromosome 21 was shown by cbNIPT on 7 fetal cells isolated from maternal blood in a male fetus with increased nuchal translucency (NT) (Figures 2 and 1) in accordance with findings by aCGH on CVS and consistent with trisomy 21 in the fetus. The parents were counseled, and on parental request the pregnancy was terminated at 13 + 3 weeks.

In case 2, high-grade mosaicism (>60%) for trisomy 13 was detected by aCGH in CVS in a male fetus giving a high risk for Patau syndrome in the fetus. cbNIPT on 2 fetal cells from maternal blood showed aneuploidy of chromosome 13 (Figure 2) with no signs of mosaicism. No subsequent ultrasound examinations or amniocentesis were carried out, and the pregnancy was terminated at 13 + 3 weeks. No subsequent analyses were carried out on aborted fetal tissue.

In case 3, high-grade mosaicism (>60%) for trisomy 2 was detected by aCGH on CVS in a male fetus. Here, cbNIPT on 4 fetal cells showed duplication of chromosome 2 (Figure 2) with no signs of mosaicism. cbNIPT also called 2 small interstitial duplications and the entire chromosome 19 that were not identified by aCGH on CVS. These duplications can be attributed to quality issues and a very high DLRS value of the specific sample indicating noisiness in log ratio data. To avoid false positive calls, samples with very high DLRS value may not be acceptable for a standard size limit of 5 Mb. Furthermore, due to high GC content, chromosome 19, which is very rarely involved in chromosomal mosaicism, is frequently called by the aCGH-analysis software. A concomitant cffNIPT analysis showed by visual review of WGS results increased coverage (>4SD) for chromosome 2 indicating maternal or fetal chromosome 2 abnormality (data not shown). Trisomy 2 most likely represents a confined placental mosaicism, and a subsequent analysis of cells from amniotic fluid revealed no signs of trisomy 2.

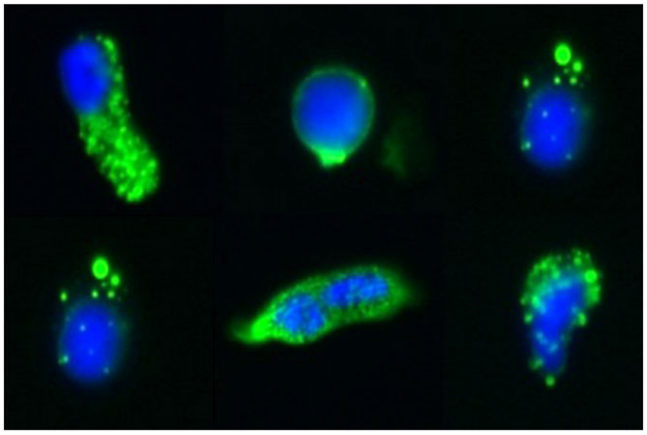

FIGURE 1 Gallery of fetal cells from case 1, enriched using ARCEDI method. These fetal cells were picked using CellCelector. WGA was performed on the cells, followed by aCGH, the result of which is shown in Figure 2 [Colour figure can be viewed at wileyonlinelibrary.com]

In case 4, which was a twin pregnancy, a partial trisomy 21 with a 12.4-Mb duplication, involving the Down critical region on 21q22.2, was detected in a male fetus by aCGH on one of the CVS. aCGH on CVS from the other fetus with a normal combined first trimester risk demonstrated a normal female karyotype. cbNIPT on 4 cells clearly demonstrated in a male fetus a duplication of 12.4 Mb on chromosome 21q22.2 (coordinates hg19 chromosome 21: 35647509–4809031) (Figure 2), which deviated from the duplication detected on CVS by app. 0.2 Mb. A concomitant cffNIPT analysis showed increased risk for trisomy 21 based on an NCV value of 4.3 for chromosome 21, which is >4 SD of the average NCV values for chromosome 21 for healthy pregnancies analyzed in our laboratory (Figure 3). Fetal fraction of DNA was 8% (VeriSeq NIPT Analysis Software).

In case 5, an unbalanced translocation including a 31.4-Mb terminal deletion on chromosome 4p and a 30.1-Mb terminal duplication on chromosome 8p in a female fetus with increased NT (5.3 mm) was detected by aCGH on a CVS. Both chromosomal abnormalities were subsequently proven by cbNIPT on 2 cells (coordinates hg19 chromosome 4: 185366–31352606 and chromosome 8: 176814–30323697)

TABLE 1 Summary of patient characteristics, indications, karyotype on CVS, and cfNIPT results in a series of fetuses presenting with aneuploidies, microduplication, and unbalanced structural rearrangement in first trimester pregnancies

Case	Age (gestational week)	BMI	Indication	NT (mm)	Karyotype [hg19] on CVS (fetal sex)	cfNIPT[a]	Analyzed cells	cbNIPT[a]
1	36 (12 + 3)	28.4	iNT	5.2	arr (21)×3 (male)	na	7	Confirmed
2	40 (12 + 1)	21.8	aFTS (1:180)	1.5	arr (13)×2~3 (65–75%) (male)	na	2	Confirmed, full
3	32 (12 + 5)	31.0	aFTS (1:37)	1.7	arr(2)×2~3 (60–70%) (male)	Suspected	4	Confirmed, full
4	23 (12 + 4)	18.6	aFTS (1:93)	3.2	arr 21q22.12q22.3(35922543–48090317)×3 dn (male)	Suspected	5	Confirmed
5	27 (12 + 3)	23.4	iNT	5.3	arr 4p16.3p15.1(71552–31517659)×1,8p23.3p12(176814–30299232)×3 (female)	na	2	Confirmed

Abbreviations: aFTS, abnormal first trimester screening; BMI, body mass index; iNT, increased nuchal translucency; na, not examined; NT, nuchal translucency.

[a]Blood samples for cfNIPT and cbNIPT were all drawn shortly before invasive testing.

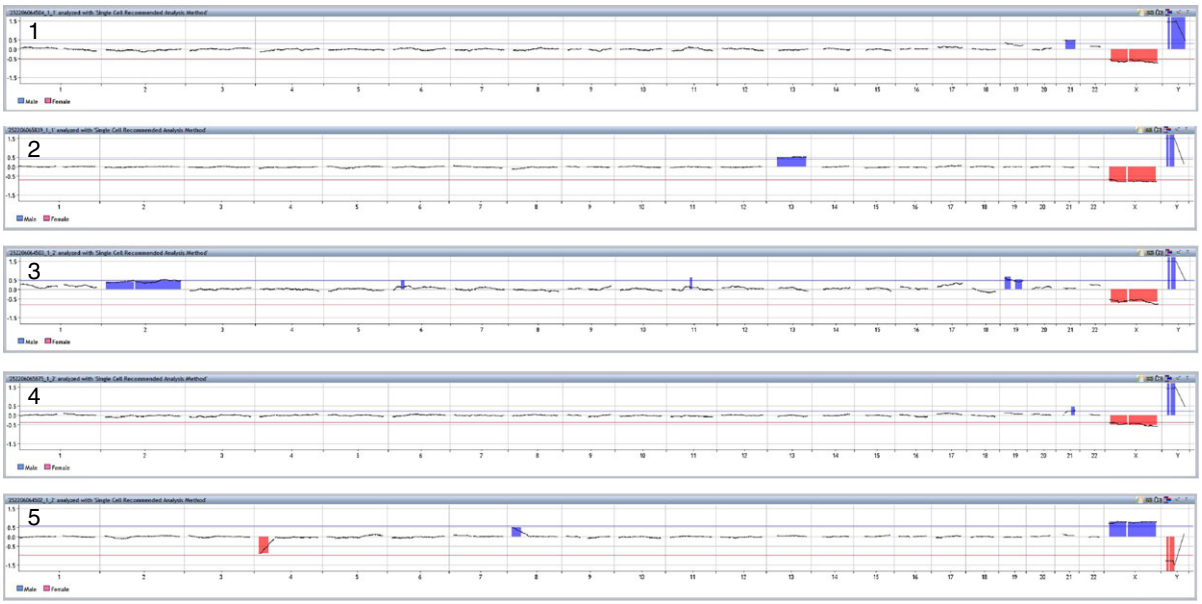

FIGURE 2 aCGH analyses on 5 cytogenetically abnormal cases. 1, Trisomy 21; 2, trisomy 13 (mosaic); 3, trisomy 2 (mosaic); 4, partial trisomy 21 (12.4-Mb duplication); 5, unbalanced translocation including 31-Mb deletion on chromosome 4p and 30-Mb duplication on chromosome 8p [Colour figure can be viewed at wileyonlinelibrary.com]

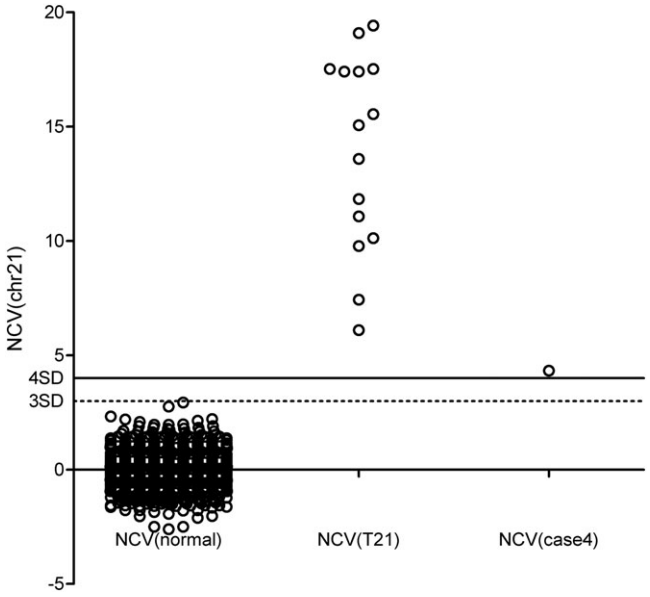

FIGURE 3 Normalized chromosome values (NCV) for chromosome 21 of healthy pregnancies (normal), of confirmed trisomy 21 pregnancies (T21), and of the reported case (case 4). SD, standard deviation for NCV (normal)

(Figure 2) deviating by 0.2 and 0.0 Mb, respectively, from the aberrations detected by aCGH on CVS.

4 | DISCUSSION

The drift from invasive testing towards cffNIPT involves the risk of diagnostic delay as well as a risk of overlooking common deletion syndromes prenatally. It is well documented that women with fetuses with large NT[3]and women with increased risk at the combined first trimester screening are at risk of atypical chromosomal aberrations in addition to the risk of aneuploidy.[4,5] These aberrations are typically not detectable by cffNIPT.[6]

cffNIPT is rapidly improving,[7,8]and in this case series we again demonstrate that cffNIPT is a sensitive analysis and may develop as a screening method for other aneuploidies than trisomy 13, 18, and 21. cffNIPT is, however, limited in nature as the fetal and maternal DNA are mixed and the DNA partly degraded, and cffNIPT is unlikely to develop into a diagnostic test. The current case with partial trisomy 21 (12.4-Mb duplication) on one hand illustrates the high sensitivity of cffNIPT based on genome-wide massive parallel sequencing for chromosome 21, in that a 12.4-Mb duplication of chromosome 21 corresponding to approximately 25% increase of DNA compared with that of a full trisomy 21 in a twin pregnancy gave a significant risk for chromosome 21 aneuploidy. But on the other hand, the case also shows the limitations of this method, because the specific chromosomal change could not be predicted based on the cffNIPT result.

More groups have tried to target fetal cells for prenatal diagnosis.[9-11] In 2014, Hatt et al published a set of markers that could specifically identify fetal cells of extravillous trophoblastic origin in the maternal circulation.[12] In a recent study, Hatt and co-workers published a method with high specificity for fetal cell enrichment and identification. This study showed that on average 12.8 fetal cells per 30 mL of maternal blood can be isolated and that the isolated fetal cells can be used to detect chromosomal changes in the fetal genome.[1] In the current study, we used pools of 2 to 7 cells for analysis, and we are working on optimizing the cbNIPT analysis for analysis of 1 to 3 fetal cells.

This case series demonstrate the current status of this technique in a clinical setting where invasive testing with CMA and cbNIPT are carried out simultaneously, and in 2 cases also included comparison with cffNIPT. The 5 cases demonstrate that cbNIPT has the potential to cover a wide range of the most prevalent clinical causes for fetal chromosomal disease: aneuploidies including mosaicism, unbalanced

translocations, subchromosomal deletions, and duplications and that the cbNIPT method has the potential to be developed into a diagnostic test. Large-scale multicenter clinical validation is however needed to determine the clinical accuracy of cbNIPT before clinical use in routine prenatal diagnostics. Fetoplacental mosaicism is a challenge in prenatal diagnosis and also in non-invasive prenatal diagnosis. Our cbNIPT method is based on the harvest and analysis of endovascular trophoblasts.[1] In mosaic pregnancies, we do not know if some or all types of abnormal endovascular trophoblasts may be released to the maternal circulation and/or degraded at altered rate as compared with normal cells resulting in potential diagnostic errors of cbNIPT. cbNIPT may also demonstrate to have reduced ability to detect mosaicism due to the low cell numbers analyzed. However, we demonstrate 2 cases where the aberrations were picked up by cbNIPT analysis without significant signs of apparent mosaicism detected by aCGH on CVS. Hereby, the more favorable prognosis of confined placental mosaicisms may be overlooked by cbNIPT most likely necessitating invasive follow-up testing for some time forward. A maternal PAPP-A level below 0.2 MOM is a strong indicator for placental aneuploidy, and low PAPP-A may indicate a need for sampling more than 2 cells from these individuals. However, the results from cbNIPT in the present cases hold great promises for the use of cbNIPT for the detection of aneuploidy, high grade mosaicism, and sub-chromosomal abnormalities. As the DNA quality retrieved from the fetal cells is suitable for CMA, other next-generation sequencing (NGS)-based methods may likely be developed to extend the diagnostic potential on fetal cells.

The screening/diagnostic landscape in prenatal diagnosis is changing rapidly. The changes call for openness, multidisciplinary skills, and hard work in translating these new techniques into clinical use.

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CONFLICT OF INTEREST

Palle Schelde is the CEO of ARCEDI Biotech, a Danish company that has developed the fetal cell enrichment and detection procedure described in this manuscript. Lotte Hatt, Ripudaman Singh, and Katarina Ravn are all employed in ARCEDI Biotech. ARCEDI Biotech has a patent pending for the markers used for enrichment and detection of circulating fetal cells.

AUTHOR CONTRIBUTIONS

E.M.V performed aCGH analysis on CVS samples, interpreted and analyzed data, and wrote the article with I.V. R.S designed and performed experiments, analyzed the data, and contributed to writing. P.S. designed and performed experiments, analyzed data, and managed the project. L.H designed and performed experiments and analyzed data. K.R. designed and performed experiments and analyzed data. R.C. performed aCGH analysis on WGA and CVS samples. D.L.L performed and analyzed non-invasive prenatal testing (NIPT) by low-coverage WGS. O.B.P. collected the clinical material in Denmark. N.U. collected the clinical material. I.V. interpreted and analyzed data, and wrote the paper with E.M.V.

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