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How does cell-based non-invasive prenatal test (NIPT) perform against chorionic villus sampling and cell-free NIPT in detecting trisomies and copy number variations? A clinical study from Denmark

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Abstract

Objectives: We aimed to compare cell-based NIPT (cbNIPT) to chorionic villus sampling (CVS) and to examine the test characteristics of cbNIPT in the first clinical validation study of cbNIPT compared to cell-free NIPT (cfNIPT).

Material and Methods: Study 1: Women ($N = 92$) who accepted CVS were recruited for cbNIPT (53 normal and 39 abnormal). Samples were analyzed with chromosomal

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microarray (CMA). Study 2: Women ($N = 282$) who accepted cfNIPT were recruited for cbNIPT. cfNIPT was analyzed using sequencing and cbNIPT by CMA.

Results: Study 1: cbNIPT detected all aberrations (32/32) found in CVS: trisomies 13, 18 and 21 (23/23), pathogenic copy number variations (CNVs) (6/6) and sex chromosome aberrations (3/3). cbNIPT detected 3/8 cases of mosaicism in the placenta. Study 2: cbNIPT detected all trisomies found with cfNIPT (6/6) and had no false positive (0/246). One of the three CNVs called by cbNIPT was confirmed by CVS but was undetected by cfNIPT, two were false positives. cbNIPT detected mosaicism in five samples, of which two were not detected by cfNIPT. cbNIPT failed in 7.8% compared to 2.8% in cfNIPT.

Conclusion: Circulating trophoblasts in the maternal circulation provide the potential of screening for aneuploidies and pathogenic CNVs covering the entire fetal genome.

Key points

What is already known about this topic?

- Circulating fetal trophoblasts can be isolated from maternal circulation and whole genome amplified DNA can be obtained.
- Case studies have demonstrated its use in detecting aneuploidies, copy number variations (CNVs) and monogenic disorders.

What does this study add?

- This is the first clinical validation study reporting high sensitivity and specificity for aneuploidies and likely also for CNVs over the entire genome.
- Known microdeletion/duplication syndromes as well as unique disease causing CNVs >1 MB can be reliably detected.

1 | INTRODUCTION

Over the last decade, cell-free DNA has provided the basis for non-invasive prenatal test for fetal trisomies, and cell-free non-invasive prenatal test (cfNIPT) has been globally implemented as a screening test in clinical practice. In many countries, cfNIPT is used as a secondary test for women identified as high risk after combined first trimester screening, and in other countries, cfNIPT is used as the primary screening test.[1] American College of Medical Genetics now recommends cfNIPT as a primary screening test for fetal trisomies 21, 18, 13 as well as for sex chromosome anormalies.[2]

Even though a large number of samples have been tested with cfNIPT, the false positive and false negative rates are uncertain for aneuploidies other than T21.[3] Confirmatory invasive testing is, thus, still recommended. Some companies offering cfNIPT have included a few pathogenic copy number variations (CNVs) (22q11, Prader Willi etc)[4] and some monogenic disorders such as cystic fibrosis.[5]

For a long time, cell-based non-invasive prenatal test (cbNIPT) using extravillous trophoblasts (EVTs) in the maternal blood has been explored[6] but it has been challenging due to the scarcity of these cells.[7] However, once isolated, every cell comes with the potential of an entire fetal genome uncontaminated by maternal DNA. Thus, they constitute an attractive source for non-invasive prenatal testing for aneuploidies, sex chromosome aberrations (SCA), and pathogenic CNVs,[8–12] as well as monogenic disorders.[13] For example, whole genome amplification from 3 harvested fetal cells rendered enough DNA for both chromosomal microarray (CMA) and cystic fibrosis screening.[13] This provides a future opportunity for a wider screening using a blood sample only from the pregnant women without the need for partner samples.

In Denmark, women with a combined first trimester risk for Down syndrome of 1:300 or above are offered a choice between invasive testing or cfNIPT, and 80% opt for invasive testing because of a wish for more comprehensive genetic information, which currently is not offered by cfNIPT.[14,15] Hence, the momentum to develop non-invasive genome-wide prenatal tests exists.

Since 2018, we have been validating cbNIPT in a clinical study at five hospitals in the Central Denmark Region where pregnant women opting for cfNIPT are also offered cbNIPT. We have previously published two cases showing how cbNIPT could detect pathogenic CNVs missed by cfNIPT.[16] As many CNVs are unique, the CMA-approach used in cbNIPT provides an opportunity to test any sizable pathogenic CNV throughout the genome. Further, single cell sorting using flow cytometry and confirmation of non-maternal origin

of EVTs using short tandem repeat (STR) analysis were implemented as part of the cbNIPT method, making this the latest significant technology change.

In this paper, we present the test characteristics of cbNIPT among women undergoing chorionic villus sampling. Furthermore, we report the test performances of both cbNIPT and cfNIPT among women opting for non-invasive prenatal test when identified as high risk at combined first trimester screening.

2 | METHODS

2.1 | Patient inclusion

In Denmark, women with a combined first trimester risk assessment resulting in a risk of more than 1:300 for trisomi 21 or more than 1:150 for trisomi 13 or 18 are considered screen positive and are offered a CVS or cfNIPT. The pretest counseling is given by the obstetrician/fetal medicine specialist as part of the everyday practice at each department and focuses on the differences between invasive sampling and NIPT concerning the information obtained and the small risk of pregnancy loss.

Study 1: Women who underwent CVS due to a high-risk result at the combined first trimester screening were enrolled for cbNIPT. Trophoblasts were harvested and whole genome amplified (WGA) DNA was stored. Once the result of the CVS was known, the trophoblasts from all the abnormal cases with CNVs larger than 1 Mb ($N = 39$) were sent for CMA. Genome-amplified DNA from trophoblasts from an equivalent sample number among the normal invasive test results ($N = 53$) were also sent for chromosomal analysis (total $N = 92$). Blood samples were retrieved in a hospital in the North or the Central Denmark Regions after written informed consent (Scientific ethics board approval 1-10-72-225-19, 69335 January 10, 2020).

Study 2: Pregnant women opting for cfNIPT after combined first trimester screening were also offered cbNIPT at any of the five hospitals in the Central Denmark Region. For each woman, 2 separate clinical reports were generated within 2 weeks and then stored in the electronic patient folder. As part of routine quality control, these data were merged into the Danish Central Cytogenetics Register. Ethical approval is waived for registry studies but are registered in internal databases (01–12 + 2022 787503 1-16-02-435-22). Patient data were retrieved from the Danish Central Cytogenetics Register (IRB DCCR and Internal Registry Number 787503, November 22, 2022) for the comparison of cbNIPT to cfNIPT. Data were included if they were analyzed after August 2020, where fluorescence activated cell sorting (FACS) was implemented along with testing for non-maternal origin of EVTs by STR. The first abnormal test result (from either cfNIPT or cbNIPT) was reported immediately to avoid clinical delay. Normal test results from either test waited for the other test, and reports were then released simultaneously to the department and the patient charts.

The patients received information from the obstetrician if one test was abnormal. A normal test result was reported to the patient by phone or letter, if both tests were normal. Lately, patients are notified of a normal result by a letter in the public digital mailbox. The women do not pay to receive the result of the cbNIPT.

For both studies, blood samples were shipped to ARCEDI Biotech for fetal cell isolation. DNA from fetal cells was sent to the Department of Clinical Genetics at Aarhus University Hospital for CMA analysis, genetic interpretation, and reporting.

2.2 | cbNIPT

Blood samples of 30 mL were collected for cbNIPT at gestational age (GA) 10 + 0 to 14 + 6 in three cell-free DNA BCT tubes (Streck Laboratories). Trophoblast isolation was performed as previously described (Jeppesen et al.[12]).

In brief, trophoblasts were enriched by magnetic activated cell sorting (Miltenyi Biotech) using markers and method previously described. Individual cells were isolated by FACS using a BD FACS-Melody™ Cell Sorter (BD Biosciences). Single cells were WGA using PicoPLEX® Single Cell WGA kit (Takara) and subsequently purified using Genomic DNA Clean & Concentrator™ kit (Zymo Research) following the manufacturer's instructions. To exclude any maternal contamination, isolated cells were validated for their EVT origin by STR analysis using the GlobalFiler kit (Thermo Fisher Scientific), analyzing 24 different STR loci in multiplex. STR analysis was run by capillary electrophoresis (ABI3500), and fragment length analysis was performed in GeneMapper ID-X Software (Thermo Fisher Scientific).

CNV analysis by CMA (array comparative genomic hybridization [aCGH]) was performed on WGA DNA from trophoblasts using SurePrintG3Human CGH 4 × 180K arrays (Agilent Technologies). Reference DNA was a pool of 10 WGA products from lymphoblastic genomic DNA (Promega). WGA DNA from up to 3 trophoblasts was pooled prior to aCGH analysis. WGA DNA from trophoblasts (1500 μg) and WGA DNA from the reference pool (1500 μg) were labeled with Cy5 (trophoblasts) or Cy3 (reference) using the SureTag Complete DNA labeling Kit (Agilent Technologies). Scanning and image acquisition were performed using a SureScan Dx Microarray Scanner (Agilent Technologies). For Study 1, an additional 1–4 single trophoblast cells were analyzed if aberrations were found or the aCGH analysis on the pool failed. In Study 2, where cfNIPT was compared with cbNIPT, no single cells were analyzed if aberrations were found or if the aCGH analysis failed. In these cases, if a cfNIPT result was available then it was prioritized to assure a quick result rather than pursuing a cbNIPT result.

Copy numbers were determined using the adm-2 algorithm. The filters used for detection of aberrations were minimum absolute average log ratio of the region of 0.3 for gains and 0.4 for losses. The analysis was performed using CytoGenomics software (Agilent Technologies). The size threshold was different for the two studies. In Study 1, where the cbNIPT samples were received from pregnant women having a CVS, the resolution of the cbNIPT test was studied, and a lower minimum size of regions of 1 Mb was applied. For the

cbNIPT samples in Study 2, a minimum size of regions of 3 Mb was applied. As no result was reported back to the pregnant women in Study 1, we could test lowering the minimum size of regions of the CNV from 3 to 1 Mb. Lowering the detection limit could potentially result in a higher degree of background noise with the risk of reporting false positives. Thus, the resolution of the algorithm in Study 1 is above 1 MB and above 3 Mb in Study 2.

CMAs on CVS or cbNIPT were interpreted using the above-mentioned settings in the CytoGenomics software for calling chromosomal deletions or duplications. Further, the CMA underwent subsequent visual inspection by a clinical laboratory geneticist for validation of chromosomal aberrations. This visual inspection was to ensure that CNVs were real and not caused by spread or noise in the data. Also, in rare cases, the inspection found clear elevation or decrementation/depression of chromosomes that were not called by the software in mosaic cases and lead to discussions with the clinical geneticist whether to include this in the report. The analyses and reporting of the microarray data both from CVS and cbNIPT are handled in the same way as the clinical context at the Department of Clinical Genetics. cbNIPT, as sometimes seen with clinical samples from spontaneous abortions (not in this paper), has an increased derivative spread compared to CVS, reducing the likelihood of detecting mosaicism and small aberrations compared to fresh CVS.

2.3 | CfNIPT

CfNIPT was performed at the Department of Clinical Genetics, Aarhus University Hospital. For cfNIPT analysis, 20 mL blood was sampled in cell-free DNA BCT tubes (Streck Laboratories) at GA 10 + 0 to 14 + 6, and cell-free DNA extraction from plasma, genome-wide massive parallel sequencing, and data analysis were conducted following the manufacturer's instructions (Illumina©; San Diego) for TG TruSeq® Nano DNA LT Library Preparation kit v1.1, and TG NSQ 500/550 HighOutput Kit v2.5. VeriSeq NIPT Analysis Software v1 was used for analysis of the fetal fraction and aneuploidy status.

2.4 | CVS analysis by CMA

CMA (SurePrint G3 Human CGH 4 × 180K arrays, Agilent Technologies) was performed on DNA from uncultured CVS. Chorionic villus samples were dissected microscopically to remove any contaminating maternal decidua prior to DNA extraction. DNA was extracted immediately from uncultured chorionic villi using the Maxwell 16 LEV Blood DNA kit (Promega). Sample (500 ng) and reference (500 ng) genomic DNA were labeled with Cy5 (sample) or Cy3 (reference) using the SureTag Complete DNA labeling Kit (Agilent Technologies). Scanning and image acquisition were carried out using a SureScan Dx microarray scanner (Agilent Technologies), and quantification of microarray image files and data analysis were performed using the CytoGenomics software (Agilent Technologies). Copy number was determined using the adm-2 algorithm, and profile deviations consisting of four or more neighboring oligonucleotides were considered genomic aberrations. Thus, the resolution was approximately 50 kb.

2.5 | Statistics

Statistical analyses were performed with R (R Foundation). Test characteristics of the cbNIPT compared with the results of confirmatory genetic tests (e.g., CVS) were calculated including the modified Wilson 95% intervals because of estimates close to 0 or 1 (BinomCI from the DescTools package).

3 | RESULTS

3.1 | Study 1—cbNIPT compared to CVS

WGA DNA from 1 to 3 trophoblasts could be analyzed with aCGH, and aneuploidies and pathogenic CNVs could be determined using the CytoGenomics software. The derivative spread was increased when compared to the analyses of high-quality DNA derived from CVS (Figure 1), but it still allowed for aneuploidy and CNV detection (Figure 2).

3.2 | Resolution, sensitivity and specificity

From all 92 (53 normal and 39 abnormal, 1 case with two aberrations) singleton pregnancies, 35/40 aberrations were subsequently detected using cbNIPT. Five mosaic aberrations in CVS were not detected by cbNIPT (Table 1). Table 1 lists all the atypical aberrations, and Table 2 shows two-by-two tables and the test characteristics obtained when comparing cbNIPT to CVS. Aberrations in the 35 cases were predominantly trisomies, with 19 T21, three T18, and one T13, where sensitivity for trisomies was 100% (23/23, sensitivity 100% [95% CI 85–100], Table 2). 6/6 pregnancies were diagnosed with fetal pathogenic non-mosaic CNVs by both CVS and cbNIPT; 3 CNVs were larger than 5 Mb and 3 CNVs were smaller than 5 Mb. The sensitivity (95% CI 54–100) and specificity were 100% (CI 96–100) for all non-mosaic CNVs. Three cases of non-mosaic SCA were correctly identified by cbNIPT (sensitivity 100% [95% CI 29–100], Table 2). Fetal sex (interpreted as the presence of Y-chromosome material) was correctly identified by cbNIPT in all samples (Table 2). Eight aberrations were mosaic in CVS, and of these, cbNIPT detected three (3/8, sensitivity of 38% [95% CI 9–75], Table 2). One case had a non-mosaic T21 as well as a pathogenic mosaic CNV, and cbNIPT detected the trisomy but not the CNV.

CVS and cbNIPT were both normal in 53 cases.

Figure 1a. CVS

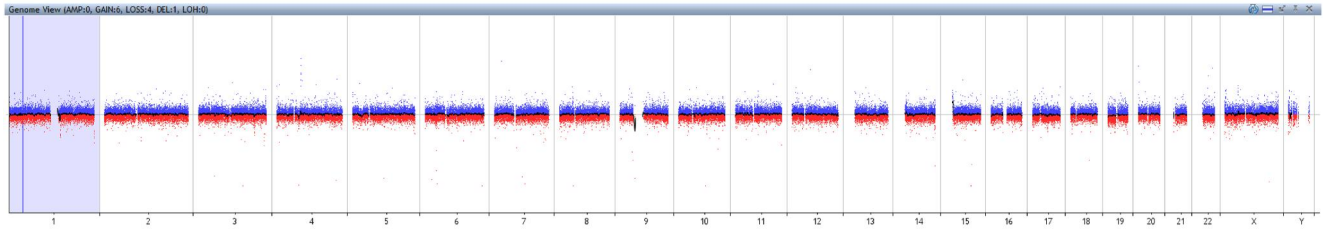

Figure 1b CbNIPT

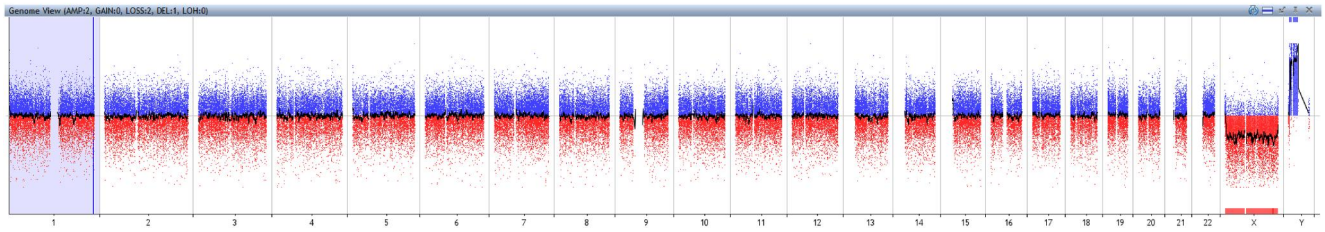

FIGURE 1 CVS and cbNIPT CMA. (A) CVS CMA from a normal male fetus using aCGH. CVS was compared to a male reference DNA sample. (B) cbNIPT genetic analysis from a normal male fetus was performed by aCGH on a pool of WGA DNA from three trophoblasts. The DNA used as a reference for trophoblast WGA product was a pool of 10 WGA reactions from female lymphoblast genomic DNA. The use of PCR-based WGA introduces an increased derivative spread in the data in the cbNIPT analysis compared to CVS analysis, but still supports whole genome interpretation. aCGH, array comparative genomic hybridization; cbNIPT, cell-based NIPT; CMA, chromosomal microarrays; CVS, chorionic villus sampling; WGA, whole genome amplification. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 2a CbNIPT

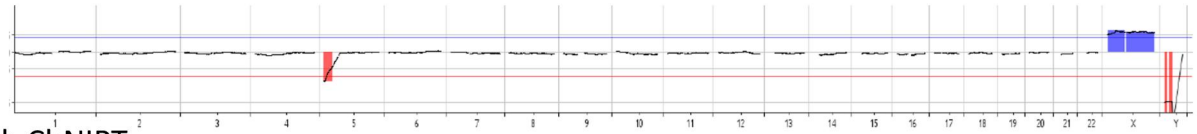

Figure 2b CbNIPT

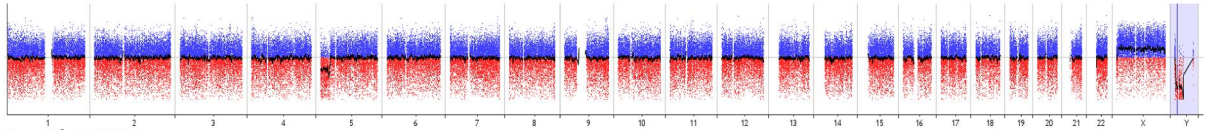

Figure 2c CbNIPT

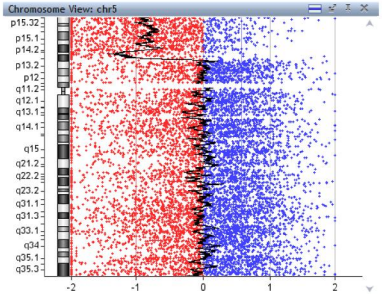

FIGURE 2 Example of cbNIPT chromosomal microarray identifying sub-chromosomal copy number variation. cbNIPT CMA analysis from a female fetus with a 29-Mb deletion at chromosome 5 resulting in Cri du Chat syndrome. Performed by aCGH on a pool of WGA DNA from three trophoblasts. The DNA used as a reference for trophoblast WGA product was from male lymphoblast. The analysis was performed in CytoGenomics software and copy numbers were determined using the adm-2 algorithm. The filters used for detection of aberrations were minimum absolute average log ratio of the region of 0.3 for gains and 0.4 for losses and a minimum size of regions of 3 Mb was applied. Though derivative spread is increased when compared to the analyses of ample and high-quality DNA derived from chorionic villus samples (Figure 1), this does not hamper interpretation. (A) Genome view average. (B) Genome view scatter plot. (C) Chromosome view of chromosome 5. aCGH, array comparative genomic hybridization; cbNIPT, cell-based NIPT; CMA, chromosomal microarrays; WGA, whole genome amplification. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Atypical cbNIPT and CVS molecular karyotypes.

Category	ID	cbNIPT molecular karyotype [GRCh37]	CVS molecular karyotype (or postnatal blood) [GRCh37]	Mb	Concordance
CNV >5 Mb	1	arr11q14.3q22.1(91427076_99027943)x1	arr11q14.3q22.1(91427076–99097341) x1mat	7.7	Yes
CNV >5 Mb	2	arrXp22.33p21.1(2334220_32834749)x1	Postnatal blood 46,X,del(Xp)	30.5	Yes
CNV >5 Mb	3	arr6q14.1q15(82880600_90550914)x1	arr6q14.1q15(82770129_90562883)x1dn	7.8	Yes
CNV <5 Mb	4	arr14q32.2q32.33 (100750123_104216266)x3,14q32.33 (104234220_107278770)x1	arr14.32.2q32.33(100744929_104223926) x3dn,14.32.2q32.31 (100744929_101482909)x4 dn,14q32.33 (104234220_107278770)x1dn	3.0	Yes
CNV <5 Mb	5	arr15q13.3(31234158_32357081)x1	arr15q13.2q13.3(30943903_32914140)x1dn	2.0	Yes
CNV <5 Mb	6	arrXq28 (153706398_155086538)x1	Xq28(153681174_155233098)x1	1.6	Yes
SCA	7	arr(X)x1	Postnatal blood 45,X[10]/46,X,+mar[3]		Yes?
SCA	8	arr(X)x3	arr(X)x3		Yes
SCA	9	arr(X)x1	arr(X)x1		Yes
Trisomy + CNV mosaicism	10	arr(21)x3	arr(21)x3,7q11.23(72401086_75916089) x1~2	3.5	Yes and no
CNV mosaicism	11	arr(X,Y)x1,(1–22)x2	arr4q23(98945431_100424583)x2~3dn	1.5	No
CNV mosaicism	12	arr(X,1–22)x2	arr3p26.3(73914_1948007)x2~3,6p25.3p24.3 (213630_8040676)x1~2	1.9	No
CNV mosaicism	13	arr[GRCh37]5p15.33-p13.3 (172622–33260213)x1	arr5p15.33p13.3(26142_33270102)x1~2	33.2	Yes
Trisomi mosaicism	14	arr(21)x2~3	arr(21)x2~3		Yes
Trisomi mosaicism	15	arr(3)x2~3	arr(3)x2~3		Yes
SCA mosaicism	16	arr(X,1–22)x2	arr(X)x1~2		No
SCA mosaicism	17	arr(X,1–22)x2	arr(X)x1~2		No

Note: The cases are divided into categories based upon cbNIPT karyotype. If the aberration is completely similar in cbNIPT and CVS then the case can only be found in Table 2, 2 × 2 table. cbNIPT and chorionic villus sampling (CVS) is performed with Chromosomal microarray (Human CGH 4 × 180K arrays). Postnatal blood is analyzed by karyotyping.

Abbreviations: cbNIPT, cell-based non-invasive prenatal test; CGH, comparative genomic hybridization; CNV, copy number variation; ID, identification number; Mb, mega base; SCA, sex chromosomal abnormality.

3.3 | Study 2—cbNIPT and cfNIPT—The clinical comparison

There were 282 samples in the Danish Central Cytogenetics Register with clinical reports on both cfNIPT and cbNIPT since August 2020. There were 22 failed analyses for cbNIPT (7.8%) and 8 for cfNIPT (2.8%) (Table 3). For cbNIPT, the failure to generate a result would have been higher if cases where no fetal cells were harvested were to be included. However, these data are not available in the register. This leaves 252 cases where both a cbNIPT and a cfNIPT report were generated, see Table 4.

Aberrations were predominantly trisomies with 4 cases of T21, 3 with T18 and none with T13 (9/9, sensitivity 100% [CI 69–100], Table 4). In three pregnancies, a mosaic CNV was detected by cbNIPT while the result of cfNIPT was normal. One CNV was confirmed in the subsequent CVS, whereas two turned out to be false positives when followed-up by CMA analysis performed on selected uncultured, untrypsinized chorion villi (Table 4). Further subsequent single-cell analysis on cbNIPT showed that they were indeed false positives caused by a cell with a suboptimal DNA quality in the poled WGA.

Fetal sex was correctly identified by cbNIPT in all samples (Table 4). Two cases of mosaic SCA were correctly identified by cbNIPT.

CbNIPT identified one case of mosaicism for T7 and one case of mosaicism for T3, and in both cases cfNIPT was normal. Both women chose to continue the pregnancy without further follow-up due to the expected benign prognosis. A mosaic case of T16 was correctly identified by both cbNIPT and cfNIPT.

Two-hundred and thirty-five samples (93%) were normal by both cbNIPT and cfNIPT.

4 | DISCUSSION

In the first clinical evaluation of cbNIPT, we found that cbNIPT correctly detected 32/32 pregnancies with trisomies, and with no false positive results. Further, 7/7 pathogenic CNVs were detected

TABLE 2 2 × 2 tables of cbNIPT detecting aberrations found by CVS (*N* = 92).

	CVS Abnormal	CVS Normal	
T21			
cbNIPT +	19	0	19
-	0	72	72
	19	72	91
	SENS 100% (CI 82-100)	SPEC 100% (CI 95-100)	
T18			
cbNIPT +	3	0	3
-	0	89	89
	3	89	92
	SENS 100% (CI 29-100)	SPEC 100% (CI 96-100)	
T13			
cbNIPT +	1	0	1
-	0	91	91
	1	91	92
	SENS 100% (CI 3-100)	SPEC 100% (CI 96-100)	
Trisomies 13, 18, 21			
cbNIPT +	23	0	23
-	0	68	68
	23	68	91
	SENS 100% (CI 85-100)	SPEC 100% (CI 95-100)	
SCA			
cbNIPT +	3	0	3
-	0	87	87
	3	87	90
	SENS 100% (CI 29-100)	SPEC 100% (CI 96-100)	
Male sex			
cbNIPT +	48	0	48
-	0	44	44
	48	44	92
	SENS 100% (CI 93-100)	SPEC 100% (CI 92-100)	
CNV			
cbNIPT +	6	0	6
-	0	82	82
	6	82	88
	SENS 100% (CI 54-100)	SPEC 100% (CI 96-100)	
Mosaic			
cbNIPT +	3	0	3
-	5	84	89
	8	84	92
	SENS 38% (CI 9-75)	SPEC 100% (CI 96-100)	

Note: If the aberration was mosaic the case was excluded from the 2 × 2 table with full aberrations and included in the mosaic 2 × 2 table. Confidence intervals for sensitivity and specificity are Clopper-Pearson confidence intervals.

Abbreviations: cbNIPT, cell-based non-invasive prenatal test; CNV, copy number variation; SCA, sex chromosomal abnormality; SENS, sensitivity; SPEC, specificity.

TABLE 3 2 × 2 tables of cbNIPT and cfNIPT failure rates (*N* = 282).

	cfNIPT +	cfNIPT -	
cbNIPT +	252	8	260
-	22	0	22
	274	8	282

Note: 282 women participated, but cbNIPT failed in 7.8% and cfNIPT failed in 2.8% to generate a result. Thus for 252 women both a cfNIPT and a cbNIPT result were available for comparison. In the failed cbNIPT cases, a cfNIPT result was available, and it was prioritized to give a quick response back to the pregnant woman rather than pursuing a cbNIPT result. Hence, no single cell analysis or redraw was performed to retrieve a cbNIPT result in these cases.

Abbreviations: cbNIPT, cell-based non-invasive prenatal test; cfNIPT, cell-free non-invasive prenatal test.

by cbNIPT (William Beuren syndrome, 15q13.3 deletion syndrome, Cri du Chat and individual CNV syndromes). There were two false positive CNV calls.

We suggest that with the CMA-approach, cbNIPT provides an opportunity to test for any sizable pathogenic CNV throughout the genome. As many CNVs are unique, cbNIPT may provide significant diagnostic advantage as compared to screening for a limited number of specific syndromes.

We found 32/32 trisomies, which indicates a good test quality for trisomies. However, assessing test qualities in real life is complicated and affected by for example, prevalence.[3] In cfNIPT, it has previously been reported that the proportion of false positives ranged from 2.7% (T21 in the high-risk population) to 30% (T13 in the general population). Likewise, the proportion of false negative cfNIPT is generally very low, 0.01%.[17] The current study is far too small for such a comparison but includes both a high (Study 1) and a low prevalence group (Study 2). cbNIPT is a screening test and invasive follow-up testing of abnormal cbNIPT results is offered as trophoblasts are of placental origin and may potentially, in case of confined placental mosaicism, not represent the fetus.

cbNIPT reliably detects fetal sex. Further, the sensitivity for SCA was 3/3 (100%) although three non-mosaic cases are very limited number. We have published case reports with fetal SCA including a report on cbNIPT being able to detect fetal XXY in a twin pregnancy.[12] We have also demonstrated how, because of the maternally uncontaminated fetal genome in cbNIPT, the interpretation of results can become unambiguous.[11] In contrast, the interpretation of the cfNIPT result for SCA is hampered by the mixture of maternal and fetal free DNA and the maternal age-related X-chromosome losses.

In seven pregnancies, a pathogenic, non-mosaic CNV ranging from 1.6 to 30 Mb was correctly diagnosed by cbNIPT (Tables 1 and 5). The resolution of CNV calling in cbNIPT is yet undetermined, but has been presented by others to be 1–2 Mb in size.[9,10] With the current spread in the data and the use of the CMA platform, we chose to exclude CNVs <1 Mb for Study 1. In a very recent study,

TABLE 4 2 × 2 tables of cbNIPT detecting aberrations found by cfNIPT (N = 252).

		cfNIPT			
T21		Abnormal	Normal		
	cbNIPT +	6	0	6	PPV 100% (CI : 61-100)
	-	0	246	246	NPV 100% (CI : 98-100)
		6	246	252	
		SENS 100% (CI 54-100)	SPEC 100% (CI 99-100)		
T18					
	cbNIPT +	3	0	3	PPV 100% (CI : 44-100)
	-	0	249	249	NPV 100% (CI : 98-100)
		3	249	252	
		SENS 100% (CI 29-100)	SPEC 100% (CI 99-100)		
T13					
	cbNIPT +	0	0	0	
	-	0	252	252	NPV100% (CI : 99-100)
		0	252	252	
			SPEC 100%		
Trisomies 13, 18, 21					
	cbNIPT +	9	0	9	PPV 100% (CI : 70-100)
	-	0	243	243	NPV 100% (CI : 98-100)
		9	242	252	
		SENS 100% (CI 69-100)	SPEC 100% (CI 98-100)		
SCA					
	cbNIPT +	0	0	0	
	-	0	252	250	NPV 100% (CI : 98-100)
		0	252	250	
			SPEC 100% (CI 99-100)		
Male sex					
	cbNIPT +	154	0	154	PPV 100% (CI : 98-100)
	-	0	96	96	NPV 100% (CI : 96-100)
		154	96	249	
		SENS 99% (CI 96-100)	SPEC 100% (96-100)		
CNV					
	cbNIPT +	0	3	3[a]	PPV 0% (CI: 0 – 56)
	-	0	249	249	NPV 100% (CI : 98-100)
		0	252	252	
Mosaic					
	cbNIPT +	3	2	5[b]	PPV 60% (CI: 23-88)
	-	0	247	247	NPV 100% (CI : 99-100)
		3	249	252	

Note: If the aberration was mosaic the case was excluded from the 2 × 2 table with full aberrations and included in the mosaic 2 × 2 table. As cfNIPT is performed with the Veriseq NIPT analysis software v1 from Illumina, which is accredited for chromosomal aneuploidies 13, 18, 21, X and Y, it cannot detect CNVs. Hence, CNVs detected by cbNIPT are placed in FP. Their comparison with CVS results can be seen in Table 5. Confidence intervals for sensitivity and specificity are Clopper-Pearson confidence intervals.

Abbreviations: cbNIPT, cell-based non-invasive prenatal test; cfNIPT, cell-free non-invasive prenatal test; CNV, copy number variation; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; SCA, sex chromosomal abnormality; SENS, sensitivity; SPEC, specificity.

[a]cbNIPT detected 3 CNV undetected by cfNIPT of which one was corroborated in the CVS, see Table 3.

[b]cbNIPT detected 2 mosaics undetected by cfNIPT see Table 3.

Menarini group reported a limit of detection (LOD) of 800 kb both in Coriell cell lines and in clinical samples,[9] which would cover most microdeletion and microduplication syndromes. Larger studies are needed to evaluate how to set thresholds for CNV size calling to avoid risking too many false positives and avoiding redundant referrals for invasive testing.

The cfNIPT analysis offered by the Central Denmark Region is the Veriseq NIPT analysis v1.4 from Illumina, which is accredited for

TABLE 5 Abnormal karyotypes cbNIPT versus cfNIPT.

Category	ID	cbNIPT molecular karyotype [GRCh37]	cfNIPT result	CVS, AC, FT molecular karyotype [GRCh37]	Mb	Concordance
Trisomy	A	arr(21)x3	Increased risk T21, XX	CVS arr(21)x3		Yes
Trisomy	B	arr(21)x3	Increased risk T21, XY	No follow-up analyses		Yes
Trisomy	C	arr(18)x3	Increased risk T18, XY	CVS arr(18)x2~3		Yes
Trisomy	D	arr(18)x3	Low fetal fraction (3%), increased risk T18	FT arr(18)x3		Yes
Trisomy	E	arr(21)x3	Increased risk T21, XY	arr(21x3)		Yes
Trisomy	F	arr(21)x3	Increased risk T21, XX	CVS arr(21)x3		Yes
Trisomy	G	arr(18)x3	Increased risk T18, XX	CVS arr(18)x3		Yes
Trisomy	H	arr(21)x3	Increased risk T21, XY	CVS arr(21)x3		Yes
Trisomy	I	arr(21)x3	Increased risk T21, XX	CVS arr(21)x3		Yes
CNV	J	arr2q37.1q37.3(235003618_243041364)x3	Normal, XX	CVS arr(X,1–22)x2	8.0	No
CNV	K	arr5p15.33-p13.3(307041_29394687)x1	Normal, XX	CVS 5p15.33p15.32(26142_6117526)x1, 5p15.32p13.3(6117527_29086898)x1~2,5p13.3(29102714_30741219)x2~3	29	Yes
CNV	L	arr7q11.23(72858845_75969661)x3	Normal, XX	CVS arr(X,1–22)x2	3.5	No
Trisomy mosaicisme	M	arr(16)x2~3	Increased risk for T16, XX	CVS arr(16)x2~3, 7q11.21q11.22(66766576_68607221)x3	1.8	Yes and no
Trisomy mosaicisme	N	arr(7)x3[1]/arr(X,Y)x1,(1–22)x2[2]	Normal, XY	No follow-up analyses		No
Trisomy mosaicisme	O	arr(3)x2~3	Normal, XY	No follow-up analyses		No
SCA mosaicisme	P	arr(X)x2~3	Increased risk for XXX	No postnatal had been performed		Yes
SCA mosaicisme	Q	arr(X)x1[3]/arr(X,Y)x1,(1–22)x2[1][a]	Increased risk for monosomy X	AC arr(X)x1,(Y)x0~1		Yes

Note: The cases are divided into categories based upon cbNIPT karyotype. cbNIPT is performed with chromosomal microarray (human CGH 4 × 180K arrays). cfNIPT is performed with the Veriseq NIPT analysis software v1 from Illumina. CVS, AC and FT is performed with chromosomal microarray (human CGH 4 × 180K arrays).

Abbreviations: AC, amniocentesis; cbNIPT, cell-based non-invasive prenatal test; cfNIPT, cell-free non-invasive prenatal test; CGH, comparative genomic hybridization; CNV, copy number variation; FT, fetal tissue; ID, identification number; Mb, Mega base; SCA, sex chromosomal abnormality; STR, short tandem repeat; WGA, whole genome amplified.

[a]In the specific case the STR profile revealed XY in 1 cell and only X profile in 3 cells, why single cell analysis was chosen instead of a WGA pool.

the common chromosomal aneuploidies 13, 18, 21, X and Y, and can therefore not be expected to detect CNVs. Illumina also has an upgraded version, VeriSeq NIPT solution v2, that screens for duplications and deletions ≥7 Mb for all autosomal aneuploidies.[18] Targeted cfDNA testing applying microarray analysis or SNP-based techniques can screen for a subset of specific pre-selected subchromosomal syndromes; however, PPV and NPV are often low for specific rare CNV syndromes.[4] We believe that a genome-wide CMA-approach may improve the performance of non-invasive testing and increase the detection of structural variants and unique CNVs throughout the genome.

1–3 trophoblasts from a sample are not enough for the detection of mosaic conditions. We did, however, find 38% of the mosaic cases detected in CVS, which is surprisingly decent sensitivity given the few trophoblasts sampled. This might potentially be explained if the high tolerance for mosaicism for aneuploidy known from cytotrophoblasts in the placenta[19] is also applicable for circulating trophoblasts. If mosaicism in circulating trophoblasts is a good predictor of mosaicism in the placenta, it may be important to detect mosaicism as these pregnancies may be prone to intrauterine growth restriction and preterm birth.[20] If not, one may not be interested in detecting mosaicism in cbNIPT as the majority of them will be confined to the placenta, and thus not an indicator of true fetal aberrations. Today, most countries use amniocenteses over CVS and will be unaware of the cases with placental mosaicism and their potential risks.

Some women (7.8%) did not receive a cbNIPT result, which is more than twice the failure rate compared to cfNIPT (2.8%). In the failed cbNIPT cases, a cfNIPT result was available, and it was prioritized to give a quick response back to the pregnant woman rather than pursuing a cbNIPT result. Hence, no single cell analysis or redraw was performed to retrieve a cbNIPT result in these cases. Analyzing additional single trophoblast cells would have decreased the failure rate but increased the reporting time with 3 days. Since April 2021, our cbNIPT has been launched commercially in Denmark under the name EVITA TEST Complete (www.evitatest.com), and here a failed test result is followed up by a redraw of blood and single cell analysis, reducing the failure rate to 2% (data not shown).

DNA from single trophoblasts was WGA and subsequently analyzed by STR to secure fetal origin and a final frontier to exclude maternal contamination. WGA DNA from up to three cells was pooled prior to aCGH analysis. Our experience is that pooling DNA from more cells gives a higher and more uniform DNA resolution by reducing derivative spread in the data. It does, however, come with the risk that one cell of poor quality can introduce noise to the result.[21] Optimally, several trophoblast cells from each pregnancy should be analyzed individually; however, this is costly using the aCGH platform.

In Study 2, comparing cbNIPT to cfNIPT, three CNVs were detected by cbNIPT. One 29-Mb pathogenic deletion of 5p-, resulting in Cri du Chat syndrome, was not detected by cfNIPT but by cbNIPT. The cbNIPT results were verified by CVS. However, two CNVs (3.0, 8.0 Mb) detected by cbNIPT were found to be false positives when compared to follow-up invasive testing. Subsequently, we did single trophoblast cell analysis and found that these two CNVs were indeed introduced in the pooled WGA DNA by one cell of very poor DNA quality. Consequently, we changed our procedure, so all positive CNV calls by cbNIPT were verified by single trophoblast analysis. This increases the reporting time by three additional days but also reduces the risk of false positive findings leading to redundant invasive testing. We have previously published two other cases on cbNIPT detecting CNVs leading to Prader Willi syndrome and 3p26 deletion syndrome. These cases were also not found by cfNIPT but confirmed by CVS.[16] Therefore, we are hopeful that cbNIPT will turn out to be a reliable detector of CNVs anywhere in the genome.

Currently, cbNIPT is significantly more costly than cfNIPT. This is because the isolation of trophoblasts from maternal blood requires additional steps as compared to accessing cell-free DNA from the plasma for cfNIPT. Moreover, analyzing single trophoblasts using aCGH is not cost-effective. It is likely that moving the analyses from a CMA-platform to an NGS-platform may significantly reduce costs due to the ability to multiplex NGS analyses. Others are currently developing cbNIPT analyses on NGS platforms.[9] Although the NGS platforms method is not clinically validated yet, the method seems promising both regarding costs and resolution.

Another strength of cbNIPT over cfNIPT is that the same blood sample used for aneuploidy and CNV detection can potentially be used for prenatal screening of monogenic disorders.[13] We have found that cbNIPT can also be used for screening for cystic fibrosis[13] and we are currently validating other monogenetic disorders in the same setup.

5 | CONCLUSIONS

Analysis of the entire fetal genome from a few cells and WGA-DNA provides the potential of screening for aneuploidies, CNVs and common monogenic disorders on a single maternal blood sample.

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CONFLICT OF INTEREST STATEMENT

LH, RS, KR, IBC, PS, BHN, and LDJ are all employed by ARCEDI, a Danish biotech company that holds the patented technology for the enrichment of circulating trophoblasts used in this study. Ida Vogel is a consultant for ARCEDI but is full time employed at Aarhus University Hospital.

DATA AVAILABILITY STATEMENT

Study data that are not presented in this article are available upon request directed to the corresponding author.

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REFERENCES

1. Gadsbøll K, Petersen OB, Gatinois V, et al. Current use of noninvasive prenatal testing in Europe, Australia and the USA: a graphical

presentation. *Acta Obstet Gynecol Scand*. 2020;99(6):722-730. https://doi.org/10.1111/aogs.13841
2. Dungan JS, Klugman S, Darilek S, et al. Noninvasive prenatal screening (NIPS) for fetal chromosome abnormalities in a general-risk population: an evidence-based clinical guideline of the American College of Medical Genetics and Genomics (ACMG). *Genet Med*. 2023;25(2):100336. https://doi.org/10.1016/j.gim.2022.11.004
3. Liehr T. False-positives and false-negatives in non-invasive prenatal testing (NIPT): what can we learn from a meta-analyses on > 750,000 tests? *Mol Cytogenet*. 2022;15(1):36. https://doi.org/10.1186/s13039-022-00612-2
4. Zaninović L, Bašković M, Ježek D, Katušić Bojanac A. Validity and utility of non-invasive prenatal testing for copy number variations and microdeletions: a systematic review. *J Clin Med*. 2022;11(12):3350. https://doi.org/10.3390/jcm11123350
5. Chandler NJ, Ahlfors H, Drury S, et al. Noninvasive prenatal diagnosis for cystic fibrosis: implementation, uptake, outcome, and implications. *Clin Chem*. 2020;66(1):207-216. https://doi.org/10.1373/clinchem.2019.305011
6. Hatt L, Brinch M, Singh R, et al. A new marker set that identifies fetal cells in maternal circulation with high specificity. *Prenat Diagn*. 2014;34(11):1066-1072. https://doi.org/10.1002/pd.4429
7. Singh R, Hatt L, Ravn K, et al. Fetal cells in maternal blood for prenatal diagnosis: a love story rekindled. *Biomarkers Med*. 2017;11(9):705-710. https://doi.org/10.2217/bmm-2017-0055
8. Vestergaard EM, Singh R, Schelde P, et al. On the road to replacing invasive testing with cell-based NIPT: five clinical cases with aneuploidies, microduplication, unbalanced structural rearrangement, or mosaicism. *Prenat Diagn*. 2017;37(11):1120-1124. https://doi.org/10.1002/pd.5150
9. Doffini A, Forcato C, Mangano C, et al. Isolation of single circulating trophoblasts from maternal circulation for noninvasive fetal copy number variant profiling. *Prenat Diagn*. 2022;n/a(n/a):14-27. https://doi.org/10.1002/pd.6275
10. Vossaert L, Wang Q, Salman R, et al. Reliable detection of subchromosomal deletions and duplications using cell-based noninvasive prenatal testing. *Prenat Diagn*. 2018;38(13):1069-1078. https://doi.org/10.1002/pd.5377
11. Jeppesen LD, Hatt L, Singh R, et al. Screening for fetal aneuploidy and sex chromosomal anomalies in a pregnant woman with mosaicism for turner syndrome—applications and advantages of cell-based NIPT. *Front Genet*. 2021;12. https://doi.org/10.3389/fgene.2021.741752
12. Jeppesen LD, Hjortshøj TD, Hindkjær J, et al. Cell-based NIPT detects 47,XXY genotype in a twin pregnancy. *Front Genet*. 2022;13. https://doi.org/10.3389/fgene.2022.842092
13. Jeppesen LD, Lildballe DL, Hatt L, et al. Noninvasive prenatal screening for cystic fibrosis using circulating trophoblasts: detection of the 50 most common disease-causing variants. *Prenat Diagn*. 2022;43(1):3-13. https://doi.org/10.1002/pd.6276
14. Sagi-Dain L, Singer A, Petersen OB, et al. Trends in non-invasive prenatal screening and invasive testing in Denmark (2000–2019) and Israel (2011–2019). *Front Med*. 2021;8:768997.
15. Lund ICB, Petersen OB, Becher NH, et al. National data on the early clinical use of non-invasive prenatal testing in public and private healthcare in Denmark 2013–2017. *Acta Obstet Gynecol Scand*. 2021;100(5):884-892. https://doi.org/10.1111/aogs.14052
16. Hatt L, Singh R, Christensen R, et al. Cell-based noninvasive prenatal testing (cbNIPT) detects pathogenic copy number variations. *Clin Case Rep*. 2020;8(12):2561-2567. https://doi.org/10.1002/ccr3.3211
17. Iwarsson E, Jacobsson B, Dagerhamn J, Davidson T, Bernabé E, Heibert Arnlind M. Analysis of cell-free fetal DNA in maternal blood for detection of trisomy 21, 18 and 13 in a general pregnant population and in a high risk population – a systematic review and meta-analysis. *Acta Obstet Gynecol Scand*. 2017;96(1):7-18. https://doi.org/10.1111/aogs.13047
18. Harasim T, Neuhann T, Behnecke A, Stampfer M, Holinski-Feder E, Abicht A. Initial clinical experience with NIPT for rare autosomal aneuploidies and large copy number variations. *J Clin Med*. 2022;11(2):372. https://doi.org/10.3390/jcm11020372
19. Grati F. Chromosomal mosaicism in human feto-placental development: implications for prenatal diagnosis. *J Clin Med*. 2014;3(3):809-837. https://doi.org/10.3390/jcm3030809
20. Lund ICB, Becher N, Christensen R, et al. Prevalence of mosaicism in uncultured chorionic villus samples after chromosomal microarray and clinical outcome in pregnancies affected by confined placental mosaicism. *Prenat Diagn*. 2020;40(2):244-259. https://doi.org/10.1002/pd.5584
21. Vossaert L, Chakchouk I, Zemet R, van den Veyver IB. Overview and recent developments in cell-based noninvasive prenatal testing. *Prenat Diagn*. 2021;41:1202-1214.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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