Overview and recent developments in cell-based noninvasive prenatal testing

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Abstract

Investigators have long been interested in the natural phenomenon of fetal and placental cell trafficking into the maternal circulation. The scarcity of these circulating cells makes their detection and isolation technically challenging. However, as a DNA source of fetal origin not mixed with maternal DNA, they have the potential of considerable benefit over circulating cell-free DNA-based noninvasive prenatal genetic testing (NIPT). Endocervical trophoblasts, which are less rare but more challenging to recover are also being investigated as an approach for cell-based NIPT. We review published studies from around the world describing both forms of cell-based NIPT and highlight the different approaches' advantages and drawbacks. We also offer guidance for developing a sound cell-based NIPT protocol.

INTRODUCTION

Cell-based noninvasive prenatal testing (cbNIPT) using fetal and placental cells offers unique advantages over both diagnostic invasive testing and cell-free DNA (cfDNA)-based NIPT. Chorionic villus sampling (CVS) and amniocentesis are the gold standard for prenatal diagnostic genetic testing, but they have a 0.3% to 0.1% procedure-associated risk of miscarriage.[1] Although this risk is very low, it has driven high patient and provider demand for, and rapid clinical implementation of, noninvasive prenatal screening (NIPS) analyzing circulating cfDNA in maternal plasma. However, the resolution and positive predictive value of cfDNA-based NIPS are limited, primarily because circulating cfDNA is mostly maternal in origin; only 5-20%, referred to as the fetal fraction, derives from the placenta. In contrast, if circulating fetal nucleated red cells and trophoblasts, or trophoblasts from the endocervical canal, can be consistently recovered, they provide a pure source of fetal DNA. We review

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here recent progress in using fetal DNA for cbNIPT and predict future developments in this field.

FETAL AND PLACENTAL CELLS IN THE MATERNAL CIRCULATION

Schmorl first identified placental cells in the maternal circulation in 1893, when he found clusters of trophoblasts in the pulmonary capillaries of women who had died of eclampsia.[2,3] Various studies conducted in the 1950's and 60's reported fetal cells in maternal blood samples collected mostly after delivery.[4-8] In 1969, Walknowska and colleagues suggested using these circulating fetal cells for NIPT, after they found male cells in lymphocyte cultures prepared from maternal samples collected between 14 and 37 weeks gestation.[9] Later, fetal cells in maternal blood were detected as early as 4 weeks gestation.[10]

Four different types of circulating fetal cells have been described: [1] trophoblasts, [2] fetal nucleated red blood cells (fnRBC), [3] lymphocytes, and [4] stem/progenitor cells. The latter two may build up maternal tolerance of the fetus or in the development of maternal autoimmune diseases,[11] and they can persist for many years after delivery.[12] This persistence makes fetal circulating lymphocytes and stem/progenitor cells unsuitable for non-invasive prenatal testing, as their presence in subsequent pregnancies could confound test results. In contrast, fnRBC and trophoblasts are rapidly cleared from the maternal circulation after delivery, with none detectable 8 weeks post-partum.[13] Primitive erythroblasts from the yolk sac are the first fnRBC to appear at 7-12 weeks gestation, but disappear after 12w,[14] when definitive erythroblasts, derived from the fetal liver, appear. The trophoblasts that enter the maternal circulation can originate from different trophoblast cell types. A detailed review of placental development and trophoblast lineage differentiation is beyond the scope of this text and can be found elsewhere.[15] Briefly, upon implantation, the polar trophectoderm of the blastocyst invades the decidual stroma and differentiates into the earliest trophoblast lineages, the primitive mononuclear cytotrophoblast (CTB) and the multinucleated primitive syncytium (PS). Early proliferating cytotrophoblasts (CTB) then breach the uterine capillaries and form primary villi covered by CTB. Secondary and tertiary villi acquire mesoderm-derived stroma and blood vessels. These floating chorionic villi are covered by multinucleated syncytiotrophoblasts, formed through asymmetric division and fusion of CTB[15,16]. Villi at the periphery invade deeper into the uterine wall and become anchoring villi covered with mononuclear extravillous trophoblasts (EVT). EVT further differentiate into deeply invading interstitial EVT and endovascular EVT that replace the endothelium of maternal spiral arteries.[15] These different stages of trophoblast development are marked by expression of different genes, and expression data support that primarily (endovascular) EVT are isolated from maternal blood samples.[17-19] Whether more primitive trophoblast cell types circulate at earlier gestational ages needs further study.

The relative scarcity of fetal cells makes it challenging to isolate them from maternal blood. Without selecting for a specific cell type, the number of fetal cells is estimated to vary between 1-2 to 2-6 cells/ml of maternal blood.[20,21] However, isolating cells without prior selection is labor-intensive, slow, and does not avoid cell types persisting from prior pregnancies. Thus, a cell type-specific enrichment procedure is recommended, even though it usually results in a lower fetal cell yield. The overall yield from maternal blood

depends on multiple variables including gestational age[10,22-27], pregnancy complications and pathologies[28,29], fetal abnormalities[20,30,31], maternal body mass index (BMI),[27,32] physical activity before blood collection[33], and various other factors,[34] including individual biological variation. The optimal period for circulating trophoblast recovery is between 10-14 weeks gestation, coinciding with the high placental vascularization rate at the end of the first trimester,[35] with a significantly lower yield after 15 weeks when there are fewer invading trophoblasts.[23,25-27] The same downward trend has been reported for fnRBC,[22,23] although one study found no correlation between the number of recovered fnRBC and gestational age.[36] In contrast, in two studies with no cell selection procedure the number of recovered cells increased throughout pregnancy,[10,23] indicating that although cell recovery varies and is methodology-dependent, circulating fetal cells can be isolated until advanced gestational age. As with fetal cfDNA,[37,38] higher maternal BMI also reduces trophoblast yield, although with circulating cells, this trend is not always statistically significant.[25,27,32]

Some factors are associated with an increased number of fetal cells in the maternal circulation (e.g. with fetal trisomy 21 and other aneuploidies)[20,30,31] including sex chromosome abnormalities, triploidy or +invdup(15).[20,31] In contrast, fetal trisomy 18, a few cases of trisomy 13, and (in one study) 47,XXY were not associated with increased numbers of fetal cells.[20,30,31] Trisomy 18 pregnancies usually have smaller placentas, which might release fewer trophoblasts into the maternal circulation. More circulating fetal cells have also been found with fetal growth restriction or other pregnancy complications such as eclampsia,[29] hypertensive disorders of pregnancy (unpublished data, Crovetti *et al.*), or other adverse pregnancy outcomes, supporting that circulating cells have potential as a biomarker for abnormal placentation.[28,39] One group reported that 30 minutes of moderate physical activity before blood draw led to an increased trophoblast yield.[33] The same investigators also found that pregnancies with male fetuses were associated with a higher median number of circulating trophoblasts compared to female fetuses, and that the level of a few cytokines (RANTES, IL-2 and IL-5) correlates with trophoblast count.[34]

It is not known whether amniocentesis, CVS, or another prior procedure increases the numbers of circulating trophoblasts or fetal cells. However, since data from cffDNA analysis show a small but non-significant increase in the amount of cffDNA or fetal fraction immediately after CVS,[40-42] we recommend that research on the yield of circulating trophoblast recovery be conducted with samples obtained before a prenatal diagnostic procedure or drawn at least one week afterward.

CELL ISOLATION FROM MATERNAL BLOOD

The general workflow of a cbNIPT protocol includes: [1] maternal sample collection, [2] initial sample processing and fetal cell enrichment (based on specific markers or size) with or without maternal cell depletion, [3] fetal cell identification, [4] fetal cell isolation, and [5] downstream analysis (Figure 1). Many different fetal cell isolation and detection techniques have been investigated over the years (overview in Supplementary tables). Earlier studies mainly utilized Y-chromosome detection (e.g., by fluorescence *in situ* hybridization (FISH)), which allows for broader capture of different cell types but precludes testing pregnancies

with female fetuses. Hence, investigators have also explored specific isolation protocols independent of fetal sex.

Fetal nucleated red blood cells

Maternal red blood cells can be removed by bulk separation or density gradient centrifugation using Ficoll[43-46] or Percoll[47-50] in different gradient combinations, to separate the nucleated cell fraction (including fetal cells) from the maternal RBC and plasma fraction. That said, using Ficoll could result in 60-80% fetal cell loss,[51] making the use of less aggressive methods more desirable. Lowering the pH of diluted blood samples by adding ACD (sodium citrate/citric acid/dextrose) improves retention of fnRBC during density gradient separation by increasing the mean corpuscular volume and lowering the density of the RBC, allowing use of a density gradient cut-off that better retains fnRBC.[52] Franchi and colleagues refined this low pH protocol and conducted a trial on 46 women:[53] using FISH for chromosomes 21 and 18, they noninvasively diagnosed 7 fetal aneuploidies. Alternatively, selective maternal RBC lysis based on differential erythrocyte carbonic anhydrase I and II activity can be used.[54,55] Carbonic anhydrase I is active in maternal RBC but not in fetal RBC. Using isotonic bicarbonate and ammonium chloride solutions in combination with a specific anhydrase II inhibitor, maternal cells will produce ammonium bicarbonate, which attracts water into the cells, causing them to burst. Additional depletion of maternal white blood cells (WBC) can be achieved by specifically targeting surface markers as CD45 and CD14 with magnetic-activated cell sorting (MACS).

Herzenberg *et al.* first reported in 1979 a fetal cell selection step that consisted of fluorescence-activated cell sorting (FACS) specific for paternal HLA antigens expressed on fetal cells but not maternal cells. They confirmed by Y chromosome quinacrine staining that they isolated fetal cells from the blood of women carrying male fetuses.[56,57] However, HLA-antigens require prior knowledge of the paternal HLA-genotype and optimization for each individual sample. Furthermore, as indicated above, Y-chromosome based confirmation is only applicable to male pregnancies. Hence, considerable efforts were made from the 1980's to early 2000's to find adequate, general markers for specific fnRBC selection. The surface marker CD71 (transferrin receptor) combined with CD36 and/or glycophorin A (GPA) are the most widely used targets,[48,58-60] and Bianchi and colleagues showed that adding anti-GPA antibodies to anti-CD71 or anti-CD36 substantially improves fnRBC selection and accuracy of fetal sex prediction.[61,62] Choolani *et al.* confirmed that primitive erythroblasts were the predominant cell type up to 12 weeks gestation, declining rapidly thereafter.[63] Both primitive and definitive erythroblasts were GPA+, CD47+, CD45− and CD35−, but CD71 and CD36 staining was very weak for primitive erythroblasts. Other strategies included targeting surface antigen i (anti-i),[44] CD34,[64] galactose-specific lectin[65] or using the HAE9 antibody[44], or focusing on physical fnRBC characteristics, including the use of charge flow separation based on cell-specific surface charge densities.[66,67] These latter methods were less successful and/or have not been further examined.

Investigators have attempted to incrementally improve the fnRBC yield by using embryonic (epsilon, ε and zeta, ζ) and fetal (gamma, γ, F) hemoglobin (Hb) for cell selection and/or identification. HbF expression is significantly higher in fnRBC compared to adult nRBC,[68]

but under certain conditions (e.g., for beta-thalassemia carriers), maternal HbF expression is upregulated.[60] Choolani and colleagues described decreasing ε-globin expression with increasing gestation from 7 to 14 weeks, when the number of ε-positive cells became almost negligible,[49] and similarly, Christensen *et al.* detected only one ε-positive cell in 18 samples collected between 8-12 weeks.[69] Similarly, zeta Hb, which decreases even earlier than ε-globin,[70] is an inadequate fetal cell marker.

One of the largest early cell-based NIPT trials so far, the NICHD (National Institute of Child Health and Human Development) Fetal Cell Isolation Study (NIFTY), included 2,700 samples collected between 1995 and 1999.[71] Samples were first subjected to density gradient centrifugation, with or without MACS or FACS depletion based on CD14, CD15 and/or CD45. Fetal nRBC were then enriched by positive MACS-based selection (CD71+) or by FACS sorting for HbF+/CD45– cells. Confirmation of fetal origin was done with X and Y– FISH in male pregnancies, and FISH for chromosomes 13, 18 and 21, to detect possible fetal aneuploidies. The results indicated that prenatal diagnosis based on fnRBC analysis was not yet ready for clinical implementation (Figure 2), mainly due to low fetal cell yield. (XY cells were found in only 41% of all male pregnancies.)

Circulating fnRBC nevertheless remain an attractive target because they avoid confined placental mosaicism (CPM), which is an issue for trophoblasts (discussed below). However, none of the many published methods have yet been validated for clinical use, in part due to lack of specific antibodies. By screening 690 different hybridomas, Zimmerman *et al.* found two antibody clones with the desired specificity for fetal erythroblast cell membranes and that target antigens other than known markers CD71, CD36, GPA, antigen i and galactose.[72] A recent study included the use of RBC aggregation followed by RBC lysis and maternal WBC depletion with anti-CD45 and -CD66b antibodies,[73] yielding two fnRBC from 1 ml samples collected at 12 and 16 weeks gestation, and on average 48.8 cells/ml in 16 samples at 23-27 weeks. Staining included antibodies for GPA and CD45. Y-PCR was positive for pools of isolated cells, although it was unclear whether these pools also contained maternal cells. Huang *et al.* reported the recovery of both trophoblasts and fnRBC in five samples using a novel silicon immune-affinity method.[74] Trisomy was convincingly documented for three of those cases by using both array comparative genomic hybridization (aCGH) and next generation sequencing (NGS) analysis of 2-, 3-, or 10-cell pools, and STR evidence showed that fnRBC were recovered in all samples. However, this study used a complex enrichment platform not easily replicated or commercially available. The same group recently reported 14 additional cases for which 2-71 confirmed fnRBC were collected from 2 ml.[75] Zhang *et al.*[76] used a microfluidic chip coated with an anti-CD71 antibody, and identified 5–35 fnRBC per 2 ml of maternal blood starting at 7 weeks gestation, and fetal origin was demonstrated by SRY-PCR.

In 2017 and 2018, three publications with overlapping authors described similar technologies with positive selection for CD147, also known as extracellular matrix metalloproteinase inducer, which had not previously been used to recover fnRBC. Using biocompatible hydroxyapatite/chitosan nanoparticles microchips,.[77] biotin-doped polypyrrole-based microchips,.[78] or bio-conjugated SiO2 gel microbeads,[79]all three reported up to 50-75 fnRBC/ml (peaking at 16-20 weeks gestation), using "anti-ε-HbF"

staining as evidence for the cells' fetal origin. But it is not clear which antibody was used, meaning some cells could have been maternal nRBC.

Trophoblasts

Other studies focused on isolating circulating trophoblasts, by selecting cells that express HLA-G, a nonclassical major histocompatibility complex class I antigen specific to EVT. In an initial study, cells were enriched with a Percoll density gradient and stained on slides with anti-HLA-G antibody, followed by FISH with probes for chromosomes X, Y and 21.[80] One or more XY HLA-G+ cells were identified for 7/10 male pregnancies, but there was also non-specific staining of maternal cells attributed to secondary and tertiary antibodies.

Investigators in Denmark conducted microarray gene expression experiments on trophoblastic cell pools to find better markers for enrichment.[17-19] They developed a proprietary protocol using the surface markers CD105 (endoglin) and CD141 (thrombomodulin) for MACS-based trophoblast selection, and cytokeratin (CK) staining for identification, as CK expression shows a typical vesicular pattern unique to trophoblasts compared to maternal WBC (Figure 3). In collaboration with our group, 100% recovery was achieved in a series of 111 samples, with on average 12.8 cells obtained from 30 ml of maternal blood.[81] Fetal origin was confirmed by XY FISH for all included male pregnancies and demonstrated that the observed CK staining pattern was sufficiently specific for circulating trophoblasts. Downstream analysis by chromosomal microarray analysis (CMA) and/or NGS in this study and follow-up reports correctly identified fetal (mosaic) autosomal trisomies, sex chromosome abnormalities, and several large copy number changes..[82,83]

Our group developed a novel maternal cell depletion protocol in collaboration with RareCyte, by using a gradient of different high-density fluids in combination with a rubber float to lift and separate the nucleated cell fraction containing the trophoblasts above the float.[84] We selected candidate trophoblasts based on nuclear morphology and CK staining pattern, and also used an anti-CD45 antibody to identify and exclude maternal WBC.[84] After further protocol adjustments, we recovered on average 0.18 trophoblasts/ml (range: 0-1.58 cells/ml) from 30-40 ml blood samples from 125 women.[85] DNA from individual trophoblasts was analyzed by low-coverage NGS for genome-wide copy number analysis, with correct identification of fetal aneuploidy in 11 cases and a known CNV in four cases (ranging from 1.1 Mb to 19 Mb in size). A new genotyping method confirmed the fetal origin of all analyzed cells by comparing fetal single nucleotide polymorphisms (SNPs) to the maternal (and when available, paternal) SNP profile (based on 50-60 SNPs to genome-wide analysis, described in [86] and unpublished data). Our most recent method includes an enrichment strategy based on a combination of anti-HLA-G, anti-Trop2, and anti-EpCAM antibodies resulting in an average yield of 0.20 trophoblasts/ml from 95 blood samples.[25]

Moving away from targeting specific antigens, Vona *et al.* explored trophoblast selection based on size using their "Isolation by Size of Epithelial Tumor/Trophoblastic cells" (ISET) protocol.[87] The blood sample is filtered through an 8 μm-pore polycarbonate filter to retain a population of larger cells, which includes trophoblasts. After staining, trophoblasts are isolated by laser capture microdissection for downstream analysis. The authors reported

recovery of 1-7 trophoblasts per 2 ml of maternal blood from six pregnant women carrying a male fetus.

More recently, several commercial and academic groups have explored microfluidics platforms (originally developed for CTC isolation) for cbNIPT applications, with variable success. These rare cell isolation platforms capture trophoblasts by immunomagnetic enrichment,[88,89] size-based selection[90,91] or specific dielectrophoretic separation,[92] although for some only proof-of-principle data is available. Compared to manual processing, these platforms require lower sample volumes and less cell manipulation, and their approach for cell enrichment is often less harsh, lowering the risk for cell loss or inadequate cell quality.

Promising results were obtained with the anti-EpCAM NanoVelcro microfluidic chip enrichment after immunostaining for CK, HLA-G, and CD45,[88] after which putative fetal cells are isolated by laser capture microdissection and analyzed by FISH, CMA, and short tandem repeat (STR) analysis. In a study of 15 women, up to 3 trophoblasts/ml were recovered for healthy pregnancies, and 7.5 cells/ml if there was a fetal genetic abnormality.[88] Zhang *et al.* claimed recovery in 31 samples of 2-20 circulating fetal cells from only 2 ml of blood using a novel stimuli-responsive microfluidic interface with EpCAM-based capture and HLA-G/CK staining, but they also found stained cells in control and postpartum groups, suggesting this approach is less specific.[93] Winter and colleagues used spike-in experiments to optimize their size-selection inertial microfluidics system, and subsequently recovered six trophoblasts from only 7 ml of blood from a pregnant woman whose fetus had trisomy 21.[90] Gur *et al.* spiked blood samples with JEG3 cells, a choriocarcinoma cell line which expresses EVT markers. Immunomagnetic selection based on EpCAM and HLA-G through a two-tiered microchip system, resulted in high purity JEG3 cell recovery.[89] Vander Plaetsen and colleagues recently tested the VTX-1 Liquid Biopsy system, which uses laminar microscale vortices for size-based trophoblast isolation, and recovered 2-6 trophoblasts from 8 ml of blood in 7/10 women carrying male pregnancies.[91]

TROPHOBLASTS FROM ENDOCERVICAL COLLECTION

In addition to maternal blood, trophoblasts can also be collected from the endocervical canal. One group developed a protocol for trophoblast retrieval and isolation from the cervix (TRIC) by inserting 2 cm of a cytobrush into the endocervical canal, collecting cervical mucus, and performing an anti-HLA-G immunomagnetic trophoblast enrichment.[94] They reported a recovery of 500–1,500 EVT per sampling with minimal maternal cell contamination, as confirmed by β-human chorionic gonadotropin (hCG) staining for 95-100% of the cells and XY FISH for male pregnancies.[95,96] Based on protein expression analysis, they determined the TRIC protocol yields EVT. The larger number of trophoblasts obtained via endocervical sampling contrasts with the scarcity of fetal cells or trophoblasts isolated from maternal blood samples, and could therefore provide a more fruitful sample source. In addition, the degree of DNA fragmentation in these cells was comparable to that of control cells, indicating adequate quality for further genetic testing. Of note, endocervical cell collection requires trained personnel. Although it is somewhat invasive, studies reported

to date[94-99] support that it is safe; cervical cancer screening using a similar approach can be done safely during pregnancy.

While trophoblast retrieval from maternal blood samples shows a downward trend with increasing gestational age,[22,23,25-27] neither gestational age[98] nor maternal BMI[98] influences endocervical EVT recovery, though fewer EVTs were recovered from women with early pregnancy loss.[97] EVTs from these pregnancies and those with fetal growth restriction and preeclampsia had altered expression of various markers such as AFP, ENG, and FLT-1, and less PAPP-A, LGALS14, and PGF compared to controls. Hence, endocervical trophoblasts have potential as biomarkers for pregnancy complications.

Investigators in South Korea recently adjusted the TRIC protocol to include a pre-enrichment fixation step, and noted a higher number of β-hCG-positive cells post-formalin-fixation.[100] Also, the Paterlini group adjusted their size-based selection ISET protocol[87] for trophoblasts collected with a cytobrush rotated at the external os, similar to a standard PAP smear.[101] For a set of 21 samples (8-21 weeks gestation), they were able to recover 2–12 trophoblasts/sample.

CELL-BASED NIPT DATA ANALYSIS CONSIDERATIONS

Although cfDNA screening for fetal chromosomal abnormalities is well integrated into the clinic, its main disadvantage is that the fetal cfDNA (fetal fraction) is only 5-20% of all cfDNA in maternal blood.[102] Furthermore, cfDNA-based NIPT results are influenced by maternal factors such as BMI, chromosomal mosaicism, fibroids, malignancies, and by CPM. Using circulating fetal cells or endocervical trophoblasts as a fetal DNA source avoids maternal DNA contamination if cells are carefully isolated and genotyped to be fetal. This eliminates the effect of most maternal confounding factors. With pure fetal DNA, smaller copy number changes down to 1-2 Mb in size can be detected by CMA or low-coverage NGS, which is in the range of those commonly identified by CMA on CVS or amniocentesis samples.[82-85] Similar to CMA on invasively obtained samples, cbNIPT currently cannot detect copy-neutral rearrangements such as balanced translocations or inversions.

An essential step in single circulating fetal cell analysis is whole genome amplification (WGA), to ensure sufficient input DNA for the downstream analysis. Each method has advantages and drawbacks, but the goal is to use a method that performs adequately for both CNV analysis and monogenic disorder testing.[e.g.103,104] A highly uniform genome coverage is required for CNV analysis, while low allelic-drop out (ADO) and false positive rate (FPR) are essential for point mutation detection. ADO (absence of amplification of one allele for a heterozygous locus) can occur due to unequal amplification (e.g., because of DNA degradation), and will lead to inaccurate NGS results. On the other hand, suboptimal polymerase fidelity and false positive results due to sequencing and, more importantly, amplification errors, can also pose a problem. One of the studies comparing three WGA technologies (five kits)[105] showed that degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR[106]) had good uniformity and reproducibility but performed worst for other features (including ADO, FPR), while multiple displacement amplification (MDA[107]) had the lowest FPR, reasonable ADO, but low reproducibility. Multiple annealing

and looping-based amplification cycles (MALBAC[108]) demonstrated high uniformity and reproducibility, and the lowest ADO rate.

The quality of the single cells will also determine the success of the WGA reaction. For trophoblasts isolated from maternal blood, there can be considerable cell-to-cell variability in whole-genome copy number profiles, making analysis of each single cell profile separately more advantageous than pooling cells or merging profiles of multiple cells from the same individual.[25] Two main biological factors are responsible for this (Figure 4). Trophoblasts in an advanced stage of apoptosis[109] give noisy whole genome profiles because the timing and degree of DNA degradation varies across the genome. Cells presumed to be in the S-phase of the cell cycle have noisy profiles because genomic regions replicate asynchronously, resulting in apparent random copy number gains and losses.[110] Both patterns are recognizable with experience. Pooling a "good-quality" cell with a cell with the aforementioned patterns or low-quality profiles can compromise the overall result and is therefore not recommended. Yet, sometimes trophoblasts are isolated as clusters of two, three, or more cells that cannot be separated (Figure 3), and pooled analysis cannot be avoided. Of note, a higher proportion of lower quality cells might be seen for certain conditions or fetal abnormalities, such as a pregnancy affected with Turner syndrome.[25]

Given the number of trophoblasts obtained from endocervical samples, analysis of a larger pool can potentially be done, and might be less sensitive to the effect of a few low-quality cells, provided most cells are of adequate quality.[95]

Separate analysis of each individual circulating cell enables the detection of distinct genetic signatures in case of non-identical twins (and theoretically, higher multiples), and confirms the presence of different karyotypes in CPM or other forms of mosaicism, provided enough cells can be tested. CPM for aneuploidy is found in 1-2% of all CVS samples[111] and can lead to abnormal results with cfDNA-based aneuploidy screening, but mosaicism cannot be confirmed noninvasively with cfDNA. In contrast, with single trophoblast-based NIPT, single cell analysis will allow detection of aneuploid and euploid cells separately, as illustrated by five CPM cases from our group.[25,84,85] For one mosaic trisomy 13 case, one case with 46,XX/45,X mosaicism, and one with two mosaic copy number losses on chromosome 13, both euploid and aneuploid cells were found. In two additional cases (trisomy 13 and trisomy 15), only two and one aneuploid cells in total were recovered, respectively, highlighting the importance of the number of recovered cells to detect CPM. The same applies to multiple pregnancies to ensure the recovery of cells from each fetus.[25,85] Determining from which fetus an individual cell originates is not possible in same-sex twin pairs, and additional genotyping is needed to determine zygosity.

Cell-based testing for single gene disorders has also been explored. The Paterlini group published two sets of samples in which they correctly identified unaffected and affected fetuses with cystic fibrosis or spinal muscular atrophy using their ISET protocol.[112,113] Chang *et al.* reported success with cbNIPT for one pregnancy at risk for congenital deafness and one for epidermolytic ichthyosis.[114] Fetal cell analysis for each known familial mutation agreed with amniocentesis results.

In a recent report on a woman carrying monozygotic twins at risk for cystic fibrosis, from which 7 circulating trophoblasts were isolated, the twins were correctly identified as unaffected carriers, but ADO was noted for 1/3 STRs included in the genotyping analysis.[115] Because of ADO, SNP-based genotyping may be better after WGA than STR-analysis, as more SNPs can be included.[103,116-119] Chen and colleagues developed a double-negative selection protocol,[120] and subjected the recovered fetal cells first to low-coverage NGS (and subsequent high-coverage whole genome sequencing for the best quality cell) for point mutation detection. Although they noted good performance overall, they observed an ADO rate of 24.9%, and (not unexpectedly) less uniform coverage for WGA products compared to unamplified genomic DNA. Cayrefourcq *et al.* investigated trophoblast analysis for monogenic diseases caused by point mutations or triplet repeat expansion.[92] They only obtained a conclusive fetal result for one out of 7 families at risk of Huntington disease, and obtained a similar result when attempting mini-exome sequencing for 9 couples at risk for a specific monogenic disorder. They concluded that due to technical issues (including cell loss throughout sample processing) and ADO (3-fold higher in trophoblasts compared to maternal single cells), further improvements would be necessary for cbNIPT to be applicable for both single gene disorders and aneuploidies/CNV.

Huang and colleagues tweaked the TRIC protocol to replace intracellular immunostaining with endoplasmic reticulum tracker dye, which is nondestructive and potentially ensures a better DNA quality for endocervically isolated trophoblasts.[121] They accurately identified the fetal genotypes in a series of 11 pregnancies at risk for alpha- or beta-thalassemia, and noted adequate SNV detection when performing whole exome sequencing in an additional five samples. Platforms avoiding WGA could also be helpful, including a sensitive single cell digital droplet PCR method recently described by Sato and colleagues.[122]

WORKING TOWARDS A CLINICAL TEST AND FUTURE OUTLOOKS

Because it is unhindered by maternal DNA contamination, cbNIPT could potentially be offered as a noninvasive diagnostic test rather than a screening assay (Table 1). In addition, cbNIPT can be provided early on, offering flexibility for further decision-making and management of the pregnancy. Endocervical cbNIPT has been performed at 5 weeks gestation and blood-based cbNIPT can also potentially be performed before 10 weeks.[95,96] cfDNA-based NIPS is already in widespread use for detection of common fetal aneuploidies. To a more limited extent, it is used for subchromosomal copy number abnormalities associated with common deletion syndromes (in some cases across the entire genome), but the resolution and positive predictive values remain low. Since cbNIPT is based on pure fetal DNA from intact cells, it could be used for testing smaller copy number variants, currently at an approximate 1 megabase resolution.[85] For the same reason, cbNIPT may also offer more flexibility and resolution for combined analysis for copy number alterations and monogenic disorder testing on the same sample. Further studies are needed to assess the analytical and clinical utility of these approaches on both circulating fetal cells and endocervical trophoblasts.

One of the main challenges in cbNIPT on circulating trophoblasts or fnRBC is consistent recovery of a sufficient number of cells from each patient to reduce the % test failures.

Current studies indicate higher cell numbers for endocervical trophoblasts, but here the challenge ispurification from the endocervical mucus and surrounding maternal cells. Significant improvements have been made for both approaches. Other aspects to consider when evaluating a test's potential for clinical implementation are turn-around-time (TAT), scalability, throughput, and cost. The TAT of library preparation, sequencing, and data analysis would be similar to cfDNA-based NIPT, but extra time is required for sample processing, including maternal cell depletion, fetal cell enrichment, and cell isolation.

Cell-based NIPT can potentially reach a large pregnant population, including those lacking access to a larger center offering diagnostic amniocentesis or CVS. More work is needed, however, to increase the throughput of current protocols and guarantee consistent test performance. The available data on (automated) microfluidics platforms are very promising, and these assays could boost throughput while also lowering TAT for cbNIPT from circulating cells. The current cost (a few thousand dollars per sample) is prohibitive for wider commercialization, but a more affordable test can be developed through further optimization of the processing protocol, bulk production, and decreasing sequencing costs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Data availability statement

Data sharing is not applicable to this review article, as no datasets were generated during the current study.

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Bulleted statements

What is already known about this topic?

- Several different protocols have been published to date for circulating fetal cell enrichment, detection, isolation, and analysis for the purpose of cell-based noninvasive prenatal testing (NIPT).

What does this review add?

- This review offers a comprehensive and up-to-date critical analysis of published studies on cell-based NIPT, focusing on more recent developments.
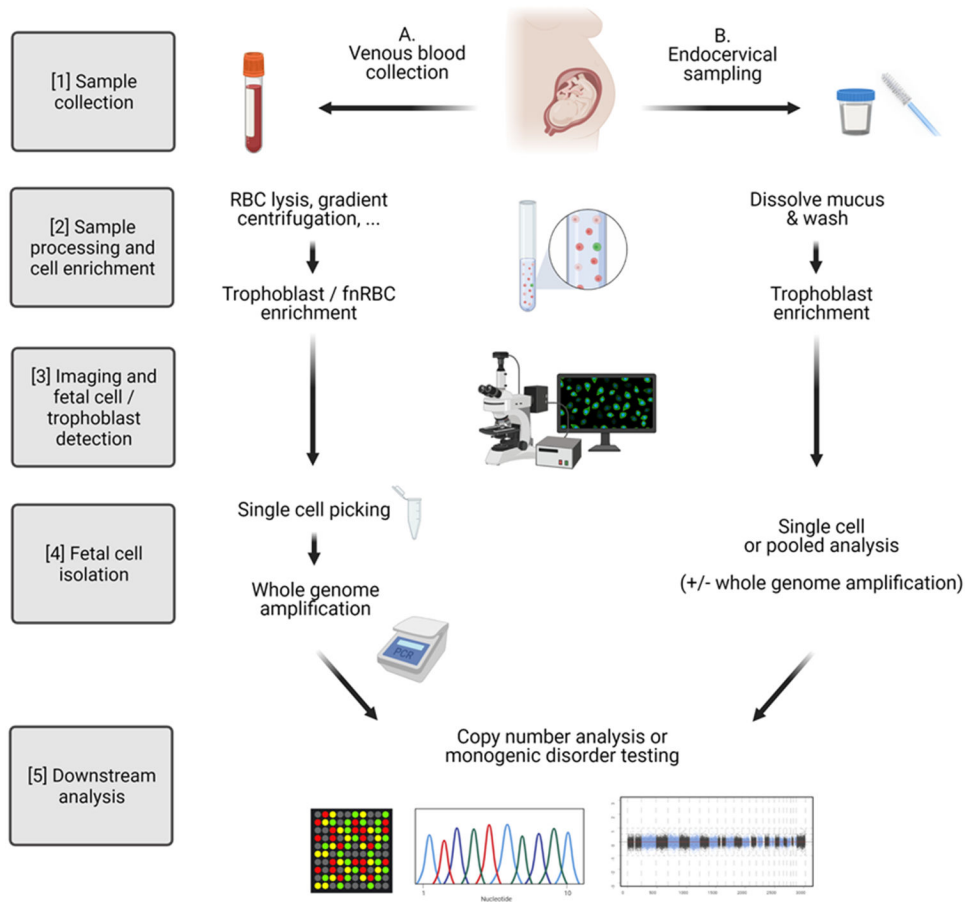
Figure 1. Cell-based NIPT workflow.
(Created with BioRender.com)
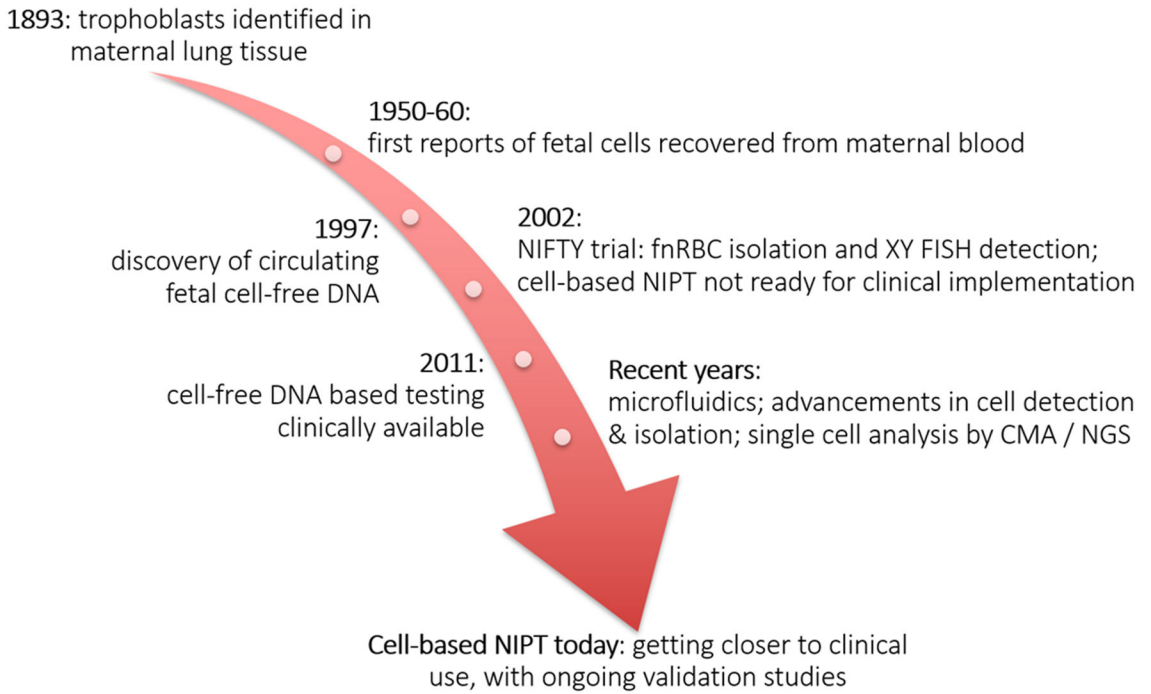
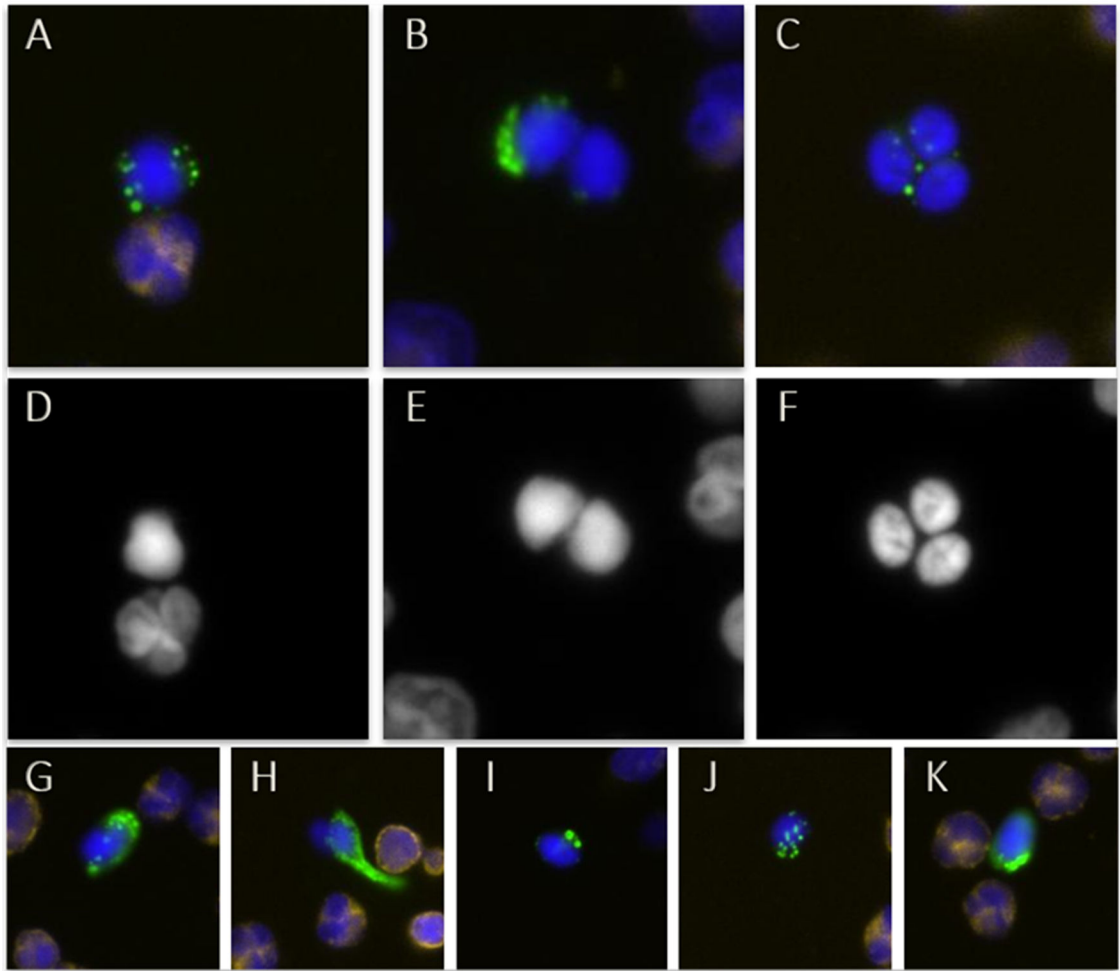**Figure 2. Timeline depicting milestones in the field of noninvasive prenatal testing.**

Figure 3. Immunostaining images of circulating trophoblasts.
The staining antibody cocktail includes anti-cytokeratin (CK; green) and anti-CD45 (yellow) antibodies, and DAPI nuclear stain. Panel A depicts a single trophoblast with a maternal cell, panel B a trophoblast doublet and panel C a cluster of three trophoblasts. Panels D, E, and F illustrate the respective DAPI staining and nuclear morphology. Panels G through K demonstrate the diversity in CK staining patterns that can be seen.
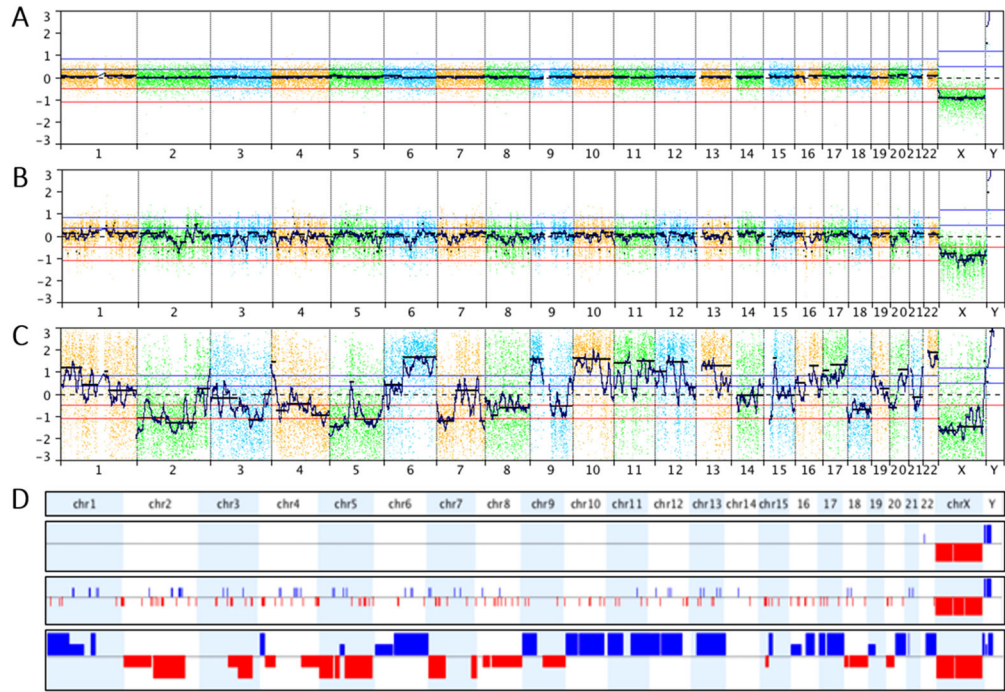
Figure 4. Whole genome profiles from circulating trophoblasts.

[A] High-quality whole genome profile of a single trophoblast obtained from a pregnancy with a male fetus. The profile was obtained by comparison to a female reference, hence the relative loss of one X chromosome and the relative gain of Y. [B] Whole genome profile of a male trophoblast presumed in S-phase. Random gains and losses can be seen throughout the genome. [C] Whole genome profile demonstrating genomic degradation of an apoptotic trophoblast. [D] Whole genome plots specifically depicting the copy number gains (blue) and losses (red) detected in all three cells above. All profiles were created in NxClinical (BioDiscovery).

Table 1.

Overview of different sample sources and their characteristics.

β-hCG: beta human choriogonadotropin, CPM: confined placental mosaicism, (f)nRBC: (fetal) nucleated red blood cell, GA: gestational age, HbF: fetal hemoglobin

Cell type	FNRBC FROM BLOOD	TROPHOBLASTS FROM BLOOD	ENDOCERVICALLY OBTAINED TROPHOBLASTS
Sampling	Maternal peripheral blood sample	Maternal peripheral blood sample	Endocervical collection with a cytobrush
Workflow	1 Remove excess of maternal cells 2 Specific fnRBC enrichment and detection 3 Single cell isolation 4 Data analysis	1 Remove excess of maternal cells 2 Specific trophoblast enrichment and detection 3 Single cell isolation 4 Data analysis	1 Remove cervical mucus 2 Trophoblast enrichment and isolation 3 Data analysis (bulk or single cell)
Major markers for enrichment and/or detection	CD71, GPA, HbF	HLA-G, EpCAM, CK	HLA-G, β-hCG
Cell recovery (normal pregnancies)	Range: 0.04-10/ml	Range: up to 500 nRBC per ml reported (not all confirmed fetal)	Range: 2-1,500/sample
Advantages	• Simple sampling method • No significant influence of BMI • Single cell analysis (fetal mosaicism detection, multiple pregnancies, quality control) • No CPM detection	• Simple sampling method • No significant influence of BMI • Single cell analysis (mosaicism detection, multiple pregnancies, quality control)	• Best cell yield (more replicates / potential pooled testing without WGA possible) • Yield not dependent on GA; early testing possible (5 weeks)
Drawbacks	• Scarcity of circulating fnRBC, WGA required • Yield dependent on GA	• Scarcity of circulating trophoblasts, WGA required • Yield dependent on GA • CPM	• More specialized personnel needed for sample collection • CPM