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Review

A Reappraisal of Circulating Fetal Cell Noninvasive Prenatal Testing

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New tools for higher-resolution fetal genome analysis including microarray and next-generation sequencing have revolutionized prenatal screening. This article provides commentary on this rapidly advancing field and a future perspective emphasizing circulating fetal cell (CFC) utility. Despite the tremendous technological challenges associated with their reliable and cost-effective isolation from maternal blood, CFCs have a strong potential to bridge the gap between the diagnostic sensitivity of invasive procedures and the desirable noninvasive nature of cell-free fetal DNA (cffDNA). Considering the rapid advances in both rare cell isolation and low-input DNA analysis, we argue here that CFC-based noninvasive prenatal testing is poised to be implemented clinically in the near future.

Highlights

Non-invasive prenatal testing based on circulating cell-free fetal DNA has gained broad clinical acceptance for the detection of common aneuploidies. However, it cannot currently provide information about the full spectrum of chromosomal abnormalities and single gene disorders.

While extremely rare, circulating fetal cells (CFCs) in maternal blood have a complete fetal genome.

Advances in genomic analysis techniques promise to resolve the issues associated with high coverage testing of the low amount of genomic material available in a single cell.

Recently, many technologies have been validated for the enrichment and isolation of rare cells from peripheral blood, paving the way for development of clinically applicable CFC isolation methodologies.

Now is the ideal time to reappraise the relevance and use of CFCs in prenatal diagnosis, particularly their clinical scope and benefit.

Future of Prenatal Diagnostics

The field of prenatal genetic testing has changed markedly over recent years with the introduction of new tools for genetic analysis coupled with a marked shift towards the use of noninvasive techniques. Although contemporary **chorionic villus sampling** (CVS; see Glossary) and **amniocentesis** procedures do not pose any substantial risk of miscarriage when performed by experienced health care providers [1], major professional bodies' (such as the American College of Obstetricians and Gynecologists) guidelines still stipulate that these procedures are associated with a small risk of miscarriage [2,3]. Combined with the perceived risk and as well as health economics considerations, CVS and amniocentesis are generally limited to high-risk pregnancies.

Noninvasive prenatal testing (NIPT) based on small DNA fragments shed from placental cells which circulate in the maternal blood, referred to as **cell-free fetal DNA** (cffDNA), is rapidly and dramatically changing the prenatal diagnostic landscape and has already gained broad clinical acceptance for the detection of common aneuploidies [4–6]. As a result, the global cffDNA-NIPT market is forecasted to grow from an estimated $329.6 million in 2013 to $5.5 billion by 2025 [7].

However, current cffDNA-NIPT technologies have a number of significant drawbacks. With the exception of Down syndrome (trisomy 21), cffDNA **positive predictive values** (PPVs) for other aneuploidies can be suboptimal based on the testing platform used (i.e., 76.61% for trisomy 18 and 32.84% for trisomy 13) [8,9] and are even lower for common microdeletions such as 22q11 microdeletion (DiGeorge) syndrome [10]. As a result, the Society for Maternal Fetal Medicine recommends against using cffDNA for microdeletion detection [11]. In addition, despite intensive research and notable advances [12], comprehensive and genome-wide fetal sequencing using cffDNA remains elusive due to the inherent technological difficulties in detecting submicroscopic chromosomal imbalances, issues of confined **placental**

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mosaicism, presence of unexpected maternal **chromosomal abnormalities**, as well as the high proportion of maternal cell free DNA in plasma (>90%) and problems determining the origin of the short DNA fragments.

The presence of **circulating fetal cells** (CFCs) in maternal blood has been recognized for over a century [13,14] and their isolation as a source of fetal genomic DNA for prenatal diagnosis has been a tantalizing goal since the implementation of routine prenatal diagnostic testing. CFCs are, however, extraordinarily rare in maternal blood, presenting a 'needle in a haystack' challenge that until recently impeded their use for noninvasive prenatal testing. Although some isolation techniques have been applied to detect these rare cells, all suffer from significant drawbacks including low efficiencies and specificities and have failed to provide a valid alternative to invasive sampling. Combined with the emergence of cffDNA-based NIPT, these frustrating setbacks have led to a decline in CFC-based diagnostic research. However, advances in single cell genomic testing and the recent development of more effective rare cell isolation technologies have reinvigorated research in the field. This is demonstrated by a number of recent reports suggesting that what was once an elusive dream might be within reach [15–17]. The aim of this article is to critically review the field and to make the case that, pending further interdisciplinary research, fetal cell-based, noninvasive prenatal diagnosis has the potential to further revolutionize the management of pregnancies at risk of genetic disorders.

The Changing Landscape of Invasive Prenatal Testing Procedures

Over the past 10 years, remarkably rapid advances in genomic methods have sparked a significant improvement in prenatal diagnosis of genetic disorders. With recent technological advances such as **chromosomal microarray analysis** (CMA) and **next-generation sequencing** (NGS), fetal genetic abnormality screening is experiencing a revolution. CMA has opened a new door towards genetic diagnosis since it does not require cell culture and provides a high detection rate [18]. The more conventional methods such as **karyotype** and **fluorescence *in situ* hybridization** (FISH) can only detect chromosomal aneuploidy (gain or loss of chromosomes) and structural aberrations with a resolution of 5–10 megabase pairs in size. The widespread use of CMA was triggered by landmark clinical studies demonstrating that **copy number variations** (CNVs) in the range of single exon to several kilobase pairs can be detected by CMA in 1–2% of pregnancies with either normal karyotype or no evident fetal anomalies [19,20]. In fetuses with abnormal ultrasound and a normal karyotype, CMA has shown to improve the identification of abnormalities by 4–6% owing to the detection of submicroscopic chromosomal rearrangements [19,21].

Although CMA has changed the landscape of NIPT, it is still a limited technique. In fact, CMA and karyotype only provide a diagnostic result in 40% of fetuses with structural abnormalities [22]. The main reason for this is that most pathogenic phenotypes are not caused by CNV since they stem from single nucleotide variation, insertions, or deletions [23]. The application of NGS as a state-of-the-art genetic diagnostic approach is therefore being actively explored in order to provide higher sensitivity and coverage (at the level of single nucleotide resolution) in prenatal testing and is beginning to become a mainstream clinical modality. **Whole genome sequencing** (WGS) has been advocated in patients suspected to have genetic disorders not detected with standard approaches. In this setting, WGS detected 25–30% more genetic abnormalities than CMA, karyotype, and qPCR. For instance, a recent report demonstrated the detection of two novel mutations responsible for Meckel–Gruber syndrome in a 12-week fetus not detected by standard genetic analysis [24]. As well as DNA obtained invasively, the use of NGS is also beneficial for cffDNA NIPT since screening can be performed on a smaller fetal DNA fraction

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(~4%) compared to CMA [25]. WGS is, however, limited by high facility and operational cost, lack of equipment access, complicated bioinformatics, and difficulty in determining which patients would most benefit, as well as ethical implications. **Whole exome sequencing** (WES) can be a more cost-effective and clinically applicable approach that has shown promise in identifying the genetic basis of sonographic anomalies associated with Mendelian disease genes and even *de novo* mutations [21]. However, CNV calling using standard WES approaches are less reliable than using WGS. Therefore, karyotyping or CMA should also be performed along with WES to enable the detection of broader range of CNVs. It is likely that WES/WGS testing will become more relevant in the field as NGS becomes quicker and cheaper and there is a greater understanding of the clinical applicability (Box 1).

cffDNA-NIPT

The limitations of invasive approaches combined with a legitimate desire for reassurance of a normal pregnancy from prospective parents have driven the development of NIPT assays based on circulating cffDNA. cffDNA-NIPT has rapidly gained broad clinical acceptance for the detection of common aneuploidies [1–3]. cffDNA originates from apoptotic trophoblastic cells in the placenta and is found at various levels in maternal blood (4–25% at 11–13 weeks gestation) [25]. Following landmark clinical studies [26–29], the first commercial test for common autosomal aneuploidies was released in 2011 and since then, more than 2 million tests have been completed [30]. A number of commercial technologies are currently available for common aneuploidies [31,32].

cffDNA-NIPT has high sensitivity (≥99% for trisomy 21, ≥97% for trisomy 18, and for trisomy 13) and high specificity (≥99% for trisomy 21, 18, and 13) for aneuploidy detection, even for low risk pregnancies [4,9,33–36]. In turn, the implementation of cffDNA screening in prenatal testing algorithms is supported by the American College of Obstetrics and Gynecology. However, testing of cffDNA should not be considered diagnostic with invasive testing strongly recommended to guide clinical decision-making. With this in mind, the American College of Medical Genetics and Genomics advocated in 2016 changing the terminology from NIPT to noninvasive prenatal screening (NIPS) to emphasize the limitations of cffDNA testing [37]. In addition, a number of recent studies demonstrated that cffDNA should not be used as a substitute for current invasive testing after detection of a potential abnormality with biochemical or ultrasound screening. For example, in a study involving 900 pregnancies, the rate of pathogenic chromosome abnormalities missed by cffDNA-NIPT was 8% for women with fetal abnormalities detected by ultrasonography [38]. The biological causes for such discordance between cffDNA-NIPT and diagnostic fetal karyotype remain to be fully elucidated but include low fetal fractions, maternal DNA copy number variations, confined placental mosaicism and the limit of detection of cffDNA for submicroscopic CNVs [6,39].

To date, cffDNA-NIPT reliably detects only a small subset of the chromosomal abnormalities that can be diagnosed using standard invasive procedures. Although paternally derived autosomal dominant disorders and *de novo* fetal mutations can be detected using complex cffDNA analyses, it remains unclear whether these could be performed with the level of reliability and cost-effectiveness required for routine clinical use [40–42]. Moreover, autosomal-recessive and X-linked conditions present even greater challenges considering the obligate background of the maternal genomic make-up and to date, even using targeted detection, relative haplotype dosage, relative mutation dosage, and prohibitively expensive ultradeep sequencing approach, there is no clinically viable universal protocol for fetal genomic profiling [43–45]. Despite these limitations, with continued improvement in sequencing technologies and

Glossary

Allelic dropout: source of missing data in the genome, in which one or both allelic copies at a locus fail to be amplified by amplification.
Amniocentesis: invasive diagnostic technique where fetal cells are obtained from a sample of amniotic fluid surrounding the fetus, extracted using an ultrasound-guided needle.
Cell-free fetal DNA (cffDNA): small fragments (100–150 base pairs) of fetal DNA that make up a significant proportion (10–20%) of free-floating DNA in the blood of pregnant women.
Chorionic villus sampling (CVS): invasive diagnostic medical test in which a small sample of chorionic villi is removed from the placenta via a needle or biopsy forceps to detect genetic abnormalities.
Chromosomal abnormalities: changes in the structure or number of chromosomes which are directly associated with a number of physical disabilities and mental disorders.
Chromosomal microarray analysis (CMA): molecular technique to inspect copy number variations (CNVs) associated with chromosomal abnormalities on a genome-wide scale.
Circulating fetal cells (CFCs): fetal cells that are shed from the placenta or fetus into maternal peripheral blood during the pregnancy.
Copy number variations (CNVs): chromosomal imbalances resulting from the deletion and/or duplication of one or more sections of DNA.
Fluorescence *in situ* hybridization (FISH): laboratory technique that binds chromosome-specific fluorescently labelled probes to target DNA.
Invasive prenatal testing: fetal cells that are collected through an invasive means (directly obtained from the placenta or the amniotic fluid surrounding the fetus) are used to detect genetic disorders.
Karyotype: number and appearance of chromosomes in the nucleus of a eukaryotic cell.
Next-generation sequencing (NGS): state-of-the-art high-throughput technology in which the whole genome can be sequenced simultaneously through short sequencing reads.

Box 1. Genomic Investigation Techniques in Prenatal Testing

CMA has become a foundation of not only prenatal diagnosis but also the standard genetic diagnostic approach for adults as well as children who suffer from multiple congenital malformations, genetic syndromes, and developmental and intellectual disabilities [65]. The American College of Obstetricians and Gynecologists (ACOG) has suggested CMA as a first-tier method for detection of fetal abnormalities [66]. Due to the perceived risk of miscarriage associated with invasive sampling, these procedures are generally only performed on high-risk pregnancies. Therefore, the need for invasively obtained fetal cells for CMA reduces its use in low-to-medium risk pregnancies. As a result, array-based testing and other methods requiring invasively obtained fetal cells are generally only used in high-risk pregnancies.

Irrespective of the origin of the fetal DNA to be tested, there is still debate over which modern genetic technology (array or sequencing) should be used as standard for DNA testing (see Figure 1 in main text). Each method has advantages and disadvantages that make it suitable for use in different settings and with different clinical diagnostic expectations. Targeted sequencing approaches are currently being developed to facilitate clinical implementation of sequencing based testing. Targeted sequencing is aimed at identifying the most common disorders using panels of primers focused on sequencing only genes of interest, and can also be applied in the NIPT setting to target chromosomes 21, 18, and 13 [67]. Advantages and disadvantages of genome and targeted sequencing for cell free fetal DNA was discussed by Benn *et al.* [68].

Non-invasive prenatal testing (NIPT): prenatal test on maternal blood to screen pregnancies for the most common fetal chromosome abnormalities, as well as gender determination.
Placental mosaicism: discrepancy between the chromosomal number within placental cells and the cells in the fetus.
Positive predictive values (PPV): probability that a positive screening test is truly positive and the patient has the disease.
Single nucleotide polymorphism (SNP): variation in a single nucleotide occurring at a specific position in the DNA.
Whole exome sequencing (WES): high-throughput technique for sequencing all of the protein-coding genes in a genome (the exome).
Whole genome amplification (WGA): laboratory technique to increase the limited amount of DNA obtained from a cell or cells with either nanogram or picogram quantities into microgram quantities for subsequent molecular analysis.
Whole genome sequencing (WGS): high-throughput technique for sequencing the whole DNA sequence of the genome at a single time.

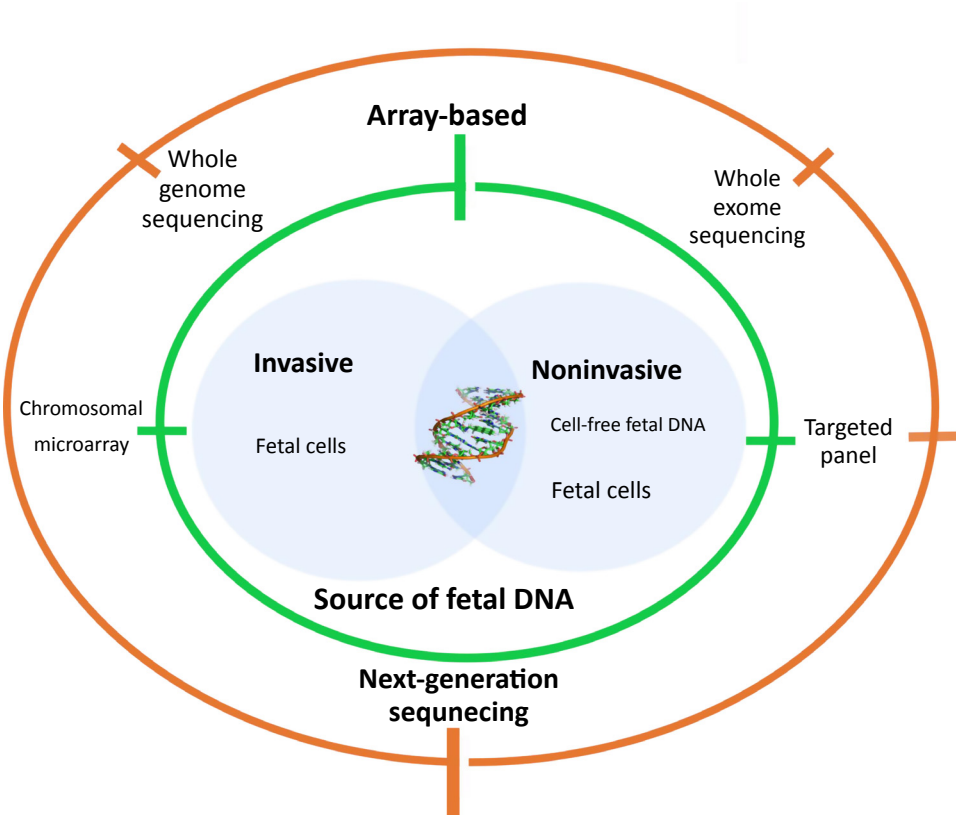

Figure 1. Source of Fetal DNA and the Two Main Types of Advanced Genetic Analysis That Can Be Applied. Sources of fetal DNA include fetal cells obtained from invasive procedures such as amniocentesis or chorionic villus sampling and DNA obtained noninvasively from either cell-free fetal DNA or from circulating fetal cells in maternal blood. The two main types of advanced genomic analysis that can be performed include array-based techniques and the more comprehensive next-generation sequencing approaches.

reduction in costs, cffDNA-NIPT will continue to play an important role in modern prenatal screening and, in turn, the global NIPT market.

CFCs

The presence of fetal cells in maternal blood was detected over a century ago [13,14]. The possibility to isolate intact fetal cells from the maternal circulation has long been recognized as an alternative to invasive procedures to obtain fetal genomic DNA for prenatal diagnosis. However, CFCs are rare in healthy pregnancies, making their implementation in genetic testing a challenge, as an ultrahigh level of enrichment is required to isolate these cells with the level of purity required for most diagnostic assays. From a purely genomic standpoint, CFCs provide a pure whole fetal genome, which is a significant advantage over cffDNA NIPT (Figure 2).

The most suitable CFCs for testing purposes in maternal blood include nucleated red blood cells (nRBCs) and trophoblastic cells. nRBCs possess the advantage of being directly derived from the fetus and have a short lifespan in the maternal circulation. Conversely, circulating fetal trophoblastic cells of placental origin have dimensions significantly larger than blood cells, which allows targeted separation based on their physical features. Syncytial nuclear aggregates (SNAs) have also been consistently observed in maternal blood. SNAs are large fragments of the outer layer of the placenta, the syncytiotrophoblast, which is formed by the fusion of progenitor cytotrophoblasts into a continuous cell layer and these could also provide a source of fetal genetic DNA using size-based isolation. However, trophoblastic fetal cells are rare in the maternal blood (1–5 trophoblasts per milliliter) [46].

Technological Advances in Rare Cell Isolation from Blood

Due to the rarity of fetal cells in maternal blood, ultraefficient enrichment technologies are required to obtain the level of purity required for most genomic assays. A number of standard rare cell isolation approaches have been tested clinically for CFCs, including density gradient centrifugation, fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), magnetophoresis, and [16]. A major challenge is the absence of highly specific markers to identify CFCs. Markers commonly used to positively isolate fetal nRBCs are not unique to this cell type (e.g., CD71 and glycophorin A). A large trial based on the most common

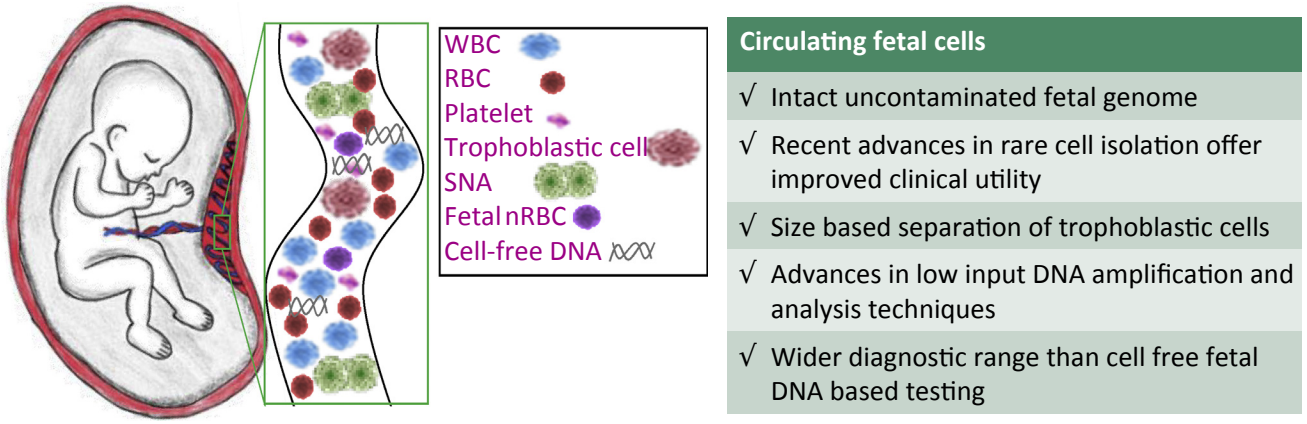

Figure 2. Advantages of Circulating Fetal Cells in Noninvasive Prenatal Testing. This includes a schematic drawing of the types of fetal material that enters into the maternal blood stream including circulating cell-free fetal DNA, fetal nucleated RBCs, trophoblastic cells including SNAs and cytotrophoblasts, as well as maternal WBCs and RBCs. Abbreviations: RBC, red blood cell; nRBC, nucleated RBC; SNA, syncytial nuclear aggregate; WBC, white blood cell.

nRBC isolation methods provided a suboptimal sensitivity of 41.4% for the detection of male fetal cells and a false-positive rate of 11.1% [47]. Similarly, there is not a single marker available for the detection/isolation of circulating trophoblastic cells [48,49]. However, the large size of trophoblastic cells, and to an even greater extent SNAs, compared to normal blood cells provides an attractive mechanism for their enrichment, and this has been exploited with some success by the isolation by size of epithelial tumor/trophoblast (ISET) filtration system. Extravillous trophoblast cells isolated using ISET were larger than 15 μm and detected as early as 5 weeks gestation and provided 100% diagnostic sensitivity and specificity for detection of cystic fibrosis or spinal muscular atrophy in a pilot study [46]. The size difference was also used in a number of studies to enrich fetal trophoblastic cells, including density gradient methods [15,16]. For example, RareCyte combined density gradient centrifugation enrichment with the RosetteSep white blood cell (WBC) depletion technique was able to recover 0.34 trophoblasts per milliliter of blood [15]. While the RosetteSep method increases the throughput, the concomitant decrease in number of recovered trophoblasts (0.74/ml without WBC depletion) demonstrates the trade-off between purity and yield that often needs to be made with rare cell isolation. A number of other approaches have also been investigated [50–52] but to date, none has provided the level of cost-effectiveness and reliability required for clinical implementation.

A wealth of technologies and methods have been developed in recent years for the isolation of circulating tumor cells (CTCs) from the peripheral blood of cancer patients via the integration of cell separation techniques with microfluidic technologies. A number of these approaches have received a CE mark towards clinical use, including VTX-1 (Vortex Bioscience) [53] and ClearCell FX1 (Clearbridge Biomedics) [54]. Since the isolation of CTCs presents similar challenges to that of CFCs, there is a significant scope to apply these approaches for the isolation of fetal cells from maternal blood. For example, nRBCs could be enriched from maternal blood by integrating microfluidic deterministic lateral displacement with magnetic separation. This approach capitalized on the intrinsic magnetic characteristics of nRBCs and could effectively eliminate ~99.99% of RBCs in the first step and deplete WBCs with an efficiency of 99.99% during hemoglobin enrichment [55]. More recently a microfluidic chip, Frequency-Enhanced Transferrin receptor Antibody-Labelled (FETAL-Chip), has been used for the enrichment and identification of circulating nRBCs from maternal blood with high enrichment efficiency (>90%) and WBCs depletion (99.9%) [56]. Advanced microfluidic technologies have also been successfully used recently for the enrichment of trophoblasts from maternal blood (Figure 3). The Nano-Velcro microchips could enrich 3–25 fetal trophoblasts (Figure 3A) from 10 ml of blood in ~2 h with a device functionalized with anti-epithelial cell adhesion molecule [16]. Following separation, array- comparative genomic hybridization (CGH) was successfully performed on single fetal cells isolated after laser capture microdissection. Inertial microfluidics could also be successfully used to enrich trophoblastic cells and SNAs, recovering 79% of trophoblastic cells in a spiking model with a WBC depletion of 99.5% and recovery of six CFCs from 7 ml maternal blood from a pregnancy with confirmed fetal trisomy 21 [57].

Importantly, the downstream analysis and requirements of CFC-based prenatal testing are different to those of CTCs. In most cases, enrichment only (i.e., decrease in the number of contaminating cells) is adequate for analysis of CTCs but due to the inherent nature of prenatal testing, pure fetal cells are typically required. However, for CFCs, high purity (free from maternal contamination) is required for sequencing and essential for array technologies in order to obtain useful and relevant genomic results (particularly when detecting autosomal recessive conditions) [58]. Considering the difficulty in removing all maternal WBCs from a blood sample, single fetal cells are usually isolated with an additional step such as laser capture microdissection after fetal cell identification. This remains a significant obstacle to the development of

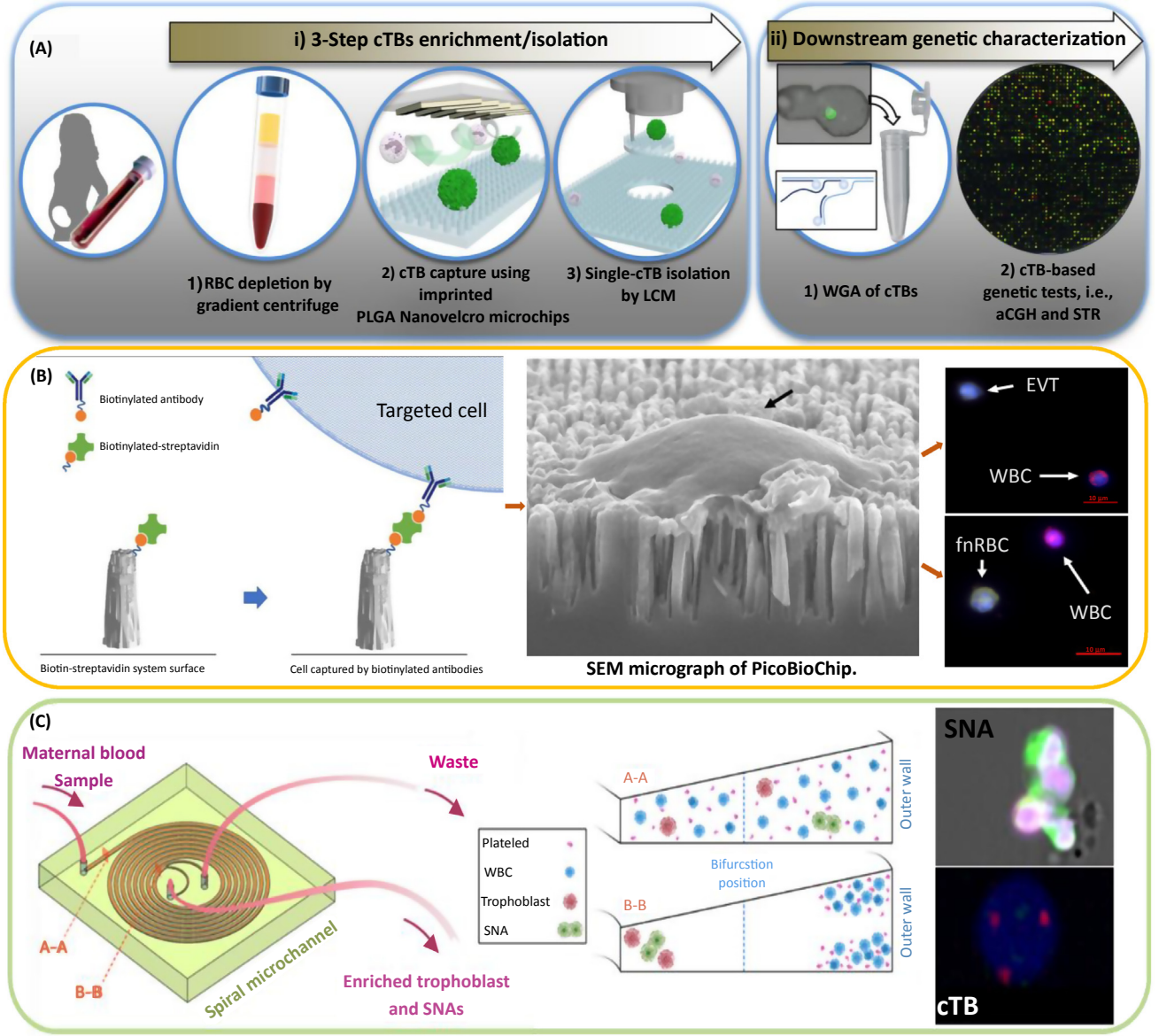

Figure 3. Three Examples of the Recent Advanced Microfluidic Technologies Used for the Detection of Circulating Fetal Cells. (A) NanoVelro cTB microfluidic workflow. (i1) RBC depletion by gradient centrifugation. (i2) Affinity capture of cTBs onto NanoVelcro microchips in the presence of anti-EpCAM capture agent. (i3) cTB isolation by LCM. For downstream genetic characterization, (ii1) three individually isolated cTBs were pooled for WGA. The resulting amplified DNA was then subjected to (ii2) aCGH and STR assay. Image adapted, with permission, from [16]. (B) Isolation of fetal cells by using silicon-based nanostructured microfluidics platform named Cell Reveal. Using immunoaffinity (antibodies) to capture the trophoblasts and the nRBCs and identifying the targeted cells through additional immunostaining of the corresponding antigens. Then the identified cells were retrieved for downstream genetic analysis. Image adapted, with permission, from [72]. (C) Inertial microfluidic schematic for enrichment of circulating fetal cells. Maternal blood was lysed before running with a trapezoidal inertial microfluidic device that enriches the larger fetal cells including cytokeratin positive SNAs and cTB (detected with fluorescence *in situ* hybridization). Image adapted, with permission, from [57]. Abbreviations: aCGH, array comparative genomic hybridization; cTB, circulating trophoblastic cell; EVT, extravillous trophoblast; LCM, laser capture microdissection; fnRBC, fetal nucleated RBC; RBC, red blood cell; SEM, Scanning electron microscopy; PLGA, poly lactic-co-glycolic acid; SNA, syncytial nuclear aggregate; STR, short tandem repeat; WBC, white blood cell; WGA, whole genome analysis.

clinically applicable cell-based-NIPT, considering the time- and resource-intensive nature of current single cell selection and manipulation procedures, as well as the added requirement for amplification that is not required for cffDNA-NIPT. Moreover, accurate fetal cell identification is essential but challenging in itself. In terms of trophoblastic cells that have varied expression of epithelial and mesenchymal markers, various cocktails of antibodies are typically used for their detection with microscopy, although the ability to detect all trophoblastic cells remains unknown [48,49].

CFC-Based Prenatal Diagnostics: an Elusive Goal or the Final Prenatal Diagnostic Frontier

The exact number of CFCs in the maternal bloodstream remains unknown. For example, there are potentially upwards of two or three fetal trophoblastic cells per milliliter [16]. Nonetheless, the rarity of CFCs severely limits the quantity of genetic material available for testing. However, state-of-the-art, high-resolution genetic analysis techniques, including microarray and next generation sequencing, are compatible with low amounts of fetal genomic materials when combined with modern targeted and/or **whole genome amplification** (WGA) methods and bioinformatics [59]. Consequently, these approaches not only improve the diagnostic sensitivity when combined with standard invasive prenatal procedures but also provide powerful platforms to build upon for the development of practical and high coverage cell-based NIPT (Box 2).

The cell-based-NIPT field is, therefore, at the convergence of significant advances in both enrichment technologies, genomics, and bioinformatics, and a number of initiatives worldwide are revisiting the potential clinical relevance of cell-based NIPT. A small number of proof-of-concept studies published since 2016 has confirmed the feasibility of performing genetic testing on circulating trophoblastic fetal cells isolated from maternal blood with whole genome amplification (Table 1). For instance, CFCs have been used for CMA/array CGH and single cell NGS to analyze the DNA of fetal cells using RareCyte technology [15]. Moreover, another recent study has demonstrated array-CGH and NGS with the ARCEDI Biotech technology with copy number differences of whole chromosome and subchromosomal aberrations after WGA [17]. Fetal cells have also been isolated with MACS and picked using CellCelector (ALS Automated Lab Solutions) and have then undergone WGA with array-CGH and successfully demonstrated

Box 2. Advanced Single Cell Genomic Analysis

Advances in single cell genome amplification and testing promise to resolve the issues associated with the inherent difficulties of analyzing only ~6 pg of DNA contained in a single cell [69]. With preimplantation testing of embryos for *in vitro* fertilization (IVF) in mind, many techniques have been developed for low input DNA amplification. However, genome amplification kits and techniques have different depth of coverage, allelic dropout, and variability in fidelity for **single nucleotide polymorphism** (SNP) calls [59]. In turn, there are difficulties in applying a specific whole genome amplification (WGA) method to the range of downstream genomic techniques that may be used, as amplification needs to be optimized for each technique (e.g., final amplified product results in different fragment lengths). It is also important to note that with increasing depth of sequencing, the number of false-positive calls generated during WGA and sequencing will increase for single/low cell numbers. Moreover, the need for WGA in single and low cell numbers may introduce potentially significant issues including allele dropout and biased amplification. However, the sensitivity and specificity of genomic diagnostic techniques may improve when multiple isolated fetal cells are pooled. It is therefore essential that test limitations are well understood and explained to patients by genetic counselors and that the accuracy of the fetal gene map must be taken into consideration before result interpretation [37].

Advanced amplification approaches based on microfluidics have strong potential to improve amplification. For example, single droplet multiple displacement amplification (MDA) within microfluidics enabled massively parallel amplification of single cell genomes, maintaining sequence specificity and coverage with improved sequence quality [70]. Nanoliter-volume single-cell MDA generated through microfluidics has also been demonstrated with 80% coverage of single genomes and 5 × sequencing depth for single nucleotide variant detection with targeted sequence and detection of copy number variants as small as 30 kb [71].

Table 1. Isolation of CFCs from Maternal Blood Advanced with Genetic Analysis[a]

Study	No of patients	Enrichment methods	Isolation methods	Analysis methods	Counts (cells/ml)	Diagnostic outcomes	Refs
Breman (2016)	37	AccuCyte Blood Fractionation System	CytePicker	STR/Array CGH/ NGS	0.36–0.74	Trisomy 13,18,21/ XXY/Chr 15 del (2.7 mb)	[15]
Kølvraa (2016)	111	MACS	CellCellector/ CytePicker	Array CGH/NGS	0.42	Trisomy 21/45X/ 46,X,r(X)	[17]
Vestergaard (2017)	5	MACS	CellCellector	Array CGH	0.42	Trisomy 21/13/2/ subchromosomal aberration	[60]
Chen (2017)	14	Double negative selection (DNS)	Manually picking	STR analysis/NGS	Not reported	Disease-associated variants	[61]
Hou (2017)	6	NanoVelcro Microchips	Laser capture microdissection	Array CGH	2–3	Trisomy 21/13/18 XXXXY/Del (9p)Dup (14q)	[16]
Huang (2017)	5	Nanostructured microfluidics (Cell Reveal)	Micromanipulator	STR/Array CGH/ NGS	2.38–7.25 (fnRBC + EVT)	Trisomy 13/18/21	[72]

[a]Abbreviations: CGH, comparative genomic hybridization, Chr, chromosome; Del, deletion, Dup, duplication; EVT, extravillous cytotrophoblast; fnRBC, fetal nucleated red blood cells; MACS, magnetic-activated cell sorting; NGS, next-generation sequencing; STR, short tandem repeat.

aneuploidy, unbalanced translocations, and subchromosomal deletions and duplications [60]. Lastly, for the first time a study has demonstrated combined fetal cell isolation (with double negative selection) and deep sequencing (towards screening for monogenic disorders) with WGS at high coverage (86.8%) and **allelic dropout** rate of 24.90% [61]. Trophoblastic cells have also been recovered from the endocervical canal and cervix (using trophoblast retrieval and isolation from the cervix; TRIC) [62]. Fetal cells isolated using the TRIC approach were successfully sequenced using the Illumina ForenSeq platform, targeting 59 short tandem repeats and 94 single nucleotide variants across all 24 chromosomes with 100% correct fetal haplotyping [63]. Another study also demonstrated the feasibility of using syncytiotrophoblasts isolated from the cervical mucus for prenatal diagnosis [64]. However, the reliance on endocervical sampling is a significant limitation when compared with the acceptance of providing peripheral blood samples in pregnancy.

All of the aforementioned studies had a small sample size and require larger clinical validation to help determine sensitivity, specificity, cost-effectiveness, and reliability, particularly in the detection of conditions with low incidence. However, these reports validate the concept that if whole CFCs can be isolated, prenatal diagnosis can be achieved with a number of advantages compared to cffDNA-NIPT. A fetal cell contains a whole pure genome, in contrast to highly diluted short fragments of cffDNA that are the current fetal genomic source for NIPT. Single-cell DNA sequencing is more cost-effective at identifying small CNVs compared with deep sequencing of cffDNA [12]. Moreover, WGS/WES on single fetal cells is possible using simple protocols, with the prospect of allowing the reliable identification of the full range of chromosomal abnormalities and single gene disorders, which account for over 6000 known diseases. While fetal-cell-based NIPT may take longer to develop, optimize, and implement clinically than cffDNA-NIPT has, it has a strong potential to improve diagnostic scope especially for single gene disorders.

One potential drawback of cell-based NIPT based on trophoblastic cells in maternal circulation (derived from the placenta) is the issue of placental mosaicism. While it has been demonstrated

in a small study that analysis of 3–5 fetal cells overcame discordant results due to placental mosaicism [15], this is unlikely to be the case for all samples considering the nature of mosaicism and larger studies are required to investigate this point (see Outstanding Questions). Moreover, placental mosaicism is primarily an issue for chromosomal abnormalities and *de novo* mutations but not inherited monogenic disorders. Another issue associated with cell-based NIPT warranting further investigation is the potential for false-positive CNVs, leading to unnecessary invasive procedures (as discussed by Breman *et al*. [15]). However, this drawback is not unique to cell-based NIPT since the source of cffDNA is also predominately placental, and therefore, also at risk of false positives due to placental mosaicism.

Roadmap to the Development and Implementation of Cell-Based NIPT

Comprehensive genetic testing of CFCs, using further advances in cell isolation technology and low input DNA genomic methods, could allow for the noninvasive prenatal detection of the full spectrum of rare but serious mutations associated with severe physical and intellectual disabilities. For example, *de novo* intragenic mutations are five times more frequent than trisomy 21, affecting as many as one in 200 pregnancies, and result in potentially severe conditions such as epilepsy and intellectual disability. In the not-too-distant future, sequencing of CFCs could enable the noninvasive detection of these single gene mutations.

Significant ethical issues would inevitably arise from clinical implementation of cell-based NIPT, particularly broad approach exome/genome analysis rather than targeted mutation or gene panel screening, as identification of variants of uncertain significance (VUSs) and incidental findings such as predispositions to adult onset conditions could have serious implications not only for the child but also immediate and extended family members. The provision of genetic counseling will, therefore, be critical to ensure informed decision-making and consent is possible. To this end, a thorough understanding of the performance and technical limitations of such a test will be required. Careful decision-making regarding the most appropriate approach will be required to balance the effectiveness of the test performed, against the potential for harm related to increased anxiety due to VUSs and incidental findings, particularly if the technology is ultimately extended to universal screening of all pregnancies.

Finally, if the isolation of CFCs can provide the genetic 'holy grail' for NIPT, allowing an increased screening capacity in a noninvasive and cost-effective fashion; for what maternal population would this test be most useful and for which conditions should it be used to screen? Hui and Bianchi recently discussed different screening models based on cffDNA-NIPT [30], and this discussion would obviously need to be expanded for cell-based testing. In the short term and providing sufficient validation, cell-based NIPT for single gene disorders could follow the same indications as recommended for invasive prenatal testing for women identified as high risk of having an affected fetus, that is, women known to be at increased risk based on family history, carrier testing, or detection of fetal abnormalities suggestive of a specific monogenic condition. This would likely improve the diagnosis of fetal genetic disorders without the risk of compromising the pregnancy through an invasive procedure. In the longer term, and pending confirmation in large-scale studies that diagnostic accuracy can reliably be achieved through a simple blood test, cell-based NIPT could be offered to all pregnancies, including low risk ones (Figure 4, Key Figure).

Concluding Remarks

Is it time for a reappraisal of cell-based NIPT? At this stage, current methods would be difficult to implement routinely in the clinical setting. However, although further research and development are still needed to develop reliable, integrated, and cost-effective CFC approaches, we

Outstanding Questions

Can CFCs be reliably and cost-effectively isolated from healthy pregnancies for genomic noninvasive testing purposes?

For what maternal population would cell-based NIPT be most useful and for which conditions should it be used to test?

Which genomic technology should be implemented (array or sequencing, whole exome, whole genome, targeted) to provide comprehensive and accurate testing?

Especially considering the use of WGS, including VUSs and incidental findings, how do we ensure that patients undergoing NIPT (particularly if implemented as a population screening) receive adequate levels of genetic counseling?

Key Figure

Cell-based NIPT and Potential Clinical Situations

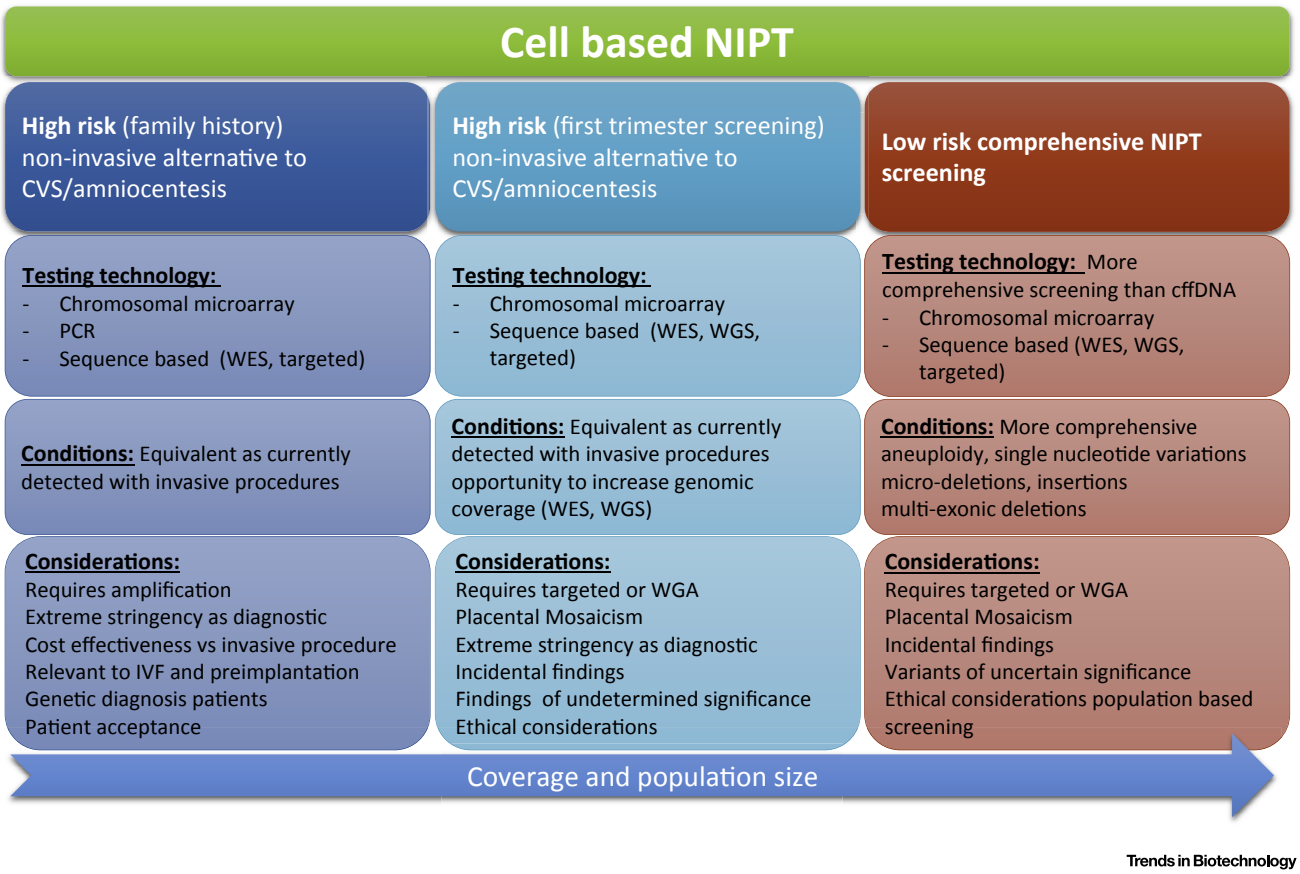

Figure 4. For a Figure360 author presentation of Figure 4, see the figure legend at https://doi.org/10.1016/j.tibtech.2018.11.001.
Abbreviations: cffDNA, cell-free fetal DNA; CVS, chorionic villus sampling; IVF, *in vitro* fertilization; NIPT, noninvasive prenatal testing; WES, whole exome sequencing; WGA, whole genome amplification; WGS, whole genome sequencing.

argue that it is no longer a question of if CFC-based testing will be implemented clinically but when and in which patient cohorts (see Outstanding Questions and Figure 360). This will be driven by the ever-growing and legitimate desire for reassurance of a normal pregnancy from prospective parents. In addition, owing to the substantial market associated with the field of prenatal testing (even independent of the clinical need), there is considerable interest from the commercial market to offer new techniques that can give patients a broad range of information about their unborn fetus. Acknowledging this consumer- and market-driven pull, as well as the rapid technological advances in the field, it will be important to make sure that there exist adequate guidelines, education, and an understanding of the sector prior to the implementation of new, more comprehensive prenatal testing technologies. It is also essential to ensure patients are effectively counseled and the ramifications of a positive result explained, especially considering the potential for incidental findings.

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