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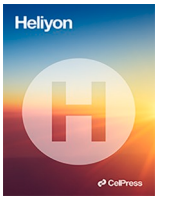

Review article

Reappraisal of evolving methods in non-invasive prenatal screening: Discovery, biology and clinical utility

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ABSTRACT

Non-invasive prenatal screening (NIPS) offers an opportunity to screen or determine features associated with the fetus. Earlier, prenatal testing was done with cytogenetic procedures like karyotyping or fluorescence in-situ hybridization, which necessitated invasive methods such as fetal blood sampling, chorionic villus sampling or amniocentesis. Over the last two decades, there has been a paradigm shift away from invasive prenatal diagnostic methods to non-invasive ones. NIPS tests heavily rely on cell-free fetal DNA (cffDNA). This DNA is released into the maternal circulation by placenta. Like cffDNA, fetal cells such as nucleated red blood cells, placental trophoblasts, leukocytes, and exosomes or fetal RNA circulating in maternal plasma, have enormous potential in non-invasive prenatal testing, but their use is still limited due to a number of limitations. Non-invasive approaches currently use circulating fetal DNA to assess the fetal genetic milieu. Methods with an acceptable detection rate and specificity such as sequencing, methylation, or PCR, have recently gained popularity in NIPS. Now that NIPS has established clinical significance in prenatal screening and diagnosis, it is critical to gain insights into and comprehend the genesis of NIPS de novo. The current review reappraises the development and emergence of non-invasive prenatal screen/test approaches, as well as their clinical application, with a focus, on the scope, benefits, and limitations.

1. Introduction

Pregnancy is a biological process accompanied by numerous physiological and biochemical changes. These changes are visible in both the fetus and the expecting mothers as the pregnancy progresses. During pregnancy, for example, placenta shedding generates certain cells that enter the maternal circulation via feto-maternal trafficking [Fig. 1] and serve as a source of fetal entities in maternal circulation [1]. Nucleated cells, free nucleic acids, and serum proteins are examples of these entities [2]. They are isolated from the maternal circulation and used in prenatal screening using genomic, proteomic or conventional analytical methods. Non-invasive prenatal screening (NIPS) is one such clinically available screening method that interrogates cell-free DNA or fetal cells in the maternal circulation to determine features like fetal gender, chromosomal abnormalities, Rhesus-D (RhD) status and other characteristics [3].

Prior to the development of non-invasive procedures, prenatal diagnosis was accomplished through invasive methods such as

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amniocentesis, fetal blood sampling (FBS), or chorionic villus sampling (CVS). Because they are explicitly dependent on fetal tissue samples, these procedures have a safety concern for the fetus [4,5]. To overcome the limitation of fetal harm perplexed by these invasive procedures, modem prenatal medicine is constantly working to develop safe, reliable and affordable prenatal diagnostic test with minimal or no risk. Non-invasive approaches have significantly reduced the use of invasive procedures because they use fetal material such as free nucleic acids or cells present in the maternal circulation, without causing harm to fetal development.

In recent years, significant data on the use of NIPS has been published. Extensive data in the form of reviews depicting the importance of NIPS in clinical practice is available; however, much of it is either scattered or single-topic oriented. In this review, we attempt to provide a comprehensive and cumulative picture of the origins of NIPS, its methods, and its application in fetal diagnosis. The review also discusses modern techniques, their limitations, and their benefits. The goal of this review is to lay the groundwork for future validation of the NIPS's diagnostic role. Furthermore, the scope of this review may be useful to policymakers or clinicians seeking a more comprehensive understanding of this expanding field.

2. Expanding scope of non-invasive prenatal screening

Earlier prenatal genetic testing was dominated by purely looking at and observing the chromosomes, a method widely known as karyotyping [6]. Since the introduction of newer techniques such as amniotic fluid cell culture, chromosome banding, fluorescence in-situ hybridization (FISH) or arrays aimed at assessing chromosomal or developmental delay in the fetus, karyotyping and its associated technique(s) expanded the scope of data interpretation and visualization. These techniques provided additional information with which to predict the overall genetic condition of the developing fetus [7–10]. Karyotyping and FISH have become the low-cost gold standard in prenatal screening of numerous chromosomal anomalies [11,12]. However, karyotyping and FISH exhibit certain limitations such as the need of a skilled analysts like cytogeneticists or clinical specialists who can obtain a tissue sample safely without harming the fetus. Due to this limitation, there has been an ongoing need for DNA-based molecular genetics techniques that could use maternal circulation fetal entities as fair and feasible molecules for NIPS. Because of advances in molecular biology, the last three decades have produced a slew of modern genomic procedures that have significantly aided in the evolution of the prenatal genetic screening arena (Fig. 2).

2.1. The emergence of cell-free fetal DNA: beginning of a new era in NIPS

Cell-free fetal DNA (cffDNA) was discovered in 1997 [13]. This discovery was reported in the Lancet as an early report, and it is from this point that the use of cffDNA in prenatal screening became certain and feasible. Lo et al. [14] later discovered discrete packets of DNA in maternal plasma that vanish from the maternal circulation within hours of delivery [14]. Following this discovery, free fetal DNA became a new research focus of investigators.

cffDNA is a small-sized fragmented DNA (<200 bp) of placental origin that accounts for 3–20% of maternal circulation DNA [15–19]. The amount of free fetal DNA varies throughout the pregnancy [2]. The amount of cffDNA increases at a rate of about 0.1% per week during the first trimester and augments to about 1% per week in the second trimester onwards [20,21]. Apart from maternal age, factors like maternal weight, fetal anomalies, maternal morbidity, maternal body mass index, multiple pregnancies and the

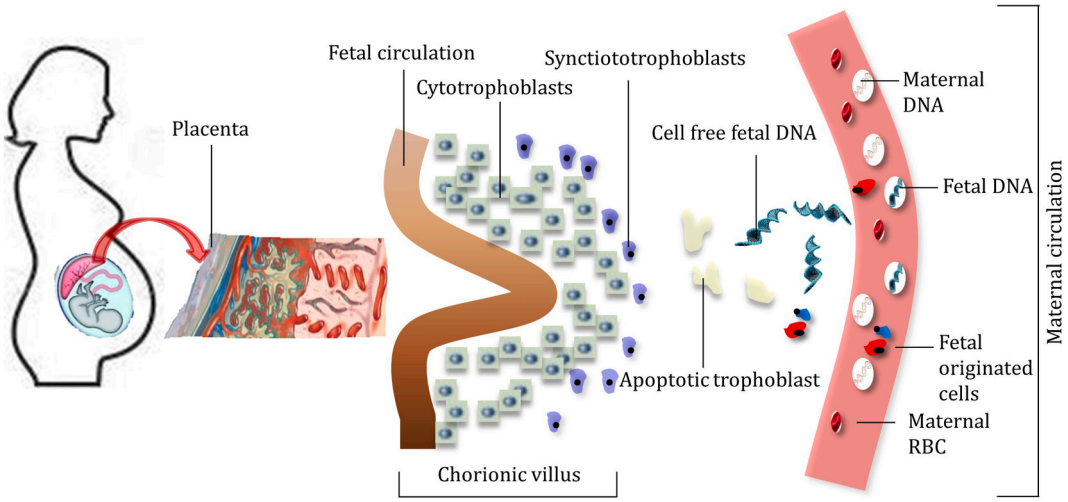

Fig. 1. The genesis of cell-free fetal DNA. During embryonic development, the placenta continuously sheds trophoblasts, some of which later transform into apoptotic trophoblasts. The fetal originated cells, free fetal DNA enters into the maternal circulation. The sprout of fetal cells like nucleated red blood cells or leukocytes also enter into maternal circulation and can be easily isolated from the peripheral blood samples. The fetal cells and DNA serve a good candidate for non-invasive prenatal screening. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

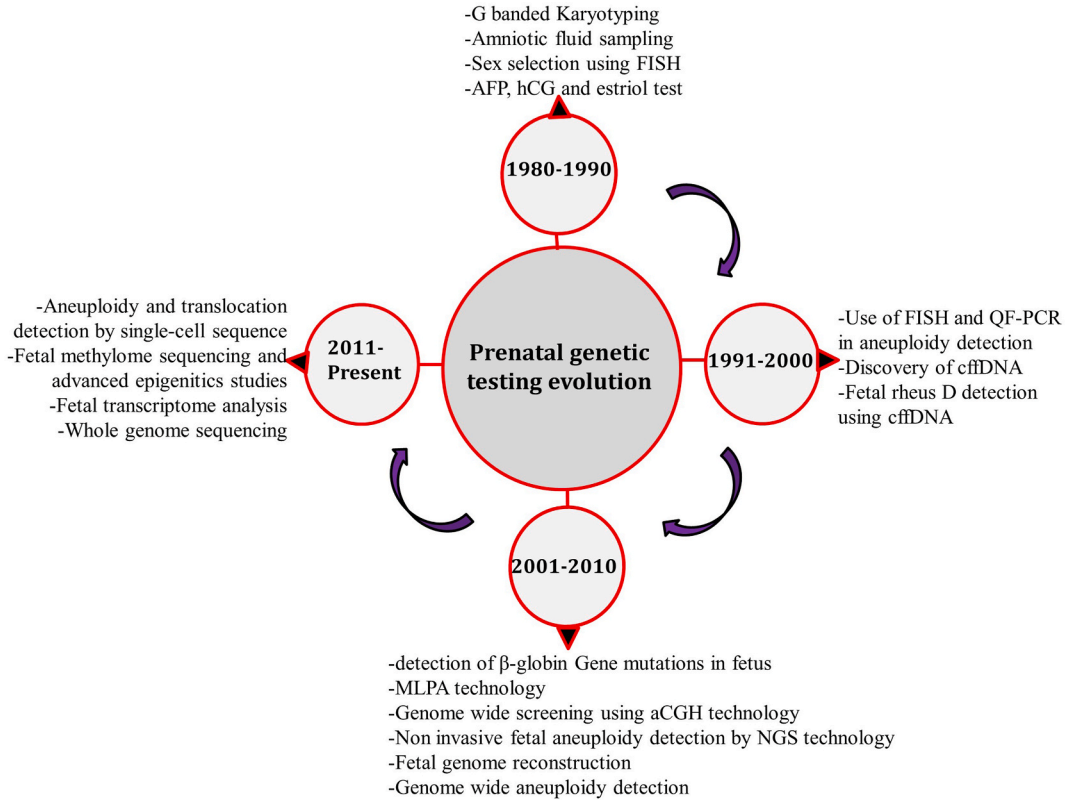

Fig. 2. Key milestones in prenatal diagnosis with associated invasive and non-invasive methods. FISH, fluorescence in-situ hybridization; QF-PCR, quantitative fluorescence Polymerase Chain Reaction; cffDNA, cell-free fetal DNA; AFP, Alpha-fetoprotein; hCG, Human chorionic gonadotropin; MLPA, multiplex ligation-dependent probe amplification; aCGH, array comparative genomic hybridization; NGS, next-generation sequencing.

number of viable fetuses present, influence the amount of cffDNA in maternal circulation [22–25]. Until now, cffDNA analysis has been used to determine fetal gender [26], genotype of fetal RhD blood group [27], and detect monogenic disorders and aneuploidy [28].

3. Detection strategies in NIPS

3.1. Cell-based detection methods

Prior to the discovery of circulating fetal DNA, NIPS was achieved by isolating and exploring fetal cells from the maternal circulation. Maternal peripheral blood contains a variety of fetal-originated cells including leukocytes, nucleated red blood cells (nRBCs) and placental trophoblasts [29]. The optimal and efficient protocol for selecting fetal cells while restricting the number of maternal cells has yet to be developed. Because of the lack of fetal-specific markers and a lower number of fetal cells, existing enrichment and isolation protocols are impacted, making the use of intact fetal cells in NIPS difficult. Though the use of advanced technology has helped to improve the use of fetal cells in prenatal screening, there are still limitations associated with fetal cell isolation, enrichment and reproducibility of the results (Table 1). Leukocytes, for example, are carried from previous to subsequent pregnancies, complicating their evaluation in the following pregnancy [30]. Furthermore, leukocytes lack distinguishing cell markers that could differentiate fetal leukocytes from maternal ones [31].

nRBC is the most focused fetal cell type used in NIPS. These are the most common types of investigative cells found in maternal circulation. These cells have a short life span (half-life less than 35 days) and are therefore unlikely to persist from preceding pregnancies or carry on to subsequent pregnancies [32]. Furthermore, they have a distinguishing morphology with restricted proliferative ability, making them unlikely to persist across pregnancies [33]. It is presumed that nRBCs are uniquely of fetal origin however, studies have demonstrated that much of the nRBCs during pregnancy are of maternal origin [34,35].

Trophoblasts are the first placental originated cells that cross into the maternal circulation. These cells have been isolated from the uterine vein, cervix and maternal lungs [36,37]. According to reports, trophoblast migration into the maternal circulation is not a common phenomenon in all pregnancies. When this happens, the maternal pulmonary circulation quickly clears the cells. Furthermore, the major challenge in using trophoblast cells for NIPS is their isolation and enrichment, which is significantly affected by a lack of placenta specific antibodies as well as the presence of multinucleated morphology [38]. The application of stem cells and haemopoietic progenitors in NIPS is still emerging. Although the culturing of mononuclear cells has previously been demonstrated, their

Table 1
Benefits and limitations of different NIPS approaches.

Approach		Benefits*	Limitations or challenges*	Type of fetal condition detected*	Reference	Implementation^
Cell-based approach (targeting cells like nRBCs, Trophoblasts, lymphocytes or Stem/Progenitor cells, exosomes)		-Genomic material remains intact hence low fragmentation -Shorter life span, for instance in nRBC makes it a good candidate cell for fetal anomaly detection	-Relatively low abundance, few of them like fetal lymphocytes and stem/ progenitor cells persist for several years following the birth -Difficult to isolate and enrich	-Aneuploidy -CNV	[29–39]	Research/ Clinically implemented
Cell-free based approach (Targeting DNA or RNA)	NGS	-Facilitates the analysis of multiple gene(s) in a single run. -Broader mutation detection range -More accurate and sensitive	-Relatively low read depths -Requires expensive equipment and infrastructure -Costly	-Beta-thalassemia and congenital adrenal hyperplasia -Aneuploidies, monogenic disorders -Microdeletion syndromes -Skeletal dysplasias	[51–55]	Research/ Clinically implemented
	Methylation	-Requires a low-cost setup and infrastructure -Cost-effective	-Bisulfite treatment may damage the cffDNA -Owing to fragmented cffDNA, methylation of target sequences is challenging -Applied to CpG islands and promoter regions	-Edwards syndrome -Downs syndrome	[63,64]	Research
	mRNA	-Direct discrimination of fetal RNA from maternal RNA -Encapsulated in a particulate matter, which protects it from degradation	-Requires a large number of informative SNPs -Limited by mRNA stability -Placental mRNA is mostly contaminated with maternal ribosomal RNA -Costly	-Trisomies	[69,71]	Research
	PCR base approaches	-Accurate quantification of DNA molecules -Low cost	-Gene- and variant-specific -Does not apply to all mutations or to cases where the causative gene is unknown	-RhD determination -Trisomies and aneuploidy -Monogenic disorder -X-linked disorders -Fetal sex determination	[74,78,82, 86,87]	Clinically implemented
Proteomic approach		-Direct discrimination of fetal proteins	-The fetal origin proteins are marred by maternal plasma proteins, making their detection complex	-Downs syndrome	[96]	Research

*The limitations, benefits and type of fetal condition detected mentioned for each technique in the table, are limited but not exhaustive.
^'Clinically implemented' refers to implementation in the clinical setting as a routine diagnostic or screening test; 'Research' denotes that the approach is still under the clinical validation study phase, or not yet implemented or recommended as a diagnostic or screening test.
NGS, next-generation sequencing; RhD, Rhesus-D; cffDNA, cell-free fetal DNA; SNPs, single nucleotide polymorphism; nRBCs, nucleated red blood cells; CNV, copy number variation.

reproducibility and application are still being investigated [39]. The intact fetal cells in maternal blood have not yet been used productively due to inconsistent recovery, low reproducibility and limited sensitivity. To overcome these limitations, cell recovery and enrichment techniques must be improved further. Even with the limitations that currently exist, fetal cells found in maternal circulation have the potential to be used as candidate markers in prenatal diagnosis.

Exosomes are essential to pregnancy, according to recent research. Exosomes are extracellular vesicles (EVs) that carry mRNAs, microRNAs (miRNAs), proteins, and other macromolecules that regulate various signaling pathways between cells. Of exosome origin, micro RNAs are the most-sought molecules, in NIPS. Peripheral blood exosome concentration rises gradually until term during a normal pregnancy, but it rises even more rapidly in pregnancy-related complications than it does in a normal pregnancy [40]. The fetus

releases EVs together with the placenta and maternal EVs. These EVs can be found in the maternal circulation as early as six weeks after conception, and their levels increase with maternal age [41]. Maternal peripheral blood exosomal content has been used to assess numerous pregnancy-related issues including gestational diabetes, preeclampsia, premature delivery, foetal growth restriction, and congenital heart disease among others [40]. Because maternal peripheral blood of pregnant women is a concoction of exosomes from various origins, identifying fetal-specific exosomes with the limited techniques available is difficult. As a result, when studying the exosomal content of pregnancy-related disorders or fetal development disorders, the aforementioned challenge creates significant uncertainty.

3.2. Cell-free based detection

NIPS's scope is not limited to cell-based detection methods. Continued research is being conducted to highlight the biological role of cell-free nucleic acids (cfNAs). Several studies have shown that free nucleic acids can be detectable in maternal circulation as early as the first trimester of pregnancy. As the pregnancy advances, cfNAs increase proportionally and reaches a maximum at the end of the pregnancy [42]. The current cell-free based detection investigations are limited to cffDNA. The role of other free nucleic acids like non-coding RNAs, miRNAs and cell-free fetal mRNA (cffmRNA) has been partially established [43–47]. Despite the fact that free fetal RNA is present in maternal circulation, its use in NIPS clinical implementation is minimal to non-existent.

Few studies have identified potential miRNAs in the placenta that are important in placentation regulation [43]. These miRNAs are divided into three types: placenta-associated, placenta specific, and placenta-derived. A few of them are arranged in clusters on chromosomes 14 and 19 [48]. Placenta specific miRNAs (miR-195, miR-278a-5p, and miR-210) have been linked to preeclampsia [49]. Non-coding RNAs like circular non-coding RNA and long non-coding RNAs are found in maternal circulation. Few studies have been conducted to investigate their role in gestational diabetes and congenital heart disease [50]. Similarly, placental cffmRNA sequences can be found in the maternal plasm. Y chromosome-specific zinc finger protein mRNA has been detected in pregnant women carrying a male fetus [51]. Barring cffDNA, role of other free fetal nucleic acids in NIPS is next to no.

4. Genomic approach in NIPS

The genomic approach can detect even a minor change happening within the cell. So what will this massive genomic breakthrough means for NIPS? Currently, most approaches (Fig. 3) are specifically aimed at investigating free fetal circulating nucleic acids of

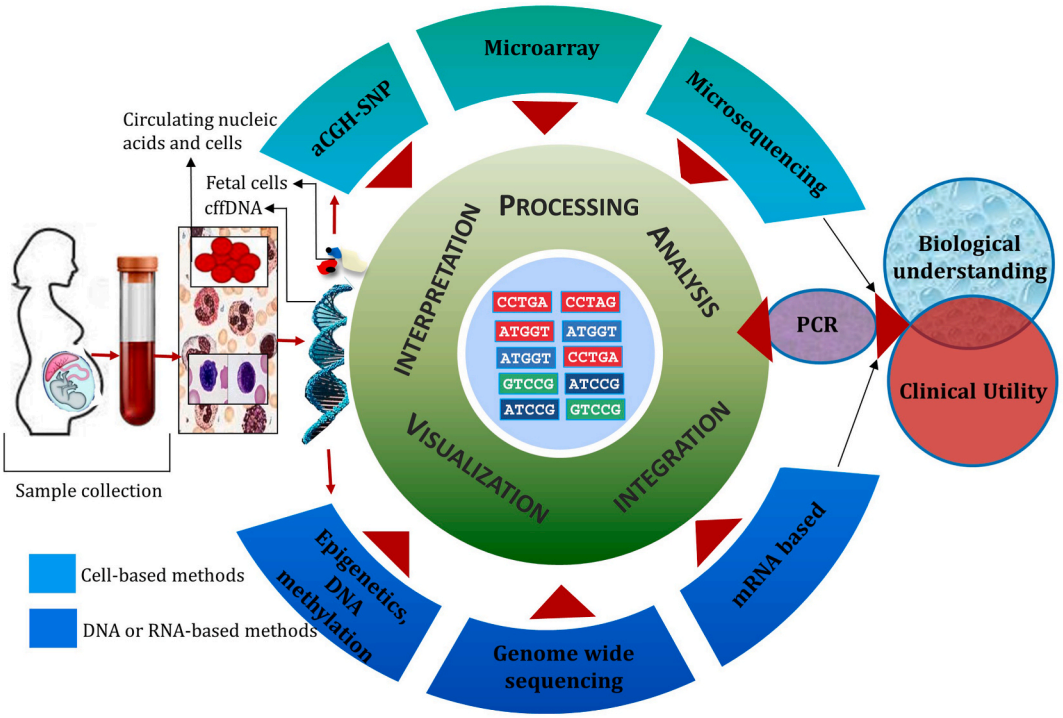

Fig. 3. Various genomic-based approaches used in prenatal screening. Maternal blood is collected from pregnant women and processed for the isolation of fetal cells or DNA. Using genomic approach study protocols, the isolated DNA or fetal cell is enriched and subjected to various gene expression studies. Data from genomic analyses are compiled and interpreted in order to discover a novel and significant clinical relevance in prenatal screening. aCGH-SNP, array comparative genomic hybridization-single nucleotide polymorphism; PCR, polymerase chain reaction; cffDNA, cell-free fetal DNA.

maternal plasma [13]. Given the difficulties encountered when working with mixed DNA molecules found in maternal blood, genomic approaches supplement and expand efforts to target the fetal fraction of maternal plasma. Modern genetic approaches may aid in understanding the genesis of fetal DNA, its origin, estimation and clinical application. Furthermore, such approaches pave the way for advances in comprehensive genome-wide analysis of genes in normal versus aberrant tissue, paving the way for efficient detection of fetal anomalies. Subsequent sections describe a few molecular genomic approaches used in NIPS, as well as their benefits and limitations. Though some of these approaches have entered routine clinical diagnosis, others are still in the development stage, and pilot-based multicenter studies are being conducted to assess their clinical efficacy and implementation (Table 1). Table 1 lists the benefits and limitations of various cell and cell-free based approaches used in NIPS.

4.1. Next-generation sequencing approach

The use of next-generation sequencing (NGS) in prenatal screening tests has broadened the testing range. The NGS approach facilitates the analysis of multiple gene(s) sequence(s) in a single run. It is now achievable to sequence the whole fetal exome or genome by using smaller or wider phenotype-specific gene panels. Earlier reports established NGS's ability to detect single base pair genomic changes with increased resolution [52]. Although fetal whole genome sequence interprets the fetus's entire genetic milieu, fetal exome sequencing (FES) has been shown to improve diagnostic yield in identifying chromosomal structural abnormalities in anomalous fetuses [52]. Nonetheless, both types aid in identification of disease variants across an exon/intron stretch.

Much literature has been published in recent years on the diagnostic efficacy, power, and utility of NGS in prenatal diagnosis. For example, two recent independent studies using relative haplotype dosage analysis to screen for beta-thalassemia and congenital adrenal hyperplasia yielded 100% true positive results [53,54]. Previously two other independent study groups demonstrated that using next-generation massively parallel shotgun sequencing (MPSS) of maternal blood, prenatal screening for aneuploidies could be accomplished [55,56]. Several validation studies have been conducted by research groups and commercial biotechnology companies to check the reproducibility of these results. For example, a study on 753 pregnancies demonstrated 100% sensitivity and 97.9% specificity in trisomy 21 (T21) detection [57]. Similarly, a biotechnology company-funded project conducted a blinded nested case-control study on 4664 pregnancies to validate their in-house laboratory developed test. The MPSS correctly tested Down syndrome in 98.6% of cases [58]. The sequencing approach has generated positive results in cases where conventional karyotyping has failed to yield correct diagnosis. For instance, a case of 16 weeks gestation was presented with ultrasound findings suggestive of skeletal dysplasia. However, a trio FES confirmed a pathogenic variant in *LEPRE1*, commonly found in osteogenesis imperfecta type VIII [59]. Apart from monogenic disorders and trisomy detection, evidence for NIPS of fetal microdeletion syndromes through sequencing has recently been explained [60]. Despite NGS's promising outlook, future improvements in accessibility and cost-effectiveness are required to expand its uses beyond trisomy detection.

4.2. DNA methylation and epigenetic approach

DNA methylation is a methyltransferase mediated chemical process in which a methyl group is added to the C5 position of cytosine dinucleotides. CpG dinucleotides (CpG islands) are distributed invariably throughout the genome and are the prime spots of DNA methylation in the human genome [61,62]. In early embryonic stage, 60% of tissue-specific differentially methylated regions are methylated however, de-methylation occurs when embryonic cells transform into adult tissues [63,64].

The primary aim of the DNA methylation-based approach is to identify the fetal-specific methylation markers that distinguish fetal DNA from the maternal DNA in maternal circulation. To locate differentially methylated regions (DMRs), three different methods, namely sodium bisulfite, restriction enzyme and methylated DNA immunoprecipitation-based methods have been investigated so far. DMRs have been mainly used for, to identify methyl-biomarkers suitable for the development of NIPS. Tong et al. reported the first attempt at NIPT screening for Edward's syndrome [65]. The authors in this study used a combination of sodium bisulfite conversion with methylated-specific PCR utilizing maternal plasma samples of normal and trisomy 18 (T18) pregnancies. Later the same group tested twenty-four euploid and five T21 maternal plasma samples using an epigenetic-genetic chromosome dosage approach, utilizing the fetal-specific hypermethylated promoter region of the *HLCS* gene locus on chromosome 21 and the *ZFY* located on chromosome Y [66]. A different approach based on methylated DNA immunoprecipitation in combination with real-time quantitative PCR was also established for the quantification of selected DMRs on chromosomes. By this method, a diagnostic formula was used to calculate the DNA methylation ratio of the selected DMRs using elected numbers of normal and T21 pregnancies [67]. A recent study looked into the use of sodium bisulfite DNA treatment in combination with NGS for NIPS. This study correctly analyzed the methylation profile of maternal plasma DNA at a single-nucleotide resolution on a genome-wide scale [68].

Decoding the methylome and understanding the fundamental mechanisms that direct epigenetic modifications, are interesting fields under investigation. Its application in understanding the underlying physiological mechanisms of cancer genesis is widely studied. Despite certain potential challenges, the application of epigenetics in NIPS is still developing. Bisulfite treatment for example, may damage the cffDNA, which is present in small amounts in maternal circulation. Furthermore, the circulating fetal DNA of maternal plasma is already fragmented, increasing the analytical challenge in sample processing.

4.3. mRNA detection approach

Fetal mRNA is present in pregnant women's maternal circulation from early gestation [51]. The majority of mRNA molecules in the maternal circulation are of placental origin [45,69]. Unlike cffDNA, placental mRNA is encapsulated in a particulate matter that

protects it from degradation [70]. This property makes it a suitable candidate for NIPS.

A panel of fetal-specific mRNA molecules that can be detected in maternal circulation and then used to investigate fetuses non-invasively, has been established using the oligonucleotide microarray method [71]. Fetal-specific mRNA of genes located on chromosome 21 has been detected in maternal plasma [72]. For example, *PLAC4* mRNA was used to develop NIPS for T21 using an RNA-SNP strategy with mass spectroscopy [73]. This method identified informative single nucleotide polymorphisms in ten T21 subjects, with diagnostic yield of 96.5% and 90% in terms of sensitivity and specificity respectively. Likewise, Tsui and colleagues used an RNA-sequencing protocol to perform serial profiling of the fetal transcriptome in maternal plasma. According to the findings of this study, the fetal transcriptome increases from 3.7% in the first trimester to 11.28% in the third trimester [74]. Later another independent research group that also found RNA transcripts in the maternal plasma, validated these results. Their sequencing and microarray analysis studies found an increase in fetal originated mRNA with increasing gestation age and suggested that these mRNA entities can be used as a screening marker for aneuploidy and other developmental defects like Angelman syndrome or congenital adrenal hyperplasia [75].

Research with fetal-originated mRNA is full of challenges. First, a major portion of the plasma RNA is ribosomal RNA rather than mRNA. Second, RNA extracted from plasma is of lower quality than RNA extracted from tissue. Third, gene expression levels have a significant impact on the amount of fetal origin RNA transcripts released into the maternal circulation. These difficulties have a significant impact on data quality. As a result, a better algorithm to mitigate these challenges is required.

4.4. PCR based approaches

The most widely used and successful method in NIPS is PCR based approaches. To date, diverse PCR approaches have been utilized in NIPS. PCR based tests are used in countries that have implemented NIPS into routine clinical practice for determining fetal RhD status [76–81]. These tests are performed using real-time PCR (rt-PCR), which targets either Y chromosome-specific or RHD-specific exons. rt-PCR produces high-quality results with acceptable sensitivity and specificity. The subsequent use of the rt-PCR approach in the detection of monogenic disorders indicated a broader application of the approach in the development of NIPS. For example, rt-PCR in combination with restriction enzyme digestion correctly identified a paternally inherited cystic fibrosis mutation [82] and Fibroblast growth factor receptor 3 related achondroplasia [83].

The extensive use of rt-PCR in NIPS has given this technique new dimensions and innovation. As a result, numerous changes and improvements to NIPS were implemented. For example, for NIPS of fetal aneuploidy, a new absolute quantification strategy, digital PCR (dPCR), has been proposed [84]. dPCR quantifies cffDNA by direct analysis of DNA extracted from maternal plasma [85]. This approach has been described by two other independent research groups that used placental DNA to calculate the amount of fetal-originated DNA present in maternal circulation [86,87]. Due to the presence of a small fraction of target DNA (fetal DNA), dPCR is yet to achieve the reliability paradigm. To overcome this limitation, droplet digital-PCR (ddPCR) has been recently developed. Studies have reported the high accuracy of ddPCR for diagnosing α- β-thalassemia and sickle cell anemia [88,89]. The use of ddPCR in screening maternally inherited sequences is well established [90] whereas its use in detecting monogenic X-linked or autosomal recessive disorders has increased the sensitivity and specificity [91]. ddPCRs approach is based on partitioning of template sample into thousands of separate reactions (droplets). Each droplet contains a distinct quantity of target DNA either zero, one, or several copies, following a Poisson distribution [87]. ddPCR is advantageous in comparison with qPCR as it does not require a calibration curve to quantify target DNA and yields quantification output with higher sensitivity. Modified PCR methods like Co-amplification at Lower Denaturation temperature PCR (COLD-PCR) for the detection of β-thalassemia [88]; 3′-Modified Oligonucleotide PCR (MEMO-PCR) for monogenic disorders [92]; Quantitative fluorescent (QF-PCR) for fetal aneuploidies [93] or Methylation DNA Immunoprecipitation coupled PCR for Down syndrome have been utilized [94,95].

4.5. Chromosomal microarray analysis

NIPS has adopted precision methods such as NIPT-Plus that identify chromosomal structural abnormalities at various stages of fetal development. Although conventional NIPT detects chromosomal variations in a limited manner, NIPT-Plus's covering range is broader, more sensitive and specific. Chromosomal variations may come from deletion, duplication or insertion of the sequences within the chromosome. These variations are commonly known as copy number variations (CNVs). The CNVs cause a copy number gain or loss in the genome. Some CNVs can cause fetal microdeletion and microduplication syndromes (MMSs) like 1p36 deletion syndrome, Prader–Willi/Angelman syndromes, DiGeorge syndrome or cri-du-chat, regardless of the pregnant woman's age [96,97], hence, detecting pathogenic fetal CNVs or MMSs in all pregnancies irrespective of the maternal age, is significant in pregnancy management.

CNVs are detected using traditional cytogenetic analysis methods like G-banded karyotype, microarray-based methods (e.g., chromosome microarray analysis (CMA)), or next-generation sequencing (NGS). CMA is considered the first-tier technique in CNV detection as it detects chromosomal imbalances (DNA copy number) in the kilobase range, making it more sensitive than standard karyotyping [98]. According to the American College of Obstetricians and Gynecologists guidelines, CMA is recommended as the first-tier genetic test in pregnancies showing fetal abnormalities on an ultrasound scan [99]. Using CMA to identify copy number change during early pregnancy is predominantly accommodative in the evaluation of developing fetuses with congenital structural or neurocognitive disorders [100,101]. This evaluation increases the overall diagnostic yield of clinically relevant cases. A consensus statement and guidelines published in 2010 suggest that CMA identifies a genetic cause in affected children with greater precision as compared with standard karyotyping [102]. Wapner and colleagues during a multicenter cohort study indicated that CMA enhances

about 6% of diagnostic yield as compared to conventional karyotyping [103].

Even though CMA has increased the diagnostic yield of NIPS, pregnant women who have clinically significant copy number variants on CMA must go further for invasive methods to corroborate CMA results. Despite its high cost, this is the most advanced method for detecting fetal chromosomal aneuploidies and single-gene disorders caused by genomic deletions and duplications.

5. Genomic versus proteomic approach

As a part of the Special Non-Invasive Advances in Fetal and Neonatal Evaluation Network program, trials have been undertaken to carry out a series of proteomic experiments using maternal plasma samples. Besides pregnancy-associated plasma protein A estimation, Quad screen, a serum-based protein test, is used to screen Down syndrome or neural tube defects [104]. This biochemical test estimates the concentration of alpha-fetoprotein, human chorionic gonadotrophin, unconjugated estriol, and inhibin-A in the mother's blood. Studies have been conducted to estimate the levels of DSCR-4, maternal serum placental growth factor, CA 19-9 and CA 15-3 in maternal plasma using a novel proteomic approach [105–108]. 2-D gel electrophoresis, Surface-enhanced laser desorption ionization-time of flight, or Matrix-assisted laser desorption ionization-time of flight followed by mass spectrometry are the main proteomics-based approaches used in NIPS. Several study groups have used these techniques to screen chromosomal abnormalities like Down syndrome [106,107]. Due to certain limitations, not much has progressed beyond these studies. For instance, the plasma represents the dynamic range of individual proteins, making it the most composite human proteome. The fetal origin proteins, which are present in a small quantity in the maternal plasma, get masked due to the presence of the most abundant maternal plasma proteins, hence complicating their detection. Under these circumstances, the identification or the concentration of the protein under observation is doubtful.

Although the use of genomics in NIPS is at the forefront of proteomics, the potential for investigating protein biomarkers through a proteomic approach is promising. It is necessary to improve the protein purification process in order to uncover a panel of new biomarkers with diagnostic potential in prenatal screening.

6. Challenges and future perspective

The major challenge for researchers in implementation of NIPS in the clinical setting is the concentration and purity issue of cffDNA. The diagnostic yield of NIPS depends on the concentration of fetal fraction in the mother's plasma. The concentration of fetal fraction at or less than 4% reduces the detection rate up to 62.1%. Its concentration above 10% increases the sensitivity and detection limits [108]. Hence, the amount of cffDNA or fetal fraction in maternal plasma is the key determining factor for the reliability of the NIPS test. Secondly, the use of positive controls for the detection of fetal-originated DNA is still under development. There is currently no low-cost direct method available for producing a gold standard control for the confirmation of cffDNA in maternal plasma. The insufficient availability of true positive controls or markers makes NIPS a discordant trial. Though the challenges mentioned above are not exhaustive, there are number of operational and technical challenges associated with each approach used in NIPS that are beyond the scope of this review.

Despite various challenges and ethical issues, many developed countries have widely adopted NIPS as a routine clinical prenatal screening test, leading to a significant reduction in invasive prenatal genetic testing. Major Biotechnological companies like SEQUENOM Inc., Aria Diagnostics Inc., Illumina Inc., Invitae Inc., Medicover., and Natera Inc. have launched different NIPS diagnostic panels for the screening of aneuploidies and monogenic disorders. This way possibility for clinical implementation is increasing and moving closer to reality. With the reduction in sequencing cost, advances in fetal DNA downstream process, algorithmic and resolution advances, the scope of NIPS in pregnancy management, is promising. The era of NIPS has opened new potential for the implementation of genomic technologies into the clinical setting.

7. Conclusions

NIPS is of significant medical importance because of its effective and safer approach. It uses fetal-derived DNA or cells to screen or diagnose various abnormalities associated with the fetus. Although the use of NIPS in fetal aneuploidy detection is well known, its application in detecting Mendelian or X-linked disorders is still evolving. Various Biotechnology companies have developed NIPS tests under different trade names. These test panels are commercially available for detecting fetal aneuploidy, determining sex, and RhD status. NIPS is becoming a reality after decades of research. Advanced and multiple genomic methods have facilitated progress and improvement towards the implementation of NIPS in clinical practice. Though the current methods have challenges and limitations, better understanding and advances have made NIPS's implementation possible in clinical practice. Such restrictions are notably linked with the cost or the infrastructure necessary for assay performance. The implementation of NIPS in clinical practice will improve and secure feto-maternal health by minimizing ethical, social, and legal implications, as well as other limitations.

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

NIPS	Non-invasive prenatal screening
cffDNA	cell-free fetal DNA
RhD	Rhesus-D
FBS	fetal blood sampling
CVS	chorionic villus sampling
FISH	fluorescence in situ hybridization
nRBC	nucleated red blood cells
cfNAs	cell-free nucleic acids
cffmRNA	cell-free fetal mRNA
miRNAs	microRNAs
NGS	next-generation sequencing
MPSS	massively parallel shotgun sequencing
DMRs	differentially methylated regions
FES	fetal exome sequencing
rt-PCR	real-time PCR
dPCR	digital PCR
ddPCR	droplet digital-PCR
COLD-PCR	Co-amplification at Lower Denaturation temperature PCR
QF-PCR	Quantitative fluorescent PCR
CMA	chromosome microarray
CNVs	copy number variations
EV	extracellular vesicle

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