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**Comparison of next generation sequencing-based and methylated DNA immunoprecipitation-based approaches for fetal aneuploidy non-invasive prenatal testing**

Christopoulou G *et al*. Present and future of aneuploidy NIPT

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**Abstract**

Over the past few years, many researchers have attempted to develop non-invasive prenatal testing methods in order to investigate the genetic status of the fetus. The aim is to avoid invasive procedures such as chorionic villus and amniotic fluid sampling, which result in a significant risk for pregnancy loss. The discovery of cell free fetal DNA circulating in the maternal blood has great potential for the development of non-invasive prenatal testing (NIPT) methodologies. Such strategies have been successfully applied for the determination of the fetal rhesus status and inherited monogenic disease but the field of fetal aneuploidy investigation seems to be more challenging. The main reason for this is that the maternal cell free DNA in the mother’s plasma is far more abundant, and because it is identical to half of the corresponding fetal DNA. Approaches developed are mainly based on next generation sequencing (NGS) technologies and epigenetic genetic modifications, such as fetal-maternal DNA differential methylation. At present, genetic services for non-invasive fetal aneuploidy detection are offered using NGS-based approaches but, for reasons that are presented herein, they still serve as screening tests which are not readily accessed by the majority of couples. Here we discuss the limitations of both strategies for NIPT and the future potential of the methods developed.

**Key words:** Non-invasive prenatal testing; Fetal aneuploidy; Next generation sequencing; Epigenetics; Differential methylation; Methylation dependent immunoprecipitation

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**Core tip:** Non-invasive prenatal screening and diagnosis of fetal aneuploidies has been a challenging field for many researchers. Different methodologies have been developed, mainly based on next-generation sequencing and epigenetic modifications. At present, non-invasive prenatal testing services are offered using next generation sequencing-based technologies which have great potential, but currently they present with certain limitations. Epigenetic approaches may overcome some of these limitations and seem to have promising potential for wider applications.

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**INTRODUCTION**

Invasive procedures such as chorionic villus sampling (CVS) and amniocentesis are a prerequisite for the prenatal diagnosis of fetal chromosomal abnormalities, either by conventional and/or molecular fetal karyotyping, or other molecular cytogenetic methods. Although these approaches yield accurate results, the rate of pregnancy loss attributed to CVS or amniocentesis is estimated to be 1.0% to 2.0%[1]. This considerable procedure-related risk of pregnancy loss has motivated researchers to try to develop non-invasive approaches in in order to provide safer healthcare service.

Since the discovery that fetal cells circulate in the maternal blood during pregnancy[2], numerous researchers worldwide have put great effort towards exploring the possibility of non-invasive prenatal investigation of the fetal genetic constitution. Initially, the focus of investigation was on circulating fetal nucleated cells, where detection of fetal gender and aneuploidies was made possible, mainly by applying FISH subsequent to cell sorting[3-5]. Even though preliminary results were promising, the development of a commercially available application has failed at least to date, mainly due to certain inherent limitations of the method. Firstly, the rarity of fetal cells in the maternal circulation made it very difficult to isolate a satisfactory number for investigation[4-6]. Secondly, the poor quality of the isolated fetal cells made the application of FISH on the nuclei problematic, resulting in less reliable results. Most importantly, the observation that fetal cells may remain in the maternal circulation for several years after their release, presents a serious problem for non-invasive prenatal investigation of subsequent pregnancies[7,8]. Nevertheless, researchers have not given up this approach entirely and attempts are still being made to overcome limitations[9-13].

The discovery of cell free fetal DNA (cffDNA) in maternal plasma during pregnancy by Dennis Lo *et al*[14] in 1997, gave rise to a whole new opportunity in the field of non-invasive prenatal testing (NIPT). Its origin is proven to be either trophoblastic or from embryonic cells in the maternal blood which have undergone apoptosis[15]. It has also been demonstrated that cffDNA is cleared from maternal plasma within a few hours after delivery[16], making its study specific to the current pregnancy. Although cffDNA is detectable from the early stages of pregnancy[17] and increases during its progression[18,19], it is demonstrated to account only for 3.0% to 6.0%[14,20] of total free DNA in maternal plasma. A more recent study utilizing microfluidics, re-estimated the cffDNA fraction to a median of 9.7% in the first trimester[21,22]. The relatively small amount of fetal DNA in maternal plasma presents one of the most serious technical challenges for whichever technology is implemented for investigation. Furthermore, the fact that fetal DNA is 50% identical with that of the mother makes the attempts for fetal aneuploidy testing even more challenging.

**CURRENTLY APPLIED METHODS**

During recent years independent teams from all over the world have focused on developing methods for NIPT using cffDNA, mainly testing for fetal aneuploidy[23]. Despite applying different strategies including SNP and allelic ratio analyses, none have managed to produce a widely available test, mainly because they depend on informative genotypes or fetal gender[24-26]. On the other hand, next-generation sequencing (NGS) technologies have made great progress in the field, resulting in commercially available NIPT services. In recent years, the use of commercially available tests for NIPT for trisomy 13, 18, 21 and sex chromosome aneuploidies has been introduced into routine antenatal care. Massively parallel direct sequencing (MPS) reads from a tested chromosome are compared to others with the aid of sophisticated bioinformatics software, resulting in a relative chromosome dose. NGS-based methods are polymorphism independent and have the ability to detect aneuploidies In a recently published meta-analysis of clinical validation and implementation studies the pooled weighted detection rate for trisomy 21 is reported to be > 99% and the false positive rate to be < 0.01%[27]. Commercially available tests based on NGS technologies have been validated on large numbers of cases and have a very high sensitivity and specificity[28-34] as well (Table 1).

Another promising prospective in NIPT is provided by methylated DNA immunoprecipitation-based (MeDIP-based) approaches. The discovery of fetal-maternal differentially methylated regions (DMRs)[35] has facilitated the development of NIPT strategies by combining MeDIP with other downstream applications. Using the “epigenetic approach”, a NIPT method based on MeDIP combined with quantitative PCR (MeDIP-qPCR) which proved to be of high precision in a proof of principle (100% sensitivity, 100% specificity)[36] and larger validation study[37] (100% sensitivity, 99.2% specificity) was developed.

**ADVANTAGES AND DISADVANTAGES**

Approaches based on NGS are extremely powerful. Besides detecting whole chromosome aneuploidy, they have the potential to detect smaller chromosomal imbalances allowing for microdeletion/microduplication syndrome NIPT[38]. However, although NIPT is already commercially available for the detection of a certain number of microdeletion/microduplication syndromes, further validation studies are needed[39]. Taking into account the vast amount of data that NGS is capable of producing, it could be potentially be combined with other methodologies to generate non-invasive fetal whole genome sequencing[40]. As impressive as this may seem at present, it is quite possible that this will materialize in the near future.

The impressive developments of NGS technologies are accompanied however by certain drawbacks. One important limitation is the low level of fetal DNA which is available for testing. This is overcome in MeDIP-based technologies which are based on fetal DNA enrichment, which then increases sensitivity substantially. However, MeDIP by which cffDNA hypermethylated regions are selectively enriched is a stage wherein bias may be introduced, influencing the test results. Therefore, it is very important to carefully select DMRs, optimize this stage and evaluate the overall performance allowing for this. Another drawback of NGS-based approaches is that the equipment/technology required is still not available in all clinical settings, making the service feasible only in large centers, such as those in the United States and China. Furthermore, the requirements for significant infrastructure, complex laboratory procedures, highly trained personnel and challenging bioinformatics analyses make NGS-based technologies costly and complex. In contrast, the “epigenetic approach”, uses equipment that is available in most genetic diagnostic laboratories offering established genetic services, it is considerably cheaper and simpler and therefore it may be applied potentially worldwide and offered to a broader population. However, current MeDIP-based approaches focus mainly on fetal trisomy 21 and at present have not yet demonstrated their ability to detect other fetal aneuploidies and submicroscopic aberrations that NGS-based technologies have proven to be capable of detecting. Moreover, large validation studies and future clinical application feedback data are awaited in order to re-evaluate the advantages and disadvantages of MeDIP-based NIPT tests.

**THE FUTURE OF FETAL ANEUPLOIDY NIPT**

Both, NGS and MeDIP-based approaches yield risk classification results at present. This means that a probability is given for each condition investigated, and depending on whether the pregnancy is assessed as being high risk or not, the couples are counseled to proceed with confirmatory invasive diagnostic testing, usually fetal karyotyping after CVS or amniocentesis. False positive results lead to unneeded invasive procedures posing an undesirable risk of pregnancy loss, while false negative results may lead to the birth of an abnormal child. There is an argument that false negative NIPT results for trisomy 18 or 13 are unlikely to result in the birth of an abnormal child because both syndromes are most likely to present with serious ultrasound findings during pregnancy. Conversely, cases with trisomy 21 (Down syndrome) may not have any indications throughout the pregnancy and consequently, NIPT false negative trisomy 21 fetuses are more likely to be born[41]. Therefore, NIPT for trisomy 13, 18 and 21 should be considered as a screening test rather than a diagnostic test, which should be robust, rapid and cost efficient. We believe that MeDIP-based tests meet these requirements for the reasons already presented, and moreover has certain advantages compared to NGS-based methods and therefore shows great potential for large scale public service access. At present, if treated as a replacement for current biochemical screening tests, the resulting risk could be combined with that derived from ultrasound markers such as nuchal translucency (NT) measurement and others. The combined NIPT-U/S risk for fetal aneuploidy may provide a safer screening strategy compared to that offered to most couples today[42]. The future aim is to eventually avoid invasive procedures and develop NIPT (testing) into NIPD (diagnosis).

For any NIPT used caution is needed when it comes to genetic counseling, in order to avoid misunderstandings concerning diagnosis. There is an ongoing debate on ethical and policy issues related to NIPT and the ESHG/ASHG invite the scientific community to contribute to setting future guidelines for NIPT[43].

**CONCLUSION**

During recent years there have been enormous advances in the field of fetal aneuploidy NIPT. Relevant genetic services are offered by academic centers and commercial companies worldwide, but not all future parents have access to this service. Our team is working towards developing a commercially available MeDIP-based test, that will be relatively inexpensive and easy to apply and from which more people can benefit. Looking ahead, we predict that epigenetic based approaches in combination with genetic-based approaches and advanced technologies (digital PCR, NGS) will contribute to the development of NIPT for more subtle fetal genetic abnormalities[44], such as point mutations, microdeletion/microduplication syndromes, *etc*.

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**Table 1 Validation and verification comparison of the most widely used commercially available non-invasive prenatal testing for trisomy 21**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Company | Sequenome | Verinata (Illumina) | Ariosa | Natera |
| Test | “Materni T21 PLUS” | “Verify” | “Harmony” | “Panorama” |
| Sensitivity | 99.6%-99%  (209/212) | > 99%  (90/90) | 100%  (81/81) | > 99%  (25/25) |
| Specificity | 99.8%  (1468/1471) | 99.8%  (409/410) | 99.97%  (2887/2888) | > 99%  (242/242) |
| False positive | 0.2%  (3/1471) | 0.2%  (1/410) | 0.03%  (1/2888) | 0 |
| No result rate | 3.4% | 5.8% | 4.7%-5.7% | 5.4% |