NONINVASIVE PREGNATAL TESTING: THE ASPECTS OF ITS INTRODUCTION INTO CLINICAL PRACTICE

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The last couple of years have witnessed the rapid development of prenatal molecular-based screening for fetal aneuploidies that utilizes the analysis of cell-free DNA circulating in the bloodstream of a pregnant woman. The present review looks at the potential and limitations of such testing and the possible causes of false-positive and false-negative results. The review also describes the underlying principles of data acquisition and analysis that the testing involves. In addition, we talk about the opinions held by the expert community and some aspects of legislation on the use of noninvasive prenatal testing (NIPT) in clinical practice in the countries where NIPT is much more widespread than in Russia.

Keywords: NIPT, NIPS, prenatal screening, fetal aneuploidy, cell-free DNA

Acknowledgment: the authors are grateful to Ekaterina Shubina of Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology for her valuable feedback.

Author contribution: Korostin DO conceived the review and supervised manuscript preparation; Plakhina DA wrote the sections about cell-free DNA and the regulatory legislation and helped to revise the manuscript; Belova VA wrote the sections about MPS-aided NIPT and the regulatory legislation and helped to revise the manuscript.

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Received: 05.10.2018 Accepted: 10.05.2019 Published online: 22.05.2019
DOI: 10.24075/brsmu.2019.036

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THE ORIGIN OF CELL-FREE FETAL DNA

Fetal chromosomal aneuploidy is one of the primary causes of spontaneous abortion, accountable for 35% of all miscarriages [1] and occurring in 0.3% of all births [2, 3]. The most common aneuploidies are trisomies 13, 18, 21 and XXY.

Trisomy 21, or Down syndrome (DS), is observed in 1 in 800 births [4]. The risk of fetal DS increases with maternal age, starting to grow exponentially once a woman turns 34 and approximating an incidence rate of 1 case per 35 births in women over 40 [5].

Until the 1980s, a woman’s age was the only reliable prognostic criterion for the risk of aneuploidy; all pregnant women over 35 were recommended to undergo an invasive diagnostic test aimed to identify the karyotype of the fetus. For younger women, the only indication for invasive diagnostic procedures was a family history [6].

Today, the 1st trimester combined ultrasound and biochemical screening test proposed back in 1997 [7] is considered to be the most reliable prognostic tool with its sensitivity of 90% for Down syndrome and the false positive rate of 5% [8].

At present, only invasive diagnostic techniques are employed to diagnose hereditary pathologies of the fetus, including chorionic villus and amniotic fluid sampling. The obtained specimens of fetal cells are analyzed by QF-PCR, MLPA, G-banding, FISH, and molecular karyotyping [9].

Origin of cell-free fetal DNA

Cell-free fetal DNA (cffDNA) transcends the placental barrier and enters the maternal bloodstream [10]. Modern technologies can detect cffDNA in the maternal blood plasma as early as the 4th week of gestation. Its concentration increases throughout pregnancy, peaking in the last 8 weeks before delivery and then dropping abruptly to almost 0 in the first hours after birth [11–15].
leaks into the maternal bloodstream following the apoptosis of trophoblast cells [16]. The placental origin of cfDNA is corroborated by its presence in anembryonic pregnancies in which no embryo is formed, but placental tissue is in place [17], as well as in women with meiotic placental mosaicism (PM).

PM, which is essentially a discrepancy between the karyotypes of a fetus and a maternal placenta, strikes 0.6–1% of women who previously underwent invasive diagnostic procedures [18]. PM can be broken down into mitotic and meiotic types. Mitotic PM results from the chromosomal nondisjunction during one of the divisions of a diploid zygote that gives rise to an aneuploid cell line and leads to confined PM. As a rule, confined PM affects only a limited region of the placenta and can be defined as a low-level mosaicism. Meiotic PM originates from an initially trisomic zygote in which a rescue event occurs: the loss of an extra chromosome copy in the early stages of fetal development. Thus, even if the placenta is partially or fully aneuploid, the fetus can still have a normal karyotype, and vice versa.

Cell-free fetal DNA characteristics

Cell-free DNA molecules circulating in the maternal blood are chopped fragments of 166 bp (maternal cfDNA) or 143 bp (fetal cfDNA) in length [19]. Such size distribution is the result of nonrandom DNA fragmentation [20], DNA is degraded by various enzymes that cut at the sites they can access. Nucleosomes represent the first level of DNA compaction. They are histone spools with DNA wound around them, spaced 20 base pairs apart. These linker regions can be easily accessed by nucleases. Therefore, we can assume that a 143 bp-long cfDNA fragment corresponds to a “linkerless” DNA coil wound and DNA fragments of a limited region of the fragment corresponds to a DNA coil containing a linker region. The nonrandom fragmentation pattern can be explained by the difference in histone H1 isoforms determined by the placental or hematopoietic origin of nucleosomes. The main function of histone H1 is to bind to a linker; apparently, the binding does not occur in the case of cfDNA, and the linker is chopped off [19, 21].

The “sawtooth”-like size distribution of shorter DNA fragments with a peak periodicity of ~10 bp suggests that cell-free DNA undergoes further nuclease cleavage in apoptotic bodies at the position of approximately every 10th nucleotide directly attached to a histone protein [19, 22]. No similar size distribution is observed during the analysis of short reads mapped onto a mitochondrial genome that lacks histones.

It has been established that hypo- and hypermethylated regions of fetal and placental genomes do not match those of the maternal genome because of epigenetic difference between tissues [23, 24]. It is hypothesized [25] that unmethylated DNA regions are more accessible for cutting. Maternal cfDNA is hypermethylated, which means tighter DNA wrapping around histones, increased compaction and nucleosome stability, and longer average cfDNA fragment lengths in comparison with fetal DNA.

NIPT aided by MPS

Fetal cells, fetal cell-free RNA and fetal cell-free DNA are potential targets for liquid biopsy. Fetal cell-free DNA has a number of advantages that allow it to be used as a basis for noninvasive prenatal testing (NIPT).

On average, the fetal fraction amounts to 10% of total cell-free DNA at the gestational age when prenatal testing is performed. This value exceeds the number of fetal cells circulating in the maternal blood by 3–4 orders of magnitude.

The contribution of maternal microchimerism is normally negligible in comparison with the fetal DNA fraction. Cell-free DNA is more stable than cell-free RNA, and the methods used for its analysis are better reproducible.

NIPT can be described as a statistical examination aimed at estimating how well each chromosome is represented in a studied sample. Normally, the number of short reads per each chromosome of a nonpregnant woman is proportional to the length of this chromosome. The same is true for women who carry a child with a normal karyotype. However, in trisomies, as is the case with trisomy 21, the proportion of reads needed to cover all copies of the chromosome of interest will be increased relative to other chromosomes. The length of chromosome 21 amounts to about 1.5% of the entire genome. Given that the cfDNA fraction makes 10% of total cfDNA present in the sample, the extra chromosome 21 will cause a 0.08% rise in this value. To assess the reliability of NIPT results, different statistical methods are used, the most common being Fisher’s Z test. It is employed to investigate whether an increase in the read count per chromosome of interest is accidental. The actual coverage is compared to the expected precalculated value with due account of the standard error. Z is calculated by the formula:

\[ Z = (x - \mu)/\delta, \]

where A is the studied chromosome; x is the number of reads mapped to A in the analyzed sample; \( \mu \) is the mean read count needed to cover A in the reference sample (normal control); \( \delta \) is the standard deviation. The resulting Z score > 3 suggests trisomy, Z < –3 suggests monosomy, whereas a range of values from –3 to 3 are indicative of a normal karyotype [26].

The expected value is calculated based on the analysis of a cell-free DNA sample obtained from a diagnosed child. During the analysis, maternal cfDNA is not separated from –3 to 3 are indicative of a normal karyotype. However, in trisomies, as is the case with trisomy 21, the proportion of reads needed to cover all copies of the chromosome of interest will be increased relative to other chromosomes. The length of chromosome 21 amounts to about 1.5% of the entire genome. Given that the cfDNA fraction makes 10% of total cfDNA present in the sample, the extra chromosome 21 will cause a 0.08% rise in this value. To assess the reliability of NIPT results, different statistical methods are used, the most common being Fisher’s Z test. It is employed to investigate whether an increase in the read count per chromosome of interest is accidental. The actual coverage is compared to the expected precalculated value with due account of the standard error. Z is calculated by the formula:

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Fetal DNA fraction can also be reliably estimated by calculating the proportion of differentially methylated genome regions in the analyzed cell-free methylome [34].

Because the lengths of fetal and maternal DNA molecules are distributed nonuniformly, the fetal DNA fraction can be determined from the ratio of fragments sized 100–150 bp to those sized 163–169 bp, since they correspond to the fetal and maternal DNA fractions, respectively [35]. This approach is effective in paired-end sequencing [36].

Another novel “nucleosome track” method of quantifying the fetal DNA fraction is underway. The idea behind it is that fetal DNA fragmentation is not random and follows a certain pattern determined by DNA packaging into nucleosomes, as described above [37].

Researchers are also starting to harness neuronal networks to estimate the fetal DNA fraction. Using large training samples (thousands of specimens with a known fetal DNA fraction), one can get reliable results by analyzing a number of certain sequencing parameters [38].

NIPT potential

NIPT is mostly used to screen for chromosomal aneuploidies, but massively parallel sequencing (MPS) technologies are capable of detecting other genome abnormalities as well.

Low and ultra-low (<1.0) coverage genome sequencing does not allow point mutations to be detected, but can be employed to screen for deletions and duplications [39]. Such strategy is used to perform prenatal genetic screening aided by high-throughput sequencing [40]. In most cases, NIPT data resolution is not sufficient to capture medium-sized (up to 5 billion bp) deletions and duplications; this problem can be solved by imprints determined per studied sample [41–44]. Unfortunately, this adds to the costs of testing. More complex bioinformatic methods of data processing are a bit less effective [45, 46]. The amount of sequencing data yielded from the sites of interest can be significantly increased through targeted enrichment of genomic DNA regions. For example, the Panorama test [47] targets about 20,000 polymorphic loci densely located in the regions prone to microdeletions. The developers believe that the detection accuracy of the test is 97.8% or higher [48].

Since the moment cffDNA was discovered, the world has seen the emergence of various approaches to the diagnosis of genetic abnormalities of the fetus. The very first of them were capable of determining the sex of the fetus [49] and its Rh factor [50]; they were designed to screen for the sequences that do not typically occur in the maternal genome and exploit different PCR types, including qPCR, ddPCR, and QF-PCR. Later, the development of methods for detecting genetic traits inherited from the father became a routine practice: X-STR markers [51], markers of autosomal dominant conditions, such as Huntington’s disease [52] and myotonic dystrophy [53] were soon discovered. However, the majority of monogenic diseases are autosomal-recessive and their development is driven by the mutations in both maternal and paternal copies of the genome. Because of that, prenatal screening typically includes 3 sequencing procedures: sequencing of maternal and paternal genomic DNA required to identify parental haplotypes and locate the mutations of interest followed by cfDNA sequencing in order to see what chromosomes the baby has inherited [54].

The analysis of the cfDNA methylome has revealed the pattern of methylation that can serve as an aneuploidy marker [55, 56]. It has been shown that the placental methylome, which is what NIPT analyzes, is dynamic; the methylation pattern can change depending on the condition of the fetus and the mother. For example, the analysis of cfDNA methylation can be used to diagnose preeclampsia [57–59].

Although there are a few disadvantages to using cell-free RNA as an analyte in screening tests (contamination by noninformative rRNA, poor preservation in the sample, low reproducibility of test results in comparison with cfDNA), changes in the expression of some RNA transcripts in the fetus can be a reliable predictor of preeclampsia long before a woman develops its symptoms [60].

NIPT validation

Like any other diagnostic technique, NIPT had to undergo clinical trials to prove its efficacy.

In 2014, a study conducted in 1,914 pregnant women from 21 US medical centers demonstrated that for NIPT the false-positive rate was significantly lower than for the standard biochemistry screening (0.3% vs. 3.6%, \( p < 0.001 \) for trisomy 21 and 0.2% vs. 0.6%, \( p < 0.03 \) for trisomy 18). The test failed in 0.9% of the participants [61].

A study published in 2015 compared the efficacy of NIPT with that of conventional diagnostic techniques [62]. It was conducted in 35 medical centers using the samples collected from 15,841 pregnancies. NIPT was able to detect all cases (38) of true aneuploidy in patients with fetal trisomy 21; in 9 patients the results were false-negative. For trisomy 21, DR was 100%, FPR was 0.06%, and PPV was 80.9% (the standard screening test used in the study returned 78.9%, 5.4%, and 3.4% for DR, FPR and PPV, respectively). NIPT performance was significantly better than that of standard screening in pregnant women with fetal trisomies 13 and 18. This means that NIPT can be used for detecting fetal trisomies in the clinical setting because it has better resolution and higher accuracy in comparison with conventional diagnostic tools.

Causes of false-positive results in NIPT

NIPT has a number of limitations that can cause false-positive results.

Maternal weight and gestational age

The amount of cfDNA correlates positively with the gestational age and is inversely proportional to the body mass index of a pregnant woman. Too few cfDNA fragments at 9–10 weeks into pregnancy do not allow NIPT results to be reliable. For women with high BMI, the test can turn to be ineffective as well, because the probability of a false-positive result remains high [15, 63] if cfDNA fraction is not estimated.

Placental mosaicism

Women who tested positive for aneuploidy by NIPT are advised to undergo an invasive diagnostic procedure to rule out placental mosaicism. Here, amniocentesis should be preferred over chorion villus sampling because the DNA in the villi has the same placentual origin as cfDNA [64–67]. It is absolutely not recommended to base the decision of pregnancy termination on NIPT results solely (see below).

Twins

Although NIPT can detect aneuploidies in twin pregnancies, it is unable to identify which of the twins has a chromosomal abnormality. Here, invasive diagnostic techniques should be
Table. Leading US manufacturers of commercial NIPR

| NIPR trade name | Manufacturer | Location |
|-----------------|--------------|----------|
| MateriTi21Plus™ | Sequenom, subsidiary of LabCorp, Inc. | San Diego, CA |
| Verifi™         | Verinata Health, now Illumina | Redwood City, CA |
| Harmony™        | Ariosa Diagnostics | San Jose, CA |
| Panorama™       | Natera       | San Carlos, CA |
for aneuploidies relied on FMF standards [29]. If the risk for aneuploidy was high (> 1 : 250), invasive diagnostic testing was carried out followed by karyotyping. The expenses were covered by health insurance. According to the recommendations published in 2017, the analysis of circulating cell-free DNA is recommended to women at high risk (from 1 : 1,000 to 1 : 51) for fetal trisomy 21 revealed by 1st trimester ultrasound and biochemistry screening. Pregnant women whose risk for aneuploidy is 1 : 50 or higher should undergo an invasive diagnostic procedure but still can opt for molecular screening first. It is emphasized that NIPT should not be regarded as a substitute for invasive diagnostic testing. The guidelines outline the need for developing a quality control and lab accreditation system. The screening strategy is to be revised in 3 years; among other things, the revision will cover the issues of screening for other aneuploidies and microdeletions.

USA

About 6.35 million pregnancies are reported annually in the USA. The NIPT market is divided between a few major players (see the Table) [86]. NIPT expenses are covered by health insurance or a patient’s personal funds. No funding is received from the state.

So far, 4 medical associations have proposed guidelines for NIPT:

• the American College of Obstetricians and Gynecologists (ACOG), May 2016 [87];
• the International Society for Prenatal Diagnosis, April 2015 [80];
• the National society of Genetic Counselors, October 2016 [88];
• the American College of Medical Genetics and Genomics (ACMG) [89].

The ACMG notes that the evolution of NIPT methods and techniques is so rapid that any currently existing clinical recommendations will become obsolete in just a couple of years. Similar to ACOG, the ACMG guidelines emphasize that all pregnant women should be informed about the possibility of undergoing NIPT and its relative advantages over conventional screening for trisomies 13, 18 and 21. Some experts and manufacturers consider these guidelines as a signal for ordering NIPT for all pregnant women regardless of the results of 1st trimester screening. This interpretation is wrong. ACMG only recommends that pregnant women should be informed of the possibility of undergoing NIPT and provided with all relevant information about the test [86]. Unfortunately, many physicians are unaware of NIPT limitations, tend to misinterpret its results or take wrong decisions. Knowing that, NIPT manufacturers provide their own genetic counselling, which raises a number of questions since the counsellors involved can be biased.

Recently, there has been a rise in the number of patients who test false-positive for sex chromosome aneuploidies. It is imperative that patients should be informed of the situation and explained that clinical outcomes for children with such aneuploidies vary. For example, although the X0 karyotype is a common cause of pregnancy loss, the quality of life of women with Turner syndrome is relatively high.

The guidelines stress that NIPT results should provide accurate information about NIPT specificity, sensitivity, FPV, NPV, and fetal DNA fraction for all types of analyzed mutations (aneuploidies of autosomes, sex chromosomes, CNV).

The most common cause of NIPT failure is low fetal DNA fraction. The low DNA fraction correlates with a number of fetal aneuploidies [62, 72], meaning that in the case of NIPT failure, the patient should be immediately offered to undergo an invasive diagnostic test instead of repeating NIPT. ACMG does not recommend to use NIPT for detecting microdeletions because no reliable assessment of its specificity and sensitivity has been made so far.

Russia

In Russia, the number of annually reported pregnancies is about 1.8 million. Screening for genetic pathology of the fetus includes biochemistry tests and ultrasound examinations conducted in the 1st trimester. If the revealed risk is 1 : 100, the woman is offered to consult a geneticist and undergo an invasive diagnostic test. All expenses are covered by health insurance and regional budgets [90]. Clinical recommendations on NIPT were published in 2016 [91]; they are largely consistent with the ACMG guidelines mentioned above.

A few obstacles impede NIPT promotion on the Russian market: NIPT is not certified in Russia and almost all MPS reagents and equipment have no marketing authorization in our country.

CONCLUSION

Incorporation of NIPT into clinical practice poses a serious dilemma. If we raise the risk threshold signaling the need for NIPT to a higher value, the doctors who perform invasive testing may lose their skills due to the lack of clients, which will lead to diagnostic inaccuracy. In this case, the detection rate may even become lower than it is now. If we start to offer NIPT to every pregnant woman, the total expenses will soar and become unacceptable even for the most affluent and developed countries. This means that the optimum risk value should be defined at which the balance between the aneuploidy detection rate and the incurred costs will be harmonious.

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