Opinion on a new and Challenging Tool in Prenatal Counseling: Non-invasive Prenatal Testing by Fetal Cell-Free DNA in Maternal Blood

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Abstract
Practicing obstetricians are constantly facing new challenges regarding prenatal diagnosis, as knowledge on this field increases exponentially. Continuous medical education in this area as in others is essential for a good clinical practice and appropriate counseling to patients.

Introduction
Congenital defects are currently the leading cause of infant mortality in the developed world. The reported prevalence of congenital defects ranges from as low as 4 per one hundred live births in France, to over 8 per hundred live births in Sudan. Worldwide, this figure is 6 per hundred live births on average [1].

In developed countries, chromosomal disorders cause one out of ten congenital defects; which means that 9 out of ten babies born with a major defect will have a normal karyotype [2]. Nevertheless, fetal karyotype analysis is an important component of prenatal diagnosis, as some entities (for example Down's syndrome) may not be adequately diagnosed by ultrasound examination biochemical screening, or a combination of both (triple screening at 11-14 weeks, quadruple screening in second trimester).

Prenatal Medicine has witnessed some major advances in the last two decades. Ultrasound imaging is the most groundbreaking among them and, to this date also the most useful tool for prenatal diagnosis.

Prenatal prevalence of chromosomal anomalies, according to Wellesley et al. [3] has the following distribution:

1. Trisomy 21: 53%
2. Trisomy 18: 13%
3. Trisomy 13: 5%
4. 5 X0 (Turner): 8%
5. Sex trisomy: 5%
6. Other rare: 16%

Both ultrasound and biochemical screening are non-invasive and present no risk to the fetus, although the former strongly depends on the expertise of the technician performing the study.

On the other hand, before 2012 DNA analysis of fetal cells required invasive techniques to obtain fetal or placental cells (amniocentesis or chorionic villus sampling - CVS). These procedures were costly and required considerable expertise, while at the same time presenting the risk of abortion and only providing small amounts of fetal chromosomes [4].

These techniques carried an abortion risk of 1 in 300 for amniocentesis and 1 in 250 for CVS, and were therefore only offered to women whose risk of karyotype alterations surpassed the risk of such an invasive procedure [5].

2012 saw a turning point in prenatal chromosome analysis due to the development of appropriate methods for studying cell-free fetal DNA in maternal blood by massive sequencing technologies.

This paper presents a review of the new diagnostic methods available for the most frequent aneuploidies (mainly Massive Sequencing of fetal cell-free DNA), and explains the implications for everyday pregnancy control of the practicing obstetrician. These technologies add challenges to our daily practice.

Classical Karyotype Analysis
Presently, a complete karyotype of the fetus can only be obtained by one of three methods, all of them requiring complete fetal cells nuclei

Chorionic villus sampling (CVS)
CVS is ideally performed at 12 weeks of gestation. Guided by ultrasound, an appropriate needle is inserted in the placenta to obtain a small amount of chorionic villus tissue. The cells of this tissue usually carry the same DNA as somatic cells, except in rare cases of mosaicism [6,7].

Among experienced professionals, the rate of complications (mainly abortion) of this invasive technique is 1 per 200-250 CVS [8].

Chorionic villi need to be washed and separated from maternal tissue (decidua and maternal blood), fetal cells are identified and their karyotype is analyzed. Results are obtained in a few days, usually less than a week. Abnormal results must be confirmed through an amniocentesis because of the chance of mosaics.

CVS has an 99.25% sensitivity for aneuploidy detection with a specificity of 98.65% [4].

Amniocentesis
This technique obtains amniotic fluid through an ultrasound guided needle. It can be performed after 13-14 weeks of gestation. Requiring the culture of fetal fibroblasts, Amniocentesis enables the analysis of the fetal karyotype. Its rate of complications (mainly abortion) among

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Limitations of Current Aneuploidy Testing

- High false positive rate (5%)
- Late information
- Prolongs uncertainty
- Involves multiple visits
- Requires specialized ultrasound
- Safety concerns (CVS and amniocentesis)

Cell-free fetal DNA in maternal blood

Simpson and Elias were able to demonstrate in 1993 that fetal cell free DNA (cfDNA) fragments were present in maternal blood [21]. In 1997 Lo et al. first isolated fragments of fetal DNA in maternal serum and plasma [22], and Fan et al. applied massive sequencing to diagnose targeted fetal aneuploidies from maternal blood in 2008 [23]. A cascade of new studies soon followed, supporting the viability of the isolation and analysis of cell free fetal DNA from maternal blood [24-29] to diagnose chromosomal anomalies.

To this end, two types of fetal DNA can be isolated from maternal blood: the one present in the nuclei of fetal cells (one in 1,000 million cells in maternal circulation), or cell-free fragments of DNA (2 to 20% of total cell-free DNA in maternal circulation) [26].

Fetal DNA is released by apoptosis into maternal blood as small fragments of 150-200 pairs of bases. Thus, maternal blood contains both maternal and fetal cell-free DNA, which can be detected as soon as 7 weeks into gestation and becomes undetectable 2 hours after delivery [28].

This paper does not attempt to describe the methodology of DNA sequencing and will focus mainly on the clinical implications of the Non Invasive Prenatal Testing (NIPT) of the most frequent aneuploidies.

Importance of fetal fraction

The fraction of fetal DNA should be above 10% of all cfDNA in maternal circulation in order for this technique to render significant results. Since, fractions below 10% can lead to erroneous interpretations [24], in these cases most laboratories offering the technique opt instead to obtain a redraw of maternal blood to perform a correct analysis [30].

Detection Rate for Down Syndrome

| False Positive Rate | 0% | 20% | 40% | 60% | 80% | 100% |
|---------------------|----|-----|-----|-----|-----|------|
| Maternal age        | 0% | 20% | 40% | 60% | 80% | 100% |
| AFP only            | 0% | 20% | 40% | 60% | 80% | 100% |
| Quad Marker Screen  | 0% | 20% | 40% | 60% | 80% | 100% |
| First Trimester Screen | 0% | 20% | 40% | 60% | 80% | 100% |
| Full Integrated Screen | 0% | 20% | 40% | 60% | 80% | 100% |

Figure 1: Evolution of trisomy assessment.

Rates based on published data

| Reference # | T21 | T18 | T13 | 45X | XXX/XXX |
|-------------|-----|-----|-----|-----|---------|
| 31          | 99.9% | 99.9% | 99.9% | 99.9% | 99.9% |
| 32          | 98.6% - 99.1% | 100% | 91.7% | N/A | N/A |
| 33          | 100% | 97.2% | 78.6% | 93.8% | N/A |
| 34          | 100% | 98.0% | N/A | N/A | N/A |

Table 1: Detection rates.
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The role of placental mosaicism

As cfDNA originates from placenta, most likely from the trophoblast, it can be likened to the direct preparation of chorionic villi. It must be noted that the chromosomal makeup of the placenta and fetus can be different, a situation occurring more frequently with chromosomes 13 and 18, as compared to chromosome 21. This can lead to false positive and false negative results [7].

Clinical implications

To this day, only five laboratories worldwide are capable of performing and commercializing the sequencing technique for cell-free fetal DNA in maternal blood. Four of them are established in California, USA (in brackets: the registered trade mark for each of the tests offered): Verinata (Verifi), Natera (Panorama), Ariosa (Harmony), and Sequenom (MaterniT21); and one in China: BGI.

Results of the different tests offered are very similar in both sensitivity and specificity to diagnose T21, T18, T13 and sexual chromosomes aneuploidies (Tables 1-3).

If a definitive diagnosis is desired, invasive procedures are required to confirm a positive non-invasive prenatal testing (NIPT) result. Test results should always be interpreted in the context of all available clinical findings. It is recommended that the healthcare provider determines the utilization of the test, including the need for genetic counseling.

NIPT is not diagnostic, since it can render false positive results (Figures 1 and 2).

There are plenty of recent publications on this subject, and a number of on-going studies that reinforce the technique's value (Table 4).

The most ambitious of these projects is the NICE study [31] (Table 5) that enrolled 50 participating clinical sites in the U.S.A. and Europe, yielding a sensitivity of 100% and a specificity of 99.97% for Down syndrome, with a false positive rate of only 0.03%.

At this point, the Committee Opinion of the ACOG (the American Congress of Obstetricians and Gynecologists) must be presented [32].

"Cell free fetal DNA appears to be the most effective screening test for aneuploidy in high risk women… is one option that can be used as a primary screening test in women at increased risk of aneuploidy". “[NIPT] should be an informed patient choice after pretest counseling”. “[NIPT] should not be offered to low-risk women or women with multiple gestations”. “A patient with a positive test result should be referred for genetic counseling and should be offered invasive prenatal diagnosis for confirmation of test results.” This opinion on NIPT can be summarized in 6 points:

* NIPT should be an informed patient choice
* It should not be part of routine prenatal laboratory assessments
* Low-risk women or women with multiple gestations should not be offered NIPT
* A negative test does not ensure an unaffected pregnancy
* A patient with a positive test result should be referred to genetic counseling
* Invasive prenatal diagnosis should be offered for confirmation of a positive NIPT result

Different situations leading to false positive results can be linked to abnormal cfDNA circulating in maternal blood from a source from the fetus, as might be the case of a woman with cancer, as recently reported by Osborne at the American College of Medical Genetics and Genomics annual Clinical Genetics Meeting in March 2013 [33]. In this report he describes a false NIPT diagnosis of both trisomies 13

| Detected Trisomies            | Natera Panorama | Verinata Verifi | Sequenom MaterniT21 PLUS | Ariosa Harmony |
|-------------------------------|-----------------|-----------------|--------------------------|---------------|
| Identified Trisomies          | 13, 18, 21      | 13, 18, 21, sex chromosomes | 13, 18, 21, sex chromosomes | 13, 18, 21, sex chromosomes |
| Identified Monosomies         | X Chromosome    | X Chromosome    | X Chromosome             | X Chromosome  |
| Method                        | Single Nucleotide Polymorphism | Massive Parallel Sequencing | Massive Parallel Sequencing | Selective Sequencing |

| Detection Rate | FPR |
|----------------|-----|
| Trisomy 21     | 0.1% |
| Trisomy 18     | 0.1% |
| Trisomy 13     | 0.1% |

Table 2: NIPT is not diagnostic, since it can render false positive results.
A personalized approach must be sought for with each patient, and informed consent must be obtained in each case. Direct interviews must be held before deciding to perform the test and it is mandatory that the results are delivered personally by a geneticist or a specialist in perinatal medicine in order to answer all possible questions regarding the test's significance.

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