IJPSR (2013), Vol. 4, Issue 4 **(Review Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 01 January, 2013; received in revised form, 30 January, 2013; accepted, 13 March, 2013

NON-INVASIVE PRENATAL DIAGNOSIS: A REVIEW

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Keywords:

Prenatal diagnosis, Biochemical screening, Non-invasive prenatal testing, Cell free fetal DNA, Aneuploides

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ABSTRACT: Aneuploidies are one of the important causes of perinatal morbidity and mortality. Initially screening for aneuploidies started with maternal age risk estimation. Later on, serum testing for biochemical markers and ultrasound markers were added. Women detected to be at high risk for aneuploidies were offered invasive testing. Recently, various methods including non-invasive prenatal testing (NIPT) by analysis of cell-free fetal DNA (cffDNA) in maternal blood has shown promise for highly accurate detection of common fetal autosomal trisomies. Incorporating these new noninvasive technologies into clinical practice will impact the current prenatal screening paradigm for fetal aneuploidy, in which genetic counseling plays an integral role. The advantage of the technique being elimination of risks such as miscarriage associated with invasive diagnostic procedures. But then this new technique has its own set of technical limitations and ethical issues at present and further research is required before implementation. Data was obtained through a literature search via Pubmed and Google as well as detailed search of our library database.

INTRODUCTION: Prenatal screening and diagnosis are routinely offered in antenatal care, and are considered to be important in managing pregnancy and allowing women to make informed choices about the continuation of pregnancies affected by developmental abnormalities [1]. The feasibility of prenatal diagnosis is something that continues to change rapidly with scientific advances, so it cannot be too strongly stressed that the person giving genetic counselling must obtain accurate information on this point before suggesting the possibility to a couple, and must be satisfied that the technique is reliably applicable as a service rather than just as a research procedure.

QUICK RESPONSE CODE

IJPSR: ICV (2011)- 5.07

Article can be accessed online on: www.ijpsr.com

Failure to do this is as reprehensible as submitting a patient to some new surgical procedure without enquiring as to its benefit and mortality. This is especially relevant when using new molecular advances, where the boundary between research discovery and established techniques can be hard to define, especially for very rare disorders, or those where the gene has been recently isolated.

When prenatal diagnosis is being considered in genetic counselling, several basic factors must be examined, but the most important is whether the couple concerned actively wish for prenatal diagnosis; all too often it is suggested simply because it may be technically feasible and without adequate information. Because most prenatal diagnostic procedures involve a large amount of worry to the parents, and a significant morbidity and mortality to the fetus (with 100 per cent mortality if the test proves abnormal and termination is requested),

prenatal diagnosis should normally be carried out only if the general criteria summarized in **Table 1** are fulfilled. These are self-evident, but as in most clinical situations, cases of real doubt may occur.

TABLE 1: CRITERIA FOR PRENATAL DIAGNOSIS

Criteria for prenatal diagnosis
• Is the disorder sufficiently severe to warrant termination of the pregnancy? • Is treatment absent or unsatisfactory? • Is termination of an affected pregnancy acceptable to the couple concerned? • Is an accurate prenatal diagnostic test available? • Is there a significant genetic risk to the pregnancy?

Women with positive-screening results will require a definitive diagnosis through amniocentesis, chorionic villus sampling (CVS) or cordocentesis. These 'invasive tests' will carry a risk for miscarriage and maternal complications. They are also expensive, anxiety provoking and likely to be completed relatively late in pregnancy. The multistep processes involved in reaching a final diagnosis requires informed consent and detailed counselling and this often needs to include providing information about other, sometimes much milder, disorders that are serendipitously identified.

Recognizing all of these difficulties, the hope has been that a set of precise, early, noninvasive prenatal diagnosis (NIPD) methods could be developed [1]. Following the discovery of substantial amounts of conceptus derived cell-free DNA (usually referred to as cell-free fetal DNA or cffDNA) and RNA (cffRNA) in maternal circulation [2, 3], expectations are now high that such routine prenatal testing will soon emerge. In this article, we review the potential impact of these developments with emphasis on the clinical use. Information was obtained through a literature search via Pubmed and Google using key words like 'aneuploidy screening', 'non invasive prenatal diagnosis' and 'cell free fetal DNA'. The internet search was accompanied by a detailed search of our library database. The articles were then reviewed and summarized in a comprehensive manner.

Genetic counselling: A couple may approach the doctor seeking preconception or early pregnancy genetic advice for a variety of reasons, including:

- A possibly heritable condition in one (or both) of the couple
- A history of infertility
- History of recurrent pregnancy loss
- A family history of one or more possibly heritable conditions
- The couple is from a population group with a high frequency of certain genetic diseases
- The couple is blood relatives (a consanguineous marriage)
- Advanced age
- The couple is anxious about reproductive risks, even though there is no specific indication that they are at increased risk.

An accurate diagnosis of the disorder is very essential for any genetic counselling. It is defined as "the process by which patients or relatives at risk of a disorder are advised of the consequences of the disorder, the probability of developing and transmitting it, the ways in which this can be ameliorated". During the genetic counselling process the counsellor should try to ensure that the consultand (an individual who seeks genetic counselling) is provide with information that enables him or her to understand:

1. The medical diagnosis and its implications in terms of prognosis and possible treatment
2. The mode of inheritance of the disorder and the risk of developing and/ or transmitting it
3. The choices or options available for dealing with the risks.

Genetic counselling should include a strong communicative and supportive element, so that those who seek information are able to reach their own fully informed decisions without undue pressure or stress. The main elements of genetic counselling can be summarized as:

- Diagnosis – based on accurate family history, medical history, examinations and investigations
- Documentation and family pedigree information
- Recognition of inheritance patterns
- Risk assessments
- Communications
- Discussions of options
- Long term contact and support

Current Screening Technologies: Maternal Serum Screening: Current screening technologies generally involve measuring biochemical markers associated with trisomy 21, as well as trisomy 18 and 13, in maternal serum during the first and/or second trimester. An ultrasound measuring fetal nuchal translucency (NT) is often included in a first trimester screen as an additional marker of trisomy 21. After first or second trimester screening, the woman typically receives a revised risk for trisomy 21 that is calculated based on her maternal age risk, the results of the serum screen, and the NT measurement (if available).

An advantage of screening is that it is non-invasive, thus posing no risk to the fetus. Only women whose revised risk exceeds a laboratory and test-dependent cut-off are candidates for diagnostic testing by chorionic villus sampling or amniocentesis. An inherent limitation of this approach is that screening only detects 85-95% of fetuses with trisomy 21, and falsely designates 3-6% of pregnancies as "positive" when they are, in fact, unaffected with trisomy 21. For every trisomy 21 fetus detected, perhaps 25 will be subjected to the risk of an invasive procedure.

Current Testing Technologies: **Chorionic Villus Sampling (CVS) or Amniocentesis:** An alternative to screening is invasive prenatal diagnosis by chorionic villus sampling (CVS) or amniocentesis which directly assesses the chromosome constitution of the fetus through cells from the pregnancy. The advantage is the diagnostic certainty of detecting trisomy 21, 18 and 13. In addition, testing fetal cells and the amniotic fluid may allow for the detection of other chromosome abnormities, genetic conditions, or open neural tube defects (**Table 2**). Although this approach to the fetal testing is gold standard and gives definitive diagnosis, the chances of miscarriage (around 1%) and invasiveness makes it inconvenient to pregnant women [4]. Thus, the need for the non-invasive methods of detection of fetal cells led to detection of these fetal cells in the cervical mucus [5, 6] and in maternal blood.

TABLE 2: PERFORMANCE OF DIFFERENT METHODS OF SCREENING AND TESTS FOR TRISOMY 21

Method of screening	Detection rate (%)	FPR (%)
MA	30	5
First trimester		
MA + fetal NT		
MA + serum free β-hCG and PAPP-A	60–70	5
MA + NT + free β-hCG and PAPP-A (combined test)	85–95	5
Combined test + nasal bone or tricuspid flow or ductus venosus flow	93–96	2.5
Second trimester		
MA + serum AFP, hCG (double test)	55-60	5
MA + serum AFP, free β-hCG (double test)	60-65	5
MA + serum AFP, hCG, uE3 (triple test)	60-65	5
MA + serum AFP, free β-hCG, uE3 (triple test)	65-70	5
MA + serum AFP, hCG, uE3, inhibin A (quadruple test)	65-70	5
MA + serum AFP, free β-hCG, uE3, inhibin A (quadruple test)	70-75	5
MA + NT + PAPP-A (11–13 weeks) + quadruple test	90-95	5
Invasive diagnostic testing		
Chorionic villus sampling	Close to 100%	
Amniocentesis	Close to 100%	

Development of NIPT: In 1997, Lo et al. first discovered cell-free fetal DNA in the plasma of pregnant women [2]. Fetal DNA can be detected from the 4th week of gestation, though only reliably from 7 weeks, and the concentration increases with gestational age-from the 16 fetal genomes per ml of maternal blood in the first trimester to 80 fetal genomes per ml in the third trimester, with a sharp

peak during the last 8 weeks of pregnancy. Fetal DNA originates trophoblast cells, and comprises around 3–6% of the total cell-free DNA in maternal circulation during early and late pregnancy, respectively (the other 94–97% being maternal cell-free DNA). Unlike cellular DNA, circulating cffDNA consists predominantly of short DNA fragments rather than whole chromosomes, of which 80% are <193 base-pairs in length. In contrast to fetal cells, cffDNA is rapidly cleared from the maternal circulation with a half life of 16 minutes and is undetectable after 2 hours of delivery [7]. In 1990, Bianchi et al first isolated intact fetal nucleated red blood cells for the purpose of prenatal diagnosis [8].

Since then, the isolation and detection of fetal cells from maternal blood has been extensively investigated by different researchers [9, 10] and various methods of fetal cell enrichment were developed [11]. In 2008, two research groups used massively parallel sequencing (MPS) of maternal plasma to detect an over representation of material from chromosome 21 in pregnancies affected with trisomy 21 [12, 13]. Other technologies for noninvasive prenatal testing for specific chromosome aneuploidies are currently being developed [14].

Three published clinical trials validated MPS to detect common aneuploidies with a high sensitivity and specificity (see **Table 3**).

TABLE 3: RESULTS FROM THREE PUBLISHED CLINICAL TRIALS THAT MEASURED MPS' SENSITIVITY AND SPECIFICITY IN DETECTING COMMON ANEUPLOIDIES

	Trisomy 21		Trisomy 18		Trisomy 13	
	Sensitivity (95% CI)	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
Palomaki *et al*., 2011	98.6% (95.9 - 99.7)	99.8% (99.4 - 99.9	-	-	-	-
Palomaki *et al*., 2012	-	-	100% (93.9 -100)	99.7% (99.3 -99.9)	91.7% (61-99)	99.1% (98.5 - 99.5)
Bianchi *et al*., 2012	100% (95.9 – 100)	100% (99.1 – 100)	97.2% (85.5 –99.9)	100% (99.2 – 100)	78.6% (49.2-99.9)	100% (99.2 – 100)

This led to the clinical availability of NIPT in high-risk pregnancies in the United States, beginning in late 2011. Palomaki et al. [15] demonstrated the ability of MPS of maternal plasma to detect fetal trisomy 21 with a near 99-percent sensitivity and specificity in high-risk pregnancies, defined by maternal age, family history, or positive serum and/or sonographic screening tests.

The group then published an analysis from the same study [16] demonstrating the detection of trisomy 18 at 100-percent sensitivity with a false-positive rate of 0.28 percent, and trisomy 13 at 91.7-percent sensitivity with a false-positive rate of 0.97 percent. The overall detection rate for trisomy 13, 18, and 21 was reported as 98.9 percent sensitivity with a false-positive rate of 1.4 percent.

Methods of detecting cffDNA: The basic principle in extracting the cffDNA is to take initially maternal plasma, separate cellular matter by centrifugation, followed by isolation and purification of all cell-free DNA, followed by exploiting the small differences between the fetal and maternal DNA sequences in order to make a specific fetal diagnosis [7].

The most common technique currently used for detection and identification of specific cffDNA sequences are: Polymerase chain reaction (PCR). Most popular types of PCR used are real-time quantitative PCR [17], nested PCR [18], pyrophosphorolysis-activated polymerization PCR [19], digital PCR which allows the exact number of original template DNA molecules to be counted [20].

Mass spectrometry [21], in which the precise mass of each DNA fragment is analysed to determine the genetic sequence, and hence detect fetal-specific alleles that differ from the maternal sequence by as little as a single base.

Multiplexed maternal plasma DNA sequencing analysis to rule out fetal trisomy 21 among high risk pregnancies [22].

Current clinical use of NIPD: First, the routine use of NIPD for fetal Rh D provides a demonstrable advantage over current Rh D detection. Briefly, Rh D is a protein on the surface of red blood cells, which is present in an overwhelming percentage of the population.

For those pregnant women who do not have the Rh D protein, or are Rh D negative, the consequences can be severe. The pregnant woman's immune system will produce antibodies against the fetal blood, especially during a second or later pregnancy, causing such life-threatening or fatal conditions as jaundice, brain damage, or heart damage.

NIPD allows for faster determinations of the Rh factor status of the mother and fetus [23]. It lessens misdiagnosis (where both the mother and fetus are Rh D negative), and the unnecessary exhaustion of medical resources. Additionally, because invasive prenatal screening and diagnostic techniques may allow fetal blood to enter the maternal blood stream needlessly, NIPD decreases the risk of anti-Rh D antibody production.

Sex determination was the first application of NIPD. Because the Y chromosome is absent in the genome of the pregnant woman, detection and measurement of fetal-derived paternally inherited DNA was the first focus of researchers in the prenatal screening field. While, detection and identification of fetal sex-linked or sex limited conditions is considered a legitimate medical reason for NIPD testing of sex, the more common use of pre-conception and prenatal technology for sex-determination is based on preference [24].

It is mainly helpful for sex-linked disease, such as haemophilia, Duchenne muscular dystrophy, X-linked mental retardation, adrenoleuko-dystrophy, Alport's syndrome, X-linked severe immunodeficiency, retinitis pigmentosa, X-linked hydrocephalus, anhidrotic ectodermal dysplasia, Hunter's syndrome, Menke's syndrome and Lesch–Nyhan syndrome [7]. Sex determination is also important in cases where development of external genitalia is ambiguous and in some endocrine disorders, such as congenital adrenal hyperplasia (CAH), where there is masculinization of the female fetus, which is preventable with antenatal treatment.

Single gene disorders can be detected by identifying a paternally inherited allele in cffDNA; Huntington's disease, achondroplasia, myotonic dystrophy, fetal carrier status in cystic fibrosis, hemoglobinopathy [7].

Noninvasive prenatal diagnosis for fetal aneuploidy: One of the applications of NIPD that appears to be close to clinical implementation is a test for fetal-chromosome abnormalities, notably Down syndrome. This testing is envisaged as being available to all women in the first trimester of pregnancy and would potentially replace current screening and diagnostic methods. Recently, noninvasive prenatal testing (NIPT) by analysis of cffDNA in maternal blood has shown promise for highly accurate detection of common fetal autosomal trisomies [25]. Analysis of cffDNA has been validated in several clinical studies utilizing next generation DNA sequencing technology [26]. Clinical studies have primarily included women identified by prior screening, with maternal age and biochemical and/or sonographic testing in the first or second trimester of pregnancy, to be at high risk for aneuploidies.

In a recently published article of noninvasive prenatal testing of fetal trisomies in a routinely screened first trimester population showed that NIPT with a chromosome-selective sequencing approach is highly accurate for fetal aneuploidy detection with very low FPR. The estimated trisomy risk score was > 99% in all cases of trisomy 21 and trisomy 18 and < 1% in 99.9% of the euploid cases [27].

There is less confidence in NIPT as a screen for trisomy 13 due to technical issues and the infrequency of the condition. Detection rates between 79-92% have been reported, meaning between 8 to 21 out of 100 pregnancies with affected fetuses will be missed. The false-positive rate may be about 1%, so 1 out of 100 unaffected pregnancies may be positive for trisomy 13, so confirmatory testing is recommended.

Implications of a positive result: NIPT is highly sensitive and specific for trisomies 21 and 18; positive results are 'near diagnostic'. However, false positives have been reported so at this time it is recommended that positive results be followed with confirmatory testing by CVS or amniocentesis [28].

Confirmatory testing can also provide important information about the cause of the trisomy; specifically, CVS or amniocentesis will identify cases of Down syndrome that are due to a 21 chromosome translocation as opposed to the more common trisomy 21. This has important recurrence risk implications for the parents and other family members. Fetal anatomic ultrasound can also be a helpful tool for pregnancies that test positive on NIPT, looking for additional ultrasound findings that support the diagnosis.

Implications of a negative result: Even though NIPT is highly sensitive and specific, it is important to remember that it is not 100%. There are false-negative results, so a negative result cannot absolutely rule out an affected fetus. A laboratory may provide a risk score, allowing the clinician to quantify risk for trisomy.

Implications of an 'unreportable' or 'no-call' result: Depending on the laboratory, 0.5-7% of women who undergo NIPT will not get a result, often because there is an insufficient amount of fetal DNA in the sample (low fetal fraction) due to various clinical reasons which may include high maternal weight or early gestational age [28].

A laboratory may decline to report results near the cutoff. In any case, the clinician must determine, in conjunction with the NIPT laboratory and the patient, whether to draw another sample later in the pregnancy, revert to conventional serum or ultrasound screening, move on to invasive testing, or decline any further testing.

Technical difficulties: There are a number of technical and clinical obstacles to achieving high diagnostic accuracy [7]:

- It is important to emphasize that complete fetal genotyping is not conceivable using cffDNA in the maternal circulation and that the genetic information derived from cffDNA is entirely restricted to the specific DNA sequence (or chromosome) detected.
- False negatives can be the result of failure to extract or detect sufficient material, due to individual variability in the amount of total cell-free DNA and the small proportion of fetal versus maternal cell-free DNA.
- False positives can be the result of either technical issues, such as contamination, or clinical abnormalities such as the presence of a nonidentical vanishing twin.

Ethical issues: Widespread clinical implementation of NIPD is likely to have significant societal consequences. One issue will be the degree of equity as to which groups will have access. NIPD may be costly and only covered by some types of medical insurance policies. If so, it could be yet another technology disproportionately available to the affluent. Indeed, a consequence of these inequalities could be the perception that NIPD constitutes a contemporary form of eugenics with the affluent, educated, or other selected groups having a greater ability to determine the genetic characteristics of their children [29].

Increased testing accompanied by a higher rate of pregnancy terminations could reduce the birth incidence and ultimately the total prevalence of at least some debilitating genetic disorders in the population. The question has already been raised as to whether trends in prenatal screening and diagnosis are likely to translate into the disappearance of births with Down syndrome [30]. The decline in the number of children born with disabilities may lead to subtle changes in public attitudes about the handicapped and their families.

Some disability advocates contend that prenatal genetic testing, with its implicit aim of preventing the birth of disabled babies, undermines the worth of individuals living with disabilities [31]. NIPD is likely to accelerate this trend.

The ethical implications of sex-selection are well documented. Sex selective breeding and sex selective abortion are most commonly associated with the nations of India and China, whose overpopulations concerns have led to growth control policies, generally targeting girl children [32].

Professional Society statements: Professional societies are beginning to make statements about the use of NIPT. ACOG (American College of Obstetricians and Gynecologists) recommends offering aneuploidy screening or invasive testing to all women, regardless of age. The ACOG and SMFM both say that cffDNA testing can be offered to pregnant women at increased risk for trisomy 13, 18, or 21.

Women age 35 and older, women with a history of a child with trisomy, and women carrying a fetus that shows abnormalities on an ultrasound are at increased risk. The cffDNA test should not be offered to low-risk women or women carrying multiple fetuses because it has not been sufficiently tested in these groups. A patient with positive result should be referred for genetic counselling and offered invasive prenatal testing for confirmation of test results.

Cell free fetal DNA does not replace the accuracy and diagnostic precision of prenatal diagnosis with CVS or amniocentesis, which remain an option for women [33].

ISPD (International Society of Prenatal Diagnosis): ISPD recognizes that NIPT can be helpful as a screening test for women who are at high risk for Trisomy 21 with suitable genetic counseling. A positive test should be confirmed through invasive testing.

NSGC (National Society of Genetic Counselors) [34]

1. NSGC recognizes NIPT as an option for aneuploidy assessment in pregnancy: Peer-reviewed data currently supports NIPT only as a screening tool for select populations. While abnormal NIPT results have a high positive predictive value, NIPT results should not be considered diagnostic at this time, and any abnormal results should be confirmed through a conventional prenatal diagnostic procedure, such as chorionic villus sampling or amniocentesis.

2. NSGC does not currently support NIPT as a routine, first-tier aneuploidy screening test in low-risk populations: To date, these technologies have been validated only in pregnancies considered to be at an increased risk for fetal aneuploidy, based on maternal age, family history, or positive serum and/or sonographic screening tests.

3. Clinical studies show that MPS effectively detects fetal trisomy 21, trisomy 13, and trisomy 18. MPS has not yet been proven efficacious in detecting other chromosomal abnormalities or single-gene disorders, and clinical trials for other technologies have not yet been published. NSGC recommends that pretest counselling for NIPT include information about the disorders that it may detect, its limitations in detecting these conditions, and its unproven role in detecting other conditions.

4. Pre- and post-NIPT genetic counselling: As with any prenatal testing, patients must have accurate, up-to-date information regarding the test, the possible results, and the available follow-up in order to make an informed choice when considering NIPT. Given NIPT's vastly superior sensitivity and specificity compared to other available aneuploidy screening –such as, first-trimester nuchal translucency and/or biochemical screening and second-trimester quad screening – it is imperative that patients understand the significant implications of a positive result prior to undergoing NIPT. NSGC recognizes that, due to limited resources, it may not be feasible for all women seeking NIPT to receive pretest counselling from a genetic counsellor. But a qualified healthcare provider should provide nondirective pretest counselling for all women considering NIPT. NSGC recommends that any patient with abnormal NIPT results should receive genetic counselling with a certified genetic counsellor and be given the option of conventional confirmatory diagnostic testing.

5. NSGC recommends that patients who have other factors suggestive of a chromosome abnormality should receive genetic counselling and have the option of conventional confirmatory diagnostic testing, regardless of NIPT results: Because NIPT does not screen for all chromosomal or genetic conditions, it does not replace standard risk assessment and prenatal diagnosis. In addition, patients who have an increased risk for genetic conditions that are beyond NIPT's scope should receive genetic counselling to discuss appropriate testing options.

FUTURE: The number of pregnancies for which there is NIPT data is still relatively small. As the population of patients who elect the test grows, experience in the performance of the test will increase, especially in sub-populations of pregnancies. Validation studies are underway in 'low risk' women and results should be available within a few years. It is expected that labs will continue to explore the number of conditions that can be detected using circulating cffDNA.

This could include using other testing technologies to interrogate the fetal DNA, such as chromosome microarray testing to detect microdeletion and duplication syndromes. Studies to assess clinical validity in the general population (e.g. low-risk women) are currently underway. As the sensitivity and specificity in the general population are better established, it is likely that NIPT will become a diagnostic test for fetal chromosomal aneuploidy for routine use in all pregnancies.

Single-gene testing will also be possible, as this is an area of ongoing research [35]. Legal issues have been raised regarding patent rights surrounding the technology being used by several laboratories. It is unclear at this time how these issues will be resolved.

Conflict of interest: The authors have no conflicts of interest.

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How to cite this article:
Dey M, Agarwal S and Sharma S: Non-Invasive Prenatal Diagnosis: A Review. *Int J Pharm Sci Res* 2013; 4(4); 1348-1355.