J Compr Ped. 2014 August; 5(3): e23254. DOI: 10.17795/compreped-23254

Published online 2014 August 25. **Review Article**

Noninvasive Prenatal Test by Cell-Free Fetal DNA in Maternal Plasma: Current Progress and Prospective Clinical Applications

Mahsa Motavaf [1]; Majid Sadeghizadeh [1,*]

[1]Department of Genetics, Tarbiat Modares University, Tehran, IR Iran

*Corresponding author: Majid Sadeghizadeh, Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, P. O. Box: 14115-175, Tehran, IR Iran. Tel: +98-2182884409, Fax: +98-2182883463, E-mail: sadeghma@modares.ac.ir

Received: August 2, 2014; **Accepted:** August 16, 2014

Context: Prenatal testing aims to identify fetal chromosomal and genetic disorders prior to delivery. Current invasive procedures such as amniocentesis and chorionic villus sampling (CVS) pose a risk to mother and fetus and such diagnostic procedures are available only to high-risk pregnancies, which limits aneuploidy detection rate. The identification of cell-free fetal DNA (cffDNA) in maternal circulation has made noninvasive prenatal testing (NIPT) possible. This review seeks to highlight the necessity of investing in NIPT and briefly summarizes the technical aspects of the NIPT and application of this method in clinical practice.

Evidence Acquisition: PubMed, OVID, SCOPUS, and the Cochrane database were searched for relevant articles published between 1995 and 2014, using appropriate keywords including prenatal screening, noninvasive testing, prenatal diagnosis, cell free fetal DNA, maternal circulation, chromosomal aneuploidies, trisomy, and sex determination. Results were restricted to systematic reviews, randomized clinical trials, meta-analysis, and observational studies.

Results: The importance of prenatal diagnosis and risks associated with current invasive techniques makes NIPT research morally and commercially beneficial. The outstanding advantages of NIPT over current prenatal diagnosis techniques include increasing detection rate, enabling earlier diagnosis, and eliminating iatrogenic fetal loss and risk to the mother due to invasive procedures. At present, two major techniques for isolating cffDNA, namely digital PCR and massively parallel sequencing (MPS), have enabled the successful implementation of NIPT into clinical practice such as fetal sex determination, RhD genotyping, and fetal chromosomal aneuploidy detection.

Conclusions: The advent of new NIPT using cffDNA has been regarded as a revolution in prenatal testing and has attracted significant commercial interest in the field. It is not overoptimistic to predict that NIPT will supplement or replace existing screening and diagnostic tools.

Keywords: Noninvasive; Genetic Testing; Prenatal Diagnosis; Cell-Free System; Fetus; DNA; Chromosomes; Aneuploidy

1. Context

Increasing maternal age increases risk of the three most common autosomal aneuploidies in live births, namely, Down's syndrome (T21), Edwards' syndrome (trisomy 18 or T18), and Patau's syndrome (trisomy 13 or T13). Antenatal screening is routinely offered to all pregnant women in the United States, England, and many parts of the developed world. The so-called combined test is usually performed at 11 to 13 weeks of gestation, using ultrasonography and/or analysis of various maternal serum biochemical markers. Thereafter, women with high-risk pregnancies are traditionally offered prenatal diagnosis including CVS and amniocentesis (AC). Both of these diagnostic tests are invasive and have miscarriage risk rates of 0.5% to 1% (1). Furthermore, the combined test per se has a 5.0% to 9.0% false positive rate (2, 3); consequently, mothers with healthy fetuses may choose to undergo further invasive testing with the risk of miscarriage. Identifying a less invasive approach to prenatal testing has been the focus of much research over recent decades. The initial attempts were based on the isolation of fetal cells in the maternal circulation (4); however, following the introduction of cffDNA in maternal plasma (5), efforts to develop NIPT turned towards the analysis of cffDNA (6). The cffDNA is a useful potential source of fetal genetic material to use for prenatal testing, as it is present in the maternal circulation from early in pregnancy and is rapidly cleared from maternal plasma shortly after delivery (7), making it pregnancy specific. However, maternal plasma cell free DNA contains both of maternal and fetal DNA, of which fetal DNA represents a minor fraction, constitutes approximately 3% to 6% of the total DNA (8). Therefore, sophisticated methods with high sensitivity and accuracy are required to detect and differentiate fetal DNA from the existing maternal DNA background. In recent years, technical advances in the molecular analysis of fetal DNA, such as digital PCR and massively parallel sequencing (MPS), have enabled the successful implementation of NIPTs such as fetal sex assessment, rhesus D (RhD) genotyping, and fetal chromosomal aneuploidy detection into clinical practice. With the ability to distinguish the

entire fetal genome from maternal plasma DNA, we foresee that an increased number of NIPTs will be available for detecting many genetic disorders in the near future. This review seeks to highlight the technical aspects of the NIPTs and their application in clinical practice.

2. Evidence Acquisition

We searched PubMed, OVID, SCOPUS, and the Cochrane database for relevant English language articles published between 1995 and 2014, using appropriate controlled vocabulary and keywords as following: prenatal screening, noninvasive testing, prenatal diagnosis, cell free fetal DNA, maternal circulation, chromosomal aneuploidies, trisomy, and sex determination. Results were restricted to systematic reviews, randomized clinical trials, meta-analysis, and relevant observational studies. Totally, 273 articles were found in primary search from which 60 articles were enrolled in this study.

3. Results

3.1. Prenatal Testing of Aneuploidies

3.1.1. Current Methods for Prenatal Testing of Fetal Aneuploidies

Prenatal testing for fetal aneuploidy includes both screening and diagnosis. At 11 to 13 weeks of gestation, a combination of tests is performed to screen for abnormalities and score the risk of the fetus having T21 and, to a lesser extent, T18. The combined test includes ultrasonography to check nuchal translucency and analyzing two biochemical markers, i.e. human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein A, in maternal blood samples. These are combined with factors such as maternal age, previous pregnancy, and the history of familial genetic abnormalities. Depending on the utilized screening approach, the accuracy of screening ranges from 75% to 96% with false-positive rates ranging from 5% to 10% (9-11). Noninvasive tests are offered to all pregnant women in the United States, England, and many other parts of developed world. Positive screening results prompt further prenatal diagnostic testing. In current diagnostic procedures, i.e. CVS and AC, the samples for karyotyping are collected through invasive methods, posing a risk of 0.5% to 1% to both mother and fetus (1). CVS can be performed early in the pregnancy, typically between 10th and 13th weeks of gestation, and involves transcervical or transabdominal tissue sampling from the placenta. By short-term direct culturing of actively dividing villous cytotrophoblastic tissue, preliminary results may be obtained within one to two days of sampling. However, following long-term culture of mesenchymal cells from the villi, the final karyotype result would take up to ten days in most cases. A general disadvantage of CVS approach lies primarily in the extra-embryonic source of this tissue; in other words, the cells come from the trophectoderm and not the fetus itself. Although fetus and placenta originate from the same zygote, in some cases (1%-2%), a divergence between the chromosomal constitution of cells in the placenta and cells in the fetus, known as chromosomal mosaicism, can occur and lead to ambiguity in diagnosis (12, 13). In addition, the risk of spontaneous abortion, following CVS may be as high as 1% (14, 15). AC, on the other hand, is the process of aspirating some amniotic fluid by inserting a needle into the amniotic sac. The amniotic fluid contains fetal cells (amniocytes), which typically originate from fetal urine, pulmonary secretions, cells shed from the respiratory track, and skin. These amniocytes are cultured, harvested, and subjected to karyotype and/or genetic analysis. AC is usually offered after 15 weeks of gestation because at this point, sufficient amniotic fluid has surrounded the fetus, making it possible to aspirate the fluid (about 20 mL) without significant risk for the fetus. Earlier AC is associated with higher rates of pregnancy loss. However, this invasive procedure is associated with a small but real risk of miscarriage as well (0.5%-1%).

3.1.2. Noninvasive Prenatal Tests for Prenatal Diagnosis of Aneuploidies

The importance of prenatal diagnosis of aneuploidies and risks associated with current invasive techniques makes NIPT research morally and commercially beneficial. First attempts to identify less invasive approaches were based on the isolation of fetal cells in maternal circulation (4). The presence of fetal cells in maternal blood was initially documented in 1969 (16) and the possibility that these cells could be recovered and analyzed during pregnancy suggested an exciting new noninvasive approach for identifying fetal genetic disorders. In normal pregnancies, different nucleated fetal cell types including trophoblasts, erythroblasts, lymphocytes, granulocytes, and possibly, mesenchymal stem cells (17) enter the maternal circulation without causing immune response. As an erythroid progenitor in adult blood is scarce in comparison with its quantitative constitution in fetal blood, most studies have focused on this cell type (18). The detection of certain fetal aneuploidies from a maternal blood sample has been accomplished using fluorescence in situ hybridization (FISH) for chromosome-specific DNA probes followed by various sorting and enrichment procedures (19-22). Although a few commercial entities continue to persevere with aneuploidy testing of intact fetal cells, utilization of approach has dismantled for a variety of reasons. The main obstacles for the use of fetal cells include lack of fetal specific markers (23), confined placental mosaicism (24), and persistence of fetal cells in the maternal circulation years after pregnancy (25). Furthermore, the presence of fetal cells in maternal blood in normal pregnancies has been estimated to be very low (at 1-2/mL), which further hampers their use

(26). In 1997, Lo et al. (5) reported the presence of cffDNA from the Y chromosome of male fetuses in the maternal plasma during pregnancy. It is present as early as fifth to seventh weeks of gestation, released continually by apoptotic cells throughout pregnancy, and is typically cleared from circulation within a matter of hours. Fetal DNA can be detected from the fourth week of gestation (27), making it possible for NIPT to be achievable earlier in pregnancy than for commercial invasive methods. However, the concentration is only reliably from seventh week, and it increases with gestational age, from the equivalent of 16 fetal genomes per milliliter of maternal blood in the first trimester to 80 in the third trimester (28), with a sharp peak during the last eight weeks of pregnancy (8). In contrast to fetal cells, cffDNA is rapidly cleared from the maternal circulation with a half-life of 16 minutes and is undetectable in the maternal circulation within two hours postpartum, which makes it specific to the current pregnancy (7). Paternally inherited alleles are detected by qualitative evaluation of maternal plasma; however, prenatal diagnosis of aneuploidies is based on dose and consequently, it is quantitative. The cffDNA constitutes only 3.0% to 6.0% of cell free DNA in maternal plasma (29), which is the main obstacle to quantitative approach for detection of aneuploidies. An aneuploid fetus bears an abnormal number of chromosomes. Due to the minor population of fetal DNA in maternal circulation, identifying the fetal chromosomal abnormalities requires stringent quantitative analysis, making NIPT of fetal aneuploidies more challenging than NIPT of paternally inherited features. For example, chromosome 21 represents approximately 1% to 1.5% of the entire human genome and an extra copy would increase the amount of DNA of this chromosome from 1.5% to 2.25%. If the proportion of cffDNA in the maternal circulation were 6%, the relative change in the total cffDNA sample would only increase from 1.5% to 1.565%. It was originally assumed that direct quantification of fetal chromosome dose in maternal plasma is unfeasible, as this small amount of fetal chromosome dose would be lost in the background of maternal one. Because conventional PCR methods are not sufficiently sensitive to measure this relatively small amount of changes in level of chromosome 21, different approaches are required. Initial attempts to overcome this obstacle were focused on the elimination of interference from the background maternal DNA by using fetal-specific markers, such as RNA (30, 31) and epigenetic markers (32). Applying fetal-specific mRNA markers in maternal plasma was based on testing fetal specific cell-free mRNA from placenta-specific 4 (*PLAC4*) gene, located on chromosome 21, which is expressed in the placenta but not in maternal blood (32). In this method, by extracting cffRNA (rather than cffDNA) from maternal plasma and testing a single nucleotide polymorphism (SNP), located in the PLAC4 fetal mRNA sequence, the chromosome 21 allelic ratios were determined to infer chromosome 21 dosages. Diagnostic sensitivity and specificity of this approach (called RNA-SNP) was indicated to be almost 100% (32, 33). However, this method has some limitations. The major drawback to SNP-based approaches is the reliance of this approach on polymorphisms within the DNA carrying the placenta-specific expression, which makes their use limited to heterozygous fetuses (32). An alternative method of using cffRNA was suggested to be the epigenetic allelic ratio (EAR) approach that has been based on differences in methylation patterns of maternal and fetal DNA. In 2009, Papageorgiou et al. (34) published a set of fetal-specific epigenetic markers for all the common chromosomal aneuploidy and subsequently, reported accurate NIPT for T21 using methylated DNA immune precipitation real-time PCR (35). However, to date, no large-scale validation study has been reported using this method. NIPT based on differential methylation has yet to find a place in clinical practice, because the use of epigenetic markers is limited by relatively labor-intensive and time-consuming bisulfite conversion or restriction enzyme digestion, which makes them less practical for use in a routine service laboratory. More recent studies are based on new sophisticated analytic methods, such as digital PCR and MPS. Digital PCR involves multiple PCR on a single DNA template from extremely diluted samples, thus, generating amplicons that are exclusively derived from one template (36) and permit counting the individual template molecules. The proportion of positive amplifications among the total number of analyzed PCRs allows an estimation of the template concentration in the original non-diluted sample. This method allows detecting less than two-fold changes in copy number. When applying this method in NIPT, the maternal plasma DNA templates are diluted to a single template molecule. Then, the target loci are amplified and quantified to allow precise measurement of DNA molecules derived from candidate chromosomes (36, 37).

MPS or Next Generation Sequencing (NGS) is a new generation sequencing technology that allows high throughput single molecule counting. Two seminal proof-of-principle experiments published in 2008 (38, 39) demonstrated the feasibility of MPS as a powerful tool for NIPT of T21. In brief, whole genome cffDNA extracted from maternal plasma is sequenced to generate millions of short sequence reads or "tags". The sequence reads are then aligned and mapped to the human genome to identify their reference human genome sequence. Thereafter, the individual uniquely mapped reads to chromosome 21 are counted and compared to the number of counts obtained from a reference euploid sample; then its genomic representation is calculated. The overrepresentation of chromosome 21 would indicate the presence of a fetus with T21. With a relatively small sample size, both of these proof-of-concept studies demonstrated 100% sensitivity and specificity for T21 detection (38, 39). Two approaches to NIPT for T21 using NGS are now commonly used in the United States, Asia, and some parts of Europe (40).

In order to increase the throughput and reduce the

cost of MPS, the alternative approaches such as multiplex sequencing (39) and genomic targeted loci sequencing (41, 42) have also been employed. The goal of multiplex sequencing is to sequence multiple patient samples simultaneously in a single run. The goal of targeted MPS is to enrich the regions from the chromosome(s) under evaluation (chromosome 21, 18, and/or 13) before sequencing. This significantly reduces the amount of required sequencing and is primarily aimed at reducing costs while increasing throughput and test performance. Regardless of the chosen approach, the sensitivity and specificity of these methods are high, ranging from 98.6% to 100% and from 99.7% to 100%, respectively. NIPT for other common aneuploidies, i.e. trisomies 13 and 18, have been reported with lower detection rates, which is caused by the larger chromosome size and higher GC content (40, 41, 43). Combined data from five studies report a sensitivity of 97.4% (188/193) for trisomy 18 (40, 41, 43); however, only three of these studies (44, 45) included data for trisomy 13 and reported a lower sensitivity of 83.3% (30/38).

3.1.3. Noninvasive Prenatal Tests of Paternally Inherited Features

The development of NIPT has allowed for the identification of paternally inherited genetic markers, which are not present in the maternal genome. The first application for NIPT of paternally inherited features is fetal sex determination. In 1997, Lo et al. (5) detected Y chromosome specific sequence (*DYS14*) in maternal plasma, using conventional Y-PCR method. Relative feasibility of distinguishing the Y chromosome of a male fetus from maternal DNA has made the fetal sex determination the most common clinical application of NIPT. In a study conducted in Iran (46), the sequences of single copy *SRY* gene and multi copy *DYS14* and *DAZ* genes on the Y chromosome of the male fetuses were detected by nested PCR. The results showed sensitivity of 95.2% in sex determination. A meta-analysis systematically combined the results from 57 independent studies from 1997 to 2011, including 3524 male-bearing and 3017 female-bearing pregnancies (47). The majority of these studies have analyzed Y chromosome specific sequences, such as *SRY* and *DYS14* in maternal plasma, applying real-time PCR. For detection of a male fetus, the overall sensitivity and specificity were 95.4% and was 98.6%, respectively. More recent publications have reported this high detection accuracy as well (48, 49). The traditional sonographic fetal sex determination is often performed after 13th gestational week (50), whereas the cffDNA-based approach can be reliably conducted between seventh and twelfth weeks of gestational (47). This is of significant value in certain clinical conditions. For instance, in congenital adrenal hyperplasia, the pregnancy of an affected female fetus would elicit the administration of dexamethasone to prevent virilization (51). Furthermore, in the case of X-linked genetic disorders such as hemophilia, Duchene muscular dystrophy, X-linked mental retardation, adrenoleukodystrophy, Alport's syndrome, retinitis pigmentosa, and X-linked hydrocephalus, early determination of fetal sex allows up to 50% of women to avoid an unnecessary invasive diagnostic test. Due to its reliable performance, a number of countries have adopted this method into clinical practice for at risk pregnancies (52, 53). Another application of NIPT of paternal inherited features is detection of fetal RhD status in RhD negative mothers. In order to eliminate the risk of all immunization, it is suggested that all RhD negative mothers receive RhD immunoglobulin prophylaxis. Consequently, a number of RhD negative mothers who carry RhD negative fetuses would receive unnecessary prophylaxis treatment. A more logical approach is to provide RhD immunoglobulin prophylaxis only to RhD negative mothers carrying RhD positive fetuses. The use of cffDNA to genotype the fetal RhD by detecting the presence of RhD sequences in RhD negative mothers was reported for the first time in 1998, by Lo et al. (54) and Faas et al. (55). Following that report, a number of large-scale clinical trials have been conducted to evaluate its performance further. A systematic review of these studies from 2006 to 2008 demonstrated high diagnostic accuracy (sensitivity, 99.5%-99.8%; and specificity, 94.0%-99.5%) (56). Due to its reliable performance and noninvasive nature, a number of countries have adopted this test in clinical practice (57).

3.1.4. Implementation of Noninvasive Prenatal Tests in to Clinical Practice

MPS-based NIPT for T21 has been recently launched by more than four commercial companies and several clinical laboratories in the United States, China, and Europe. A number of studies have been published to report the initial clinical laboratory experience in NIPT for fetal aneuploidy using maternal plasma (58-61). NIPT can be integrated into prenatal screening and diagnosis practice through three potential clinical approaches: 1) NIPT can replace the current maternal serum screening approaches; 2) NIPT can be performed as an intermediate step after the screening and before the invasive diagnostic testing; or 3) replacement of invasive diagnostic testing by NIPT is suggested as well. Adoption of each of these options is depended on multiple factors such as clinical performance (sensitivity and specificity) and practical considerations (test availability, the ease of the method, cost-effectiveness, and timeliness). Professional societies such as the International Society for Prenatal Diagnosis (ISPD), American College of Obstetricians and Gynecologists (ACOG), the Society for Maternal Fetal Medicine (SMFM), the International Society for Prenatal Diagnosis (ISPD), the National Society of Genetic Counselors (NSGC), and the Society of Obstetricians and Gynecologists of Canada (SOGC) have all published their opinions on how to implement the MPS-based NIPT for fetal aneuploidy into clinical practice (8, 41, 60, 62). Reviewing the published

data on NIPT indicated that these professional societies agree that NIPT is a safe and effective primary screening test for fetal aneuploidy in high-risk pregnancies based on their age, the presence of anomalies in ultrasonography, history of aneuploidy, and in those pregnancies at risk for aneuploidy due to the presence of a Robertsonian translocation in a parent. NIPT can also be used as a complementary test for women who have a positive maternal serum screening test.

4. Conclusions

The presence of cffDNA in maternal circulation is promising for the development of NIPT. However, detection of small amount of circulating fetal DNA in the large background of maternal DNA is technically challenging. In recent years, development of highly sensitive and precise molecular techniques such as digital PCR and MPS has overcome technical obstacles. These methods have enabled the successful adoption of NIPT into clinical practice such as fetal sex determination, RhD genotyping, and fetal chromosomal aneuploidy detection. Due to its noninvasive nature, high diagnostic accuracy, broad applications, and availability at an earlier gestational age, NIPT has the tremendous potential to become standard prenatal genetic testing.

Acknowledgements

The authors would like to thank Dr. Saeid Safari for his helping hands in preparing the manuscript.

Authors' Contributions

Mahsa Motavaf: study concept and design, critical revision of the manuscript for important intellectual content, acquisition of data, and drafting the manuscript; Majid Sadeghizadeh: critical revision of the manuscript for important intellectual content, study supervision, and drafting the manuscript.

References

1. Tabor A, Alfirevic Z. Update on procedure-related risks for prenatal diagnosis techniques. *Fetal Diagn Ther.* 2010;**27**(1):1–7.
2. Go AT, van Vugt JM, Oudejans CB. Non-invasive aneuploidy detection using free fetal DNA and RNA in maternal plasma: recent progress and future possibilities. *Hum Reprod Update.* 2011;**17**(3):372–82.
3. Hahn S, Lapaire O, Tercanli S, Kolla V, Hosli I. Determination of fetal chromosome aberrations from fetal DNA in maternal blood: has the challenge finally been met? *Expert Rev Mol Med.* 2011;**13**.
4. Bianchi DW. From Michael to microarrays: 30 years of studying fetal cells and nucleic acids in maternal blood. *Prenat Diagn.* 2010;**30**(7):622–3.
5. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet.* 1997;**350**(9076):485–7.
6. Chitty LS, Bianchi DW. Noninvasive prenatal testing: the paradigm is shifting rapidly. *Prenat Diagn.* 2013;**33**(6):511–3.
7. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet.* 1999;**64**(1):218–24.
8. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet.* 1998;**62**(4):768–75.
9. Benn PA, Fang M, Egan JF, Horne D, Collins R. Incorporation of inhibin-A in second-trimester screening for Down syndrome. *Obstet Gynecol.* 2003;**101**(3):451–4.
10. Nicolaides KH. Nuchal translucency and other first-trimester sonographic markers of chromosomal abnormalities. *Am J Obstet Gynecol.* 2004;**191**(1):45–67.
11. Acog Committee on Practice Bulletins . ACOG Practice Bulletin No. 77: screening for fetal chromosomal abnormalities. *Obstet Gynecol.* 2007;**109**(1):217–27.
12. Ledbetter DH, Zachary JM, Simpson JL, Golbus MS, Pergament E, Jackson L, et al. Cytogenetic results from the U.S. Collaborative Study on CVS. *Prenat Diagn.* 1992;**12**(5):317–45.
13. Goldberg JD, Wohlferd MM. Incidence and outcome of chromosomal mosaicism found at the time of chorionic villus sampling. *Am J Obstet Gynecol.* 1997;**176**(6):1349–52.
14. Alfirevic Z, Sundberg K, Brigham S. Amniocentesis and chorionic villus sampling for prenatal diagnosis. *Cochrane Database Syst Rev.* 2003(3):CD003252.
15. American College of O, Gynecologists.. ACOG Practice Bulletin No. 88, December 2007. Invasive prenatal testing for aneuploidy. *Obstet Gynecol.* 2007;**110**(6):1459–67.
16. Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet.* 1969;**1**(7606):1119–22.
17. Hahn S, Holzgreve W. Prenatal diagnosis using fetal cells and cell-free fetal DNA in maternal blood: what is currently feasible? *Clin Obstet Gynecol.* 2002;**45**(3):649–56.
18. Forestier F, Daffos F, Catherine N, Renard M, Andreux JP. Developmental hematopoiesis in normal human fetal blood. *Blood.* 1991;**77**(11):2360–3.
19. Elias S, Price J, Dockter M, Wachtel S, Tharapel A, Simpson JL, et al. First trimester prenatal diagnosis of trisomy 21 in fetal cells from maternal blood. *Lancet.* 1992;**340**(8826):1033.
20. Pezzolo A, Santi F, Pistoia V, De Biasio P. Prenatal diagnosis of triploidy using fetal cells in the maternal circulation. *Prenat Diagn.* 1997;**17**(4):389.
21. Ganshirt-Ahlert D, Borjesson-Stoll R, Burschyk M, Dohr A, Garritsen HS, Helmer E, et al. Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting. *Am J Reprod Immunol.* 1993;**30**(2-3):194–201.
22. Simpson JL, Elias S. Isolating fetal cells from maternal blood. Advances in prenatal diagnosis through molecular technology. *JAMA.* 1993;**270**(19):2357–61.
23. Avent ND, Plummer ZE, Madgett TE, Maddocks DG, Soothill PW. Post-genomics studies and their application to non-invasive prenatal diagnosis. *Semin Fetal Neonatal Med.* 2008;**13**(2):91–8.
24. Wapner RJ. Invasive prenatal diagnostic techniques. *Semin Perinatol.* 2005;**29**(6):401–4.
25. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A.* 1996;**93**(2):705–8.
26. Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet.* 1997;**61**(4):822–9.
27. Illanes S, Denbow M, Kailasam C, Finning K, Soothill PW. Early detection of cell-free fetal DNA in maternal plasma. *Early Hum Dev.* 2007;**83**(9):563–6.
28. Birch L, English CA, O'Donoghue K, Barigye O, Fisk NM, Keer JT. Accurate and robust quantification of circulating fetal and total DNA in maternal plasma from 5 to 41 weeks of gestation. *Clin Chem.* 2005;**51**(2):312–20.
29. Bischoff FZ, Sinacori MK, Dang DD, Marquez-Do D, Horne C, Lewis DE, et al. Cell-free fetal DNA and intact fetal cells in maternal blood circulation: implications for first and second trimester non-invasive prenatal diagnosis. *Hum Reprod Update.* 2002;**8**(6):493–500.
30. Poon LL, Leung TN, Lau TK, Chow KC, Lo YM. Differential DNA

methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin Chem.* 2002;**48**(1):35–41.
31. Papageorgiou EA, Karagrigoriou A, Tsaliki E, Velissariou V, Carter NP, Patsalis PC. Fetal-specific DNA methylation ratio permits noninvasive prenatal diagnosis of trisomy 21. *Nat Med.* 2011;**17**(4):510–3.
32. Lo YM, Tsui NB, Chiu RW, Lau TK, Leung TN, Heung MM, et al. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nat Med.* 2007;**13**(2):218–23.
33. Tsui NB, Akolekar R, Chiu RW, Chow KC, Leung TY, Lau TK, et al. Synergy of total PLAC4 RNA concentration and measurement of the RNA single-nucleotide polymorphism allelic ratio for the noninvasive prenatal detection of trisomy 21. *Clin Chem.* 2010;**56**(1):73–81.
34. Papageorgiou EA, Fiegler H, Rakyan V, Beck S, Hulten M, Lamnissou K, et al. Sites of differential DNA methylation between placenta and peripheral blood: molecular markers for noninvasive prenatal diagnosis of aneuploidies. *Am J Pathol.* 2009;**174**(5):1609–18.
35. Tsaliki E, Papageorgiou EA, Spyrou C, Koumbaris G, Kypri E, Kyriakou S, et al. MeDIP real-time qPCR of maternal peripheral blood reliably identifies trisomy 21. *Prenat Diagn.* 2012;**32**(10):996–1001.
36. Lo YM, Lun FM, Chan KC, Tsui NB, Chong KC, Lau TK, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc Natl Acad Sci U S A.* 2007;**104**(32):13116–21.
37. Fan HC, Quake SR. Detection of aneuploidy with digital polymerase chain reaction. *Anal Chem.* 2007;**79**(19):7576–9.
38. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci U S A.* 2008;**105**(42):16266–71.
39. Chiu RW, Chan KC, Gao Y, Lau VY, Zheng W, Leung TY, et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc Natl Acad Sci U S A.* 2008;**105**(51):20458–63.
40. Boon EM, Faas BH. Benefits and limitations of whole genome versus targeted approaches for noninvasive prenatal testing for fetal aneuploidies. *Prenat Diagn.* 2013;**33**(6):563–8.
41. Sparks AB, Struble CA, Wang ET, Song K, Oliphant A. Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18. *Am J Obstet Gynecol.* 2012;**206**(4):319 e1–9.
42. Zimmermann B, Hill M, Gemelos G, Demko Z, Banjevic M, Baner J, et al. Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. *Prenat Diagn.* 2012;**32**(13):1233–41.
43. Bianchi DW, Platt LD, Goldberg JD, Abuhamad AZ, Sehnert AJ, Rava RP, et al. Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. *Obstet Gynecol.* 2012;**119**(5):890–901.
44. Palomaki GE, Deciu C, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, et al. DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: an international collaborative study. *Genet Med.* 2012;**14**(3):296–305.
45. Ashoor G, Syngelaki A, Wang E, Struble C, Oliphant A, Song K, et al. Trisomy 13 detection in the first trimester of pregnancy using a chromosome-selective cell-free DNA analysis method. *Ultrasound Obstet Gynecol.* 2013;**41**(1):21–5.
46. Zargari M, Sadeghi MR, Shahhosseiny MH, Kamali K, Saliminejad K, Esmaeilzadeh A, et al. Fetal Sex Determination using Non-Invasive Method of Cell-free Fetal DNA in Maternal Plasma of Pregnant Women During 6(th)- 10(th) Weeks of Gestation. *Avicenna J Med Biotechnol.* 2011;**3**(4):201–6.
47. Devaney SA, Palomaki GE, Scott JA, Bianchi DW. Noninvasive fetal sex determination using cell-free fetal DNA: a systematic review and meta-analysis. *JAMA.* 2011;**306**(6):627–36.
48. Macher HC, Noguerol P, Medrano-Campillo P, Garrido-Marquez MR, Rubio-Calvo A, Carmona-Gonzalez M, et al. Standardization non-invasive fetal RHD and SRY determination into clinical routine using a new multiplex RT-PCR assay for fetal cell-free DNA in pregnant women plasma: results in clinical benefits and cost saving. *Clin Chim Acta.* 2012;**413**(3-4):490–4.
49. Wikman AT, Tiblad E, Karlsson A, Olsson ML, Westgren M, Reilly M. Noninvasive single-exon fetal RHD determination in a routine screening program in early pregnancy. *Obstet Gynecol.* 2012;**120**(2 Pt 1):227–34.
50. Odeh M, Granin V, Kais M, Ophir E, Bornstein J. Sonographic fetal sex determination. *Obstet Gynecol Surv.* 2009;**64**(1):50–7.
51. Nimkarn S, New MI. Prenatal diagnosis and treatment of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Mol Cell Endocrinol.* 2009;**300**(1-2):192–6.
52. Hill M, Finning K, Martin P, Hogg J, Meaney C, Norbury G, et al. Non-invasive prenatal determination of fetal sex: translating research into clinical practice. *Clin Genet.* 2011;**80**(1):68–75.
53. Hill M, Lewis C, Jenkins L, Allen S, Elles RG, Chitty LS. Implementing noninvasive prenatal fetal sex determination using cell-free fetal DNA in the United Kingdom. *Expert Opin Biol Ther.* 2012;**12 Suppl 1**:S119–26.
54. Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med.* 1998;**339**(24):1734–8.
55. Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet.* 1998;**352**(9135):1196.
56. Legler TJ, Muller SP, Haverkamp A, Grill S, Hahn S. Prenatal RhD Testing: A Review of Studies Published from 2006 to 2008. *Transfus Med Hemother.* 2009;**36**(3):189–98.
57. Finning K, Martin P, Daniels G. A clinical service in the UK to predict fetal Rh (Rhesus) D blood group using free fetal DNA in maternal plasma. *Ann N Y Acad Sci.* 2004;**1022**:119–23.
58. Lau TK, Chan MK, Lo PS, Chan HY, Chan WS, Koo TY, et al. Clinical utility of noninvasive fetal trisomy (NIFTY) test–early experience. *J Matern Fetal Neonatal Med.* 2012;**25**(10):1856–9.
59. Fairbrother G, Johnson S, Musci TJ, Song K. Clinical experience of noninvasive prenatal testing with cell-free DNA for fetal trisomies 21, 18, and 13, in a general screening population. *Prenat Diagn.* 2013;**33**(6):580–3.
60. Dan S, Wang W, Ren J, Li Y, Hu H, Xu Z, et al. Clinical application of massively parallel sequencing-based prenatal noninvasive fetal trisomy test for trisomies 21 and 18 in 11,105 pregnancies with mixed risk factors. *Prenat Diagn.* 2012;**32**(13):1225–32.
61. Futch T, Spinosa J, Bhatt S, de Feo E, Rava RP, Sehnert AJ. Initial clinical laboratory experience in noninvasive prenatal testing for fetal aneuploidy from maternal plasma DNA samples. *Prenat Diagn.* 2013;**33**(6):569–74.
62. Masuzaki H, Miura K, Yoshiura KI, Yoshimura S, Niikawa N, Ishimaru T. Detection of cell free placental DNA in maternal plasma: direct evidence from three cases of confined placental mosaicism. *J Med Genet.* 2004;**41**(4):289–92.