Down syndrome

Chapter 6

Down's Syndrome with a Particular Focus on Screening Test- circulatory DNA

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Abstract

In 1988 Wald and colleagues reported on a method of screening Down syndrome using the three biochemical markers (AFP, hCG and uE 3) together with maternal age as parameters in a single test, known as the 'triple test'. With the triple test 60% of all Down syndrome cases could be prenatally detected at a 5% false positive rate (FPR). Later on, hCG was replaced with the free beta subunit of hCG (fβ-hCG), and inhibin-A was added to get the 'quadruple test'.

The focus of prenatal screening for DS shifted towards the first trimester of pregnancy. Except for fβ-hCG, maternal serum pregnancy-associated plasma protein A (PAPP-A) was introduced in a test together with an enlarged nuchal translucency (NT) on a first-trimester ultrasound scan. Combining these three screening markers (fβ-hCG, PAPP-A and NT) with maternal age, we got the 'first trimester combined test' .With this test approximately 85-90% of all DS cases could be detected at a 5% FPR.

Currently, more and more research focuses on the genomic detection of DS in maternal blood, with the use of massive parallel genomic sequencing (MPS). This technique can identify and quantify many DNA fragments in a relatively short time, either in nuclei of foetal cells or free-floating in maternal serum and has been proven as an accurate and reliable method to detect fetal chromosome aneuploidies.

Definitive diagnosis of fetal chromosomal aneuploidies still requires the performance of invasive procedures such as chorionic villus sampling (CVS) or amniocentesis, which are associated with a risk of miscarriage.

Keywords: Down syndrome; Screening tests; Circulatory DNA

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History

For centuries, people with Down syndrome have been alluded to in art, literature and science. Although other people had previously recognized the characteristics of the syndrome, it was Down who described the condition as a distinct and separate entity.

In recent history, advances in medicine and science have enabled researchers to investigate the characteristics of people with Down syndrome.

1. Introduction- Explanation of how 21 Trisomy Develop, kinds of 21 Trisomies

Typically, the nucleus of each cell contains 23 pairs of chromosomes, half of which are inherited from each parent. Down syndrome occurs when an individual has a full or partial extra copy of chromosome 21. There are three types of Down syndrome: trisomy 21 (nondisjunction), translocation and mosaicism [1-3].

Most cases, ninety- five per cent of infants with Down's syndrome, type of Down syndrome called trisomy 21 because of nondisjunction, which results in an embryo with three copies of chromosome 21 instead of the usual two. Prior to or at conception, a pair of 21st chromosomes in either the sperm or the egg fails to separate. More than 90% of the cases of chromosomal nondisjunction are of maternal origin, mainly during meiosis I, about 5% involve an additional paternal extra chromosome and a small proportion (2%) is consequence of post-zygotic mitotic non-disjunction [3,4].

Less commonly, four per cent, translocation Down syndrome occurs when part of chromosome 21 becomes attached (translocated) to another chromosome during the formation of reproductive cells (eggs and sperm) in a parent or very early in fetal development. Usually the two long arms of chromosome 21 are fused, and the two short arms lost (Robertsonian translocation), so that there are still 46 chromosomes. Affected people have two normal copies of chromosome 21 plus extra material from chromosome 21 attached to another chromosome, for example 13,14,1 or 22 resulting in three copies of genetic material from chromosome 21 [5].

A very small percentage of people with Down syndrome, about one per cent, have an extra copy of chromosome 21 in only some of the body's cells. In these people, the condition is called mosaic Down syndrome. Mosaicism occurs when nondisjunction of chromosome 21 takes place in one - but not all - of the initial cell divisions after fertilization. When this occurs,

there is a mixture of two types of cells, some containing the usual 46 chromosomes and others containing 47. Those cells with 47 chromosomes contain an extra chromosome 21 [5].

2. Down's Syndrome Phenotype

It is believed that having extra copies of genes on chromosome 21 disrupts the course of normal development, causing the characteristic features of Down syndrome and the increased risk of health problems associated with this condition.

DS phenotype is complex and varies among individuals, who may present a combination of dysmorphic features and developmental delay. The intellectual disability is a characteristic observed in all cases and the most frequent clinical features include muscular hypotonia (99%), diastasis of the muscle rectus of abdomen (90%), upslanted palpebral fissures (90%), microcephaly (85%), flat occipital (80%), joint hyperextension (80%), broad hands with short fingers (70%), short stature (60%), clinodactyly of fifth finger (50%), epicanthal fold (40%), low-set ears (50%), single palmar crease (40%), atlantoaxial instability (15%) and label-femoral instability (10%). [3]. On average, 50-70% of children with DS have congenital heart defects, such as ventricular septal defect, atrial septal defect, tetralogy of Fallot, patent ductus arteriosus and atrioventricular septal defect [6,7]. The rest most common group of abnormalities are those of the intestinal tract, notably duodenal atresia (0,018 %), ocular problems, such as refractive errors, nystagmus, abnormalities of the retina, [7] hearing loss 80%, thyroid dysfunction, particularly hypothyroidism (0,7 %), [6] periodontal diseases, upper airway obstruction, hypogonadism. Down's syndrome is also associated with urinary tract malformations, limbs defects, immunodeficiency, [8] increased risk for hematological disorders, leukemia (acute megakaryoblastic leukaemia) (1-2 %), [9] epilepsy (9 %) and early onset of Alzheimer's disease (77 %). **Table 1** shows which genes in chromosome 21 are associated with different phenotypes of DS.

Table 1: View on the genes present in chromosome 21, associated with phenotypes of DS.

Gene symbol	Gene location	Candidate gene for
APP	21q21.3	Neurodegeneration
BACH1	21q22.11	Alzheimer's disease-like neuropathological changes
DOPEY2	21q22.2	Functional brain alterations and mental retardation
DSCAM	21q22.2	Mental retardation and the precocious dementia
DYRK1A	21q22.13	Leukemogenesis, Impaired brain development, Early onset of neurofibrillary degeneration
ERG	21q22.3	Alzheimer's disease-like neuropathological changes
OLIG2	21q22.11	Developmental brain defects
SIM2	21q22.13	Impairment of learning and memory, Pathogenesis of mental retardation
SOD1	21q22.11	Neurodegeneration
PCP4	21q22.2	Abnormal neuronal development

3. Epidemiology

One in every 691 babies in the United States is born with Down syndrome, making Down syndrome the most common genetic condition. In about 1 in 150 pregnancies, the fetus has Down's syndrome but over three-quarters of these die in a miscarriage. The incidence of miscarriage is about 43 per cent for Down's syndrome fetuses alive at 10 weeks of gestation, and 23 per cent for those alive at 16 weeks.

Down syndrome occurs in people of all races and economic levels, though older women have an increased chance of having a child with Down syndrome (see **Table 2**). There is no definitive scientific research that indicates that Down syndrome is caused by environmental factors or the parents' activities before or during pregnancy.

All 3 types of Down syndrome are genetic conditions (relating to the genes), but only 1% of all cases of Down syndrome have a hereditary component (passed from parent to child through the genes).

Table 2: Risk for DS and over all Chromosomal abnormality in relation to mother's age.

Maternal Age at Delivery (years)	*Risk of DS*	*Risk of Any Chromosomal Abnormality*
20	1/1650	1/530
25	1/1250	1/480
30	1/950	1/390
35	1/385	1/180
40	1/100	1/65
45	1/30	1/19

Since many couples are postponing parenting until later in life, the incidence of Down syndrome conceptions is expected to increase.

The likelihood of having a second child with Down syndrome is high, it is estimated that her chances of having another baby with trisomy 21 is 1 in 100 up until age 40.

The risk of recurrence of translocation is about 3% if the father is the carrier and 10-15% if the mother is the carrier. Genetic counseling can determine the origin of translocation.

4. Screening and Diagnostic Methods

There are several methods that allow the early detection of DS in prenatal phase. At this point, it is not possible avoid congenital malformations or genetic diseases, but the objective is its early detection, looking for emotional and psychological preparation for parents and family and adequate medical support and monitoring for the child's birth.

4.1 Nuchal translucency (NT)

Among the screening methods are the nuchal translucency test, the measurement of maternal serum concentrations of various fetoplacental products and fetal ultrasound. The NT test is the measurement of the fluid filled fold at the back of the fetal neck in the first trimester of pregnancy, performed through transabdominal or transvaginal sonography. The test is performed between the 11th and 13th weeks of gestation and the minimum fetal crown–rump length (CRL) should be 45 mm and the maximum 84 mm, fetal NT increases with CRL and therefore it is essential to take gestation into account [3]. Nowadays, it is well established that the measurement of fetal NT thickness provides effective and early screening for trisomy 21 and other major aneuploidies, such as Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13) besides for screening of congenital heart disease. In **Table 3** is shown the power of NT measurement. In case of abnormality in NT measurement, additional tests are needed to elucidate the cause of increased nuchal fold [3,10,11].

Table 3: A chance of normal birth dependent of NT measurement.

NT measurement	Chance of normal birth
≤ 3.4mm	95%
3.5 – 4.4mm	70-86%
4.5 – 5.4mm	50-77%
5.5 – 6.4mm	67%
≥ 6.5mm	31%

4.2 The triple test

Pregnancies with fetal aneuploidies are associated with altered maternal serum concentrations of various fetoplacental products, including alpha-fetoprotein (AFP), free chorionic gonadotropin (β-hCG), unconjugated estriol (uE3), inhibin A (INH-A) and pregnancy associated plasma protein-A (PAPP-A) [11-13]. AFP is produced in the yolk sac and fetal liver, while uE3 and hCG are produced by the placenta. Elevated β-hCG concentration and low levels of AFP and uE3 suggests the presence of a fetus with DS [14]. The triple test which consist of measurement of concentration of maternal serum AFP, β-hCG, uE3 is performed in second trimester of pregnancy and the values should be adjusted to gestational age The expected detection rate and false-positive rate are about 73 - 78% and 7.5 - 9%, respectively [15].

4.3 Quadruple test

The incorporation of INH-A into maternal serum DS screening in the second trimester, along with AFP, hCG and uE3, is named quadruple test [3,16]. INH-A is a glycoprotein mainly secreted from the corpus luteum and the placenta and its concentration is raised in the serum of pregnant women carrying a fetus with DS. The quadruple test presents expected detection

rate and false-positive rate about 79 - 82% and 6.5 - 7.8%, respectively [15]. The measurement of PAPP-A is also used as a screening gestations of fetus with DS in the first trimester, the reduced concentration of PAPP-A at 10–14 weeks of pregnancy is characteristic for fetal DS during the first trimester of pregnancy [13].

4.4 Ultrasound

The fetal ultrasound is also considered a method of screening for DS, any change in the development of organs or structures is easily visualized [17]. Besides increased nuchal translucency in the first trimester, alterations commonly detected in DS in the second trimester of gestation include lack of visualization of the nasal bone, [18] reduced femur and humerus, mild pyelectasis, hyperechoic bowel and echogenic intra cardiac focus. An ultrasound scan examination is used to estimate gestational age and should be done before the serum test is interpreted [17,19].

4.5 Amniocentesis

Among the invasive methods for obtaining fetal cells, the amniocentesis is the method indicated for obtaining fetal cells between 15 -20 weeks of gestation. If this test is performed earlier may result in fetal injury. The term "early amniocentesis" is sometimes used to describe use of the process between weeks 11 and 13. This requires taking a small sample of amniotic fluid trans abdominally under ultrasound guidance. The procedure-related fetal loss rate is about 0.4-0.8 % [3,20,21]. After 20th week of gestation, the option is percutaneous umbilical blood sampling or cordocentesis, which involves direct sampling of fetal blood from the umbilical cord. The procedure-related loss rate is about 1.0-1.5% and cordocentesis with placenta penetration had a significantly higher rate of fetal loss [22,23].

Complications of amniocentesis includepreterm laborand delivery, respiratory distress, postural deformities,chorioamnionitis, fetal trauma and alloimmunisation of the mother (rhesus disease). Amniotic fluid embolismhas been described as a possible risk [20,22,23].

This process can be used for prenatal sex discernment and hence this procedure has legal restrictions in some countries.

Amniocentesis can predict fetal lung maturity, which is inversely correlated to the risk of infant respiratory distress syndrome and also detects problems such as infection, in which detect a decreased glucose level, aGram stain showing bacteria or an abnormal differential count of white blood cells, Rh incompatibility, Decompression of polyhydramnios. Moreover, an emerging indication for amniocentesis is in the management of preterm rupture of membranes where measurement of certain amniotic fluid inflammatory markers, especially IL-6, may be helpful [24].

After obtaining fetal cells, conventional karyotype analysis has been used for the past few decades as the gold standard for the prenatal diagnosis of numerical and major structural chromosomal abnormalities such as fluorescence in situ hybridization (FISH) , polymerase chain reaction quantitative fluorescent (QF-PCR), the multiplex ligation-dependent probe amplification (MLPA) test and DNA sequencing, can also be used for a rapid diagnosis of aneuploidies of sex chromosomes and trisomies [21,13,18]. It has been showed that QF-PCR technique presents 95.4% sensitivity, 100% specificity, 99.5% efficiency and is less laborious than the FISH technique, less time consuming, and some results were obtained in eight hours. The sensitivity, specificity, and efficiency of the assay for detecting DS using this technique are about 95.4%, 100%, and 99.5%, respectively [25].

4.6 CVS

Chorionic villus sampling (CVS) entails sampling of the chorionic villus (placental tissue) and testing it for chromosomal abnormalities, usually with FISH or PCR. CVS usually takes place at 10–12 weeks' gestation, earlier than amniocentesis or percutaneous umbilical cord blood sampling. It is the preferred technique before 15 weeks [26].

Test results take about 2 weeks. An obstetrician can perform this procedure in about 5 minutes, after preparation.

Chorionic villus sampling does not detect: Neural tube defects (these involve the spinal column or brain), Rh incompatibility, Birth defects (which are problems in the way the baby's body forms). This test can help detect more than 200 disorders, but can miss some genetic defects. Abnormal results may be due to a number of different genetic conditions, including: Down syndrome, Hemoglobinopathies, Tay-Sachs disease.

The risks of CVS are only slightly higher than those of an amniocentesis. Possible complications include: Bleeding, Infection, Miscarriage, Rh incompatibility in the mother, Rupture of membranes. When CVS is performed after 9 weeks gestational age, limb problems are no more frequent than in pregnancies without such testing.

5. Newer Non-Invasive Methods

Considering the risks which accompany invasive methods for obtaining fetal cells, the use of noninvasive methods could be a good .The most recent breakthroughs have involved analyzing circulatory DNA- cell-free fetal DNA in the mother's blood. This has come to be known as 'non-invasive prenatal testing' (NIPT), because all that is needed is a maternal blood test.

Although NIPT for Down's syndrome is not completely diagnostic, large scale studies show that the test has a detection rate of over 99 and can also look for Edward's and Patau's

syndrome [27,28].

This test can be done from 10 weeks of pregnancy. The sample is then sent to a laboratory abroad to be analyzed. We can detected fetal DNA circulating freely in the maternal blood stream which can be sampled by venipuncture on the mother [29].

5.1 CFF DNA

Cell-free fetal DNA comes from the placenta, the trophoblasts. The fetal DNA is fragmented and makes its way into the maternal bloodstream via shedding of the placental micro particles into the maternal bloodstream. It is estimated that 2-6% of the DNA in the maternal blood is fetal in origin. It is first detectable from about 4-5 weeks' gestation and reaches the required level needed to test for Down syndrome by 10 weeks' gestation in most pregnancies. The cff DNA is cleared from the maternal circulation within the first hour after birth, and therefore we know that it is specific to the woman's current pregnancy. However, it is important to remember that NIPT for Down syndrome analyses both the baby's and the mother's cell-free DNA.

Cell-free fetal DNA is significantly smaller than the maternal DNA in the bloodstream, with fragments approximately 200bp in size. Many protocols to extract the fetal DNA from the maternal plasma use its size to distinguish it from the maternal DNA.

There are some new protocols for testing non-compatible RhD factors, sex determination forX-linked genetic disorders and testing for single gene disorders. Current studies are now looking at determining aneuploidies in the developing fetus. These protocols can be done earlier than the current prenatal testing methods, and have no risk of spontaneous abortion, unlike current prenatal testing methods. Non-invasiveprenatal diagnosis(NIPD) has been implemented in the UK and parts of the US; as technology continues to advance it is almost certain that we will see a shift from the current methods of chorionic villi sample(CVS) and amniocentesisto NIPD [30], which have procedure-related miscarriage risks of about 1 in 100 pregnancies and 1 in 200 pregnancies, respectively [31,32].

The general procedure is that a sample of the woman's blood is taken after 10 weeks of pregnancy but can be detected as early as the fifth week. The test measures the relative amount of free fetal DNA in the mother's blood which consists of approximately 2-6% of the total.

Limitations include the concentration of all cell-free DNA in maternal blood is low, the total amount of cell-free DNA varies between individuals, cell free fetal DNA molecules are out-numbered by maternal cell-free DNA molecules, the fetus inherits half the genome from the mother. However, there are ways around these limitations. Addition of formaldehyde to maternal blood samples increases the percentage of free fetal DNA. The purpose of formaldehyde is to

stabilize intact cells, and inhibit further release of maternal DNA. The mean percentage of free fetal DNA in maternal blood ranges from 0.32% to 40%, with a mean percentage of 7.7 [33] without formaldehyde-treatment. The mean percentage of free fetal DNA with formaldehyde treatment was 20.2%. Another way to increase the fetal DNA is based on physical length of DNA fragments. Fetal DNA is smaller in size, a standardized size fractionation can be [34] and can comprise up to 70% of total cell-free DNA.

5.2 PCR- detection

To detect fetal DNA, the majority of studies focus on detecting paternally inherited sequences. For example, primers can be designed to target the Y chromosome of male fetuses for polymerase chain reaction (PCR). A variety of methods have been used for mutation detection in fetal DNA.

Real -time PCR, or real-time PCR, also called quantitative PCR (qPCR) , either a modification of the polymerase chain reaction (called PCR, Polymerase Chain Reaction). This modification using appropriate dyes and probes, and appropriate equipment, allows you to monitor growth of the product in each successive cycle of the reaction.

In Real-time quantitative PCR Fluorescent probes are used to monitor the accumulation of amplicons produced throughout the PCR process. Thus, increase in reporter fluorescent signal is proportional to the number of amplicons generated. The appropriate real-time PCT protocol is designed according to the mutation or genotype to be detected. Point mutations are analyzed with qualitative real-time PCR with the use of allele-specific probes. Insertions and deletions are analyzed by dosage measurements using quantitative real-time PCR. Real-time PCR assays for single cell analysis have been developed for a Y-chromosome marker [35], a common Tay-Sachs disease mutation [33], the most common cystic fibrosis mutation [36] and a wide range of thalassemia mutations and Hb S [37].

The most important advantages of the use of technology Real-time PCR is its much higher sensitivity and precision. This technique allows us to analyze the material even with a low concentration of the starting DNA. Factor in favor of Real-time PCR is also the possibility of quantitative analysis indicating a specific numerical value specifying the number of copies of a particular amplicon in units of volume. Due to the fact that it does not require additional steps in order to visualize the products obtained, the method is much less time consuming. Omitting the step of transferring the samples to gel electrophoresis was also reduced risk of contamination of samples other products after the PCR reaction. Real - time PCR is also characterized by a wide range of concentrations assayed and the high reproducibility of the results [38]. In fact, the only drawbacks seem to be hardware requirements and higher cost of the analysis.

In 2010 was evaluated the use of nested PCR to detect the Y chromosome in fetal DNA of maternal plasma. The results show 96% sensitivity and 88% specificity [39].

Nested PCR involves the implementation of a two stage amplification. The products resulting from the first stage may still contain non-specifically amplified DNA fragments. Unwanted background is reduced by carrying out the subsequent reaction. The amplification product was obtained in the first PCR is duplicator in the second PCR, which significantly increases the sensitivity of the method and at the same time does not reduce its specificity. Advantages of Nested PCR amplification Less potential undesired sequences, Increased specificity, sensitivity Increased.

The other way is Digital PCR; method has been demonstrated as useful for studying variations in gene sequences. Digital PCR (dPCR) is a refinement of conventional PCR methods that can be used to directly quantify and clonally amplify nucleic acids (including DNA, cDNA, methylated DNA, or RNA). The key difference between dPCR and traditional PCR lies in the method of measuring nucleic acids amounts, with the former being a more precise method than PCR. PCR carries out one reaction per single sample. dPCR also carries out a single reaction within a sample, however the sample is separated into a large number of partitions and the reaction is carried out in each partition individually. This separation allows a more reliable collection and sensitive measurement of nucleic acid amounts.

Microfluidic devices have the ability to perform highly parallel analysis in a single PCR step [40]. Absolute quantification can be achieves rather than relative quantification compared to RT-PCR. Thus, point mutations, copy number variations, loss of heterozygosity or aneuploidy can be detected [41]. Digital PCR can differentiate between maternal blood plasma and fetal DNA in a multiplex fashion [40].

High-throughput shotgun sequencing technology from plasma of pregnant women, obtaining about 5 million sequence tags per patient sample can be done.

In this approach, genomic DNA is cut into pieces of about 150 Mb and inserted into BAC vectors, transformed into E. coli where they are replicated and stored. The BAC inserts are isolated and mapped to determine the order of each cloned 150 Mb fragment. This is referred to as the Golden Tiling Path. Each BAC fragment in the Golden Path is fragmented randomly into smaller pieces and each piece is cloned into a plasmid and sequenced on both strands. These sequences are aligned so that identical sequences are overlapping. These contiguous pieces are then assembled into finished sequence once each strand has been sequenced about 4 times to produce 8X coverage of high quality data [42].

In 2008, using this method, it was possible to identify aneuploid pregnancies; trisomy detected at gestational ages as early as 14th week. Shot-gun sequencing can be done with a

Solexa/Illumina platform. Whole fetal genome mapping by parental haplotype analysis using sequencing of cell free fetal DNA was done in 2010 [32].

6. Researches

Canick et al. conducted a study on prenatal testing for Down syndrome (trisomy 21), trisomy 18 and trisomy 13 by massively parallel shotgun sequencing (MPSS) of circulating cell free DNA in pregnant women with multiple gestations. Although study size is limited, the authors concluded that this data provides evidence that MPSS testing can be reliably used as a secondary screening test for Down syndrome in women with high-risk twin gestations [43].

Ehrich et al. evaluated a multiplexed massively parallel shotgun sequencing assay for noninvasive trisomy 21 detection using circulating cell-free fetal DNA. The overall classification showed 100% sensitivity and 99.7% specificity. The authors reported that extending the scope of previous reports, these results warrant clinical validation in a larger multicenter study [44].

Matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF MS) combined with base extension after PCR allows cell free fetal DNA detection with single base specificity and single DNA molecule sensitivity [45].

MALDI technique offers not destructive evaporation (desorption) and ionization of both small and large biomolecules.

Time-of-flight mass spectrometry (TOFMS) is a method of mass spectrometry in which an ion's mass-to-charge ratio is determined via a time measurement. Ions are accelerated by an electric field of known strength. The time that it subsequently takes for the particle to reach a detector at a known distance is measured. This time will depend on the mass-to-charge ratio of the particle (heavier particles reach lower speeds). From this time and the known experimental parameters one can find the mass-to-charge ratio of the ion.

Measurement of the molecular weight of the compound under analysis is done in a mass spectrometer (MS).

DNA is first amplified by PCR, then linear amplification with base extension reaction (with a third primer) is designed to anneal to the region upstream of the mutation site. Either 1-2 bases are added to the extension primer to produce two extension products from wild-type DNA and mutant DNA. Since single base specificity is achieved, it is better than hybridization-based techniques with Taqman probes. A study by Akolekar and colleagues genders of 90/91 males babies were confirmed, MALDI-TOF mass spectometry had a 99.1% accuracy with 98.9% sensitivity, 99.2% specificity [46].

There is also a possibility to exploid differences in gene activation between maternal DNA and fetal DNA. Most current research is focussed on using epigenetic modifications to detect cffDNA. Hypermethylated RASSF1A promoter has been reported as universal fetal marker to confirm the presence of cffDNA. cffDNA was extracted from the maternal plasma, digested with methylation-sensitive and insensitive restriction enzymes and RT-PCR analysis of RASSF1A, SRY, DYS14 was done. 79/90 (88%) of maternal blood samples were detected for hypermethylated RASSF1A [47].

In maternal plasma mRNA transcripts from genes expressed in the placenta are detectable [48]. The mixture of plasma is centrifuged and aqueous layer transferred, RNA is extracted. RT-PCR is set up for selected RNA expression. Specifically, hPL and beta-hCG mRNA is stable in maternal blood. This can help to confirm the presence of fetal DNA in the maternal plasma [49].

Reverse- transcription PCR is commonly used in molecular biology to detect RNA expression levels. RT-PCR is often confused with real-time polymerase chain reaction (qPCR) by students and scientists alike. However, they are separate and distinct techniques. While RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA. RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase. Subsequently, the newly synthesized cDNA is amplified using traditional PCR [50].

6.1 X-linked diseases, genetic disorders

Almost every woman wants to know the gender of her baby, especially if her child is a carrier of x-linked disease. Ultrasonography is unreliable during the first trimester of pregnancy, other methods for sex determination before the use of cffDNA are invasive and performed at 11 weeks of gestation. There is a small risk of miscarriage [51]. The main method is to target the SRY gene on the Y chromosome and DYS14 sequence [52,53].

In the case of X-linked diseases, if cffDNA can determine gender, invasive testing can be eliminated.

Lack of the Y chromosome in the maternal plasma suggests that the fetus is female, but could also indicate failure to detect cffDNA in the maternal plasma. Thus, paternal polymorphisms or sex independent markers are further used to detect cffDNA [54].

Invasive testing is used at 11 weeks of the pregnancy to determine the sex of the fetus. In Europe, tests for sex determination are available as early as 7 weeks of gestation;

There are also Genetic studies carried in families at high risk for inherited genetic disorders, some Severe monogenic diseases for which prenatal diagnosis is more commonly applied to include cystic fibrosis, beta-thalassemia, sickle cell anemia, spinal muscular atrophy, myotonic dystrophy, fragile-X syndrome, Duchenne muscular dystrophy and Hemophilia [55]. Both autosomal dominant and recessive disorders have been detected noninvasively by analyzing paternally inherited DNA. Limitations to single gene disorders are autosomal recessive mutation or when the fetal autosomal dominant mutation is maternally inherited. There are also large sequence mutations that include duplication, expansion, insertion of DNA sequences [56]. cff DNA is fragmented with 200-300bp in length, thus these are harder to detect. One example is achondroplasia, a common autosomal dominant form of dwarfism, caused by FGFR3 gene point mutations. Two pregnancies were examined in this study, one fetus was found to have the paternally inherited G1138A mutation and the other with a G1138A de novo mutation [57]. Another example is the Huntington's disease. It is currently diagnosed at 10–13 weeks at gestation with chronic villus sampling for polymorphic repeats. With qRT-PCR, there has been detection of CAG repeats at 17, 20 and 24, all normal levels [58].

6.2 CDN. Researches

It has been suggested that technology using cell-free fetal DNA may ultimately replace amniocentesis, invasive procedure, which In the United States still serves as the gold standard diagnostic tool for those women who require antenatal fetal blood genotyping. Rhesus blood group (D antigen) is used to determine the risk of hemolytic disease in the fetus. In hemolytic disease, the maternal antibodies destroy RhD-positive fetal red blood cells what leads to lethality for the fetus. A significant amount of blood can be exchanged between mother and infant during birth, CVS, amniocentesis and accidents.49 RHD gene determines the Rhesus D status [59]. Chinen et al. 2010 shows that 15% of Caucasian females, 3-5% of black African females and <3% of Asian females are RhD-negative. In the United Kingdom and other countries, cffDNA tests are now routinely being offered to RhD-negative patients at increased risk of isoimmunization. Anti-RhD immune globulin, a blood derivative, is only offered in the event of a RhD-positive fetus for these women [49,60]. In United States, prophylactic treatment is recommended for all RhD-negative pregnant women to prevent isoimmunization in case of RhD incompatibility [61].

Aneuploidy, which refers to abnormal number of chromosomes, can also be detected by cffDNA. A number of fetal nucleic acid molecules derived from aneuploid chromosomes can be detected including SERPINEB2 mRNA, clad B, hypomethylated SERPINB5 from chromosome 18, placenta-specific 4 (PLAC4), hypermethylatedholocarboxylasesynthetase (HLCS) and c21orf105 mRNA from chromosome 12 [62] With complete trisomy, the mRNA alleles in maternal plasmas isn't the normal 1:1 ratio, but is in fact 2:1. Allelic ratios determined by epigenetic markers can also be used to detect the complete trisomies. Massive parallel

sequencing and digital PCR for fetal aneuploidy detection can be used without restriction to fetal-specific nucleic acid molecules. Several cell-free fetal DNA and RNA technologies are under development to test a pregnancy for aneuploidy, mostly focusing on Down syndrome testing. Sampling of cffDNA from maternal blood for analysis by massively parallel sequencing (MPSS) is estimated to have asensitivity of between 96 and 100%, and aspecificity between 94 and 100% for detecting Down syndrome [63]. It can be performed at 10 weeks of gestational age. One study in the United States estimated a false positive rate of 0.3% and a positive predictive value of 45% when using cffDNA to detect Down syndrome [14].

Preeclampsia is the most prevalent pregnancy complication and predominant cause of premature birth, occurring at 2-5% of pregnancies in first world countries and 10% in developing countries. Preeclampsia refers to abnormal placental growth which can cause multiple problems including preterm labor, polyhydramnios, gravidarum, hyperemesis and fetal-maternal hemorrhage [49].

In 1999 studied quantitative variations of cffDNA in maternal blood for preeclampsia.64 cffDNA was measured by using PCR to the SRY gene. The levels were fivefold more in preeclampsia pregnancies than normal. This is consistent with subsequent studies [64,65]. The largest study compared 120 women with preeclampsia pregnancies and 120 gestational age matched normal women. The cffDNA concentrations were 176 vs 75 genome equivalents/ml at 29 weeks of gestations. Cff DNA concentration can be used as a screening tool.

In the largest and most comprehensive study to date, Palomaki et al. evaluated the analytic validity of a noninvasive prenatal screening test for Down syndrome that measures circulating cell-free DNA in maternal plasma. Test results were compared to those obtained after chorionic villus sampling or amniocentesis. Down syndrome detection rate was 98.6% (209/212), the false-positive rate was 0.20% (3/1471) and the testing failed in 13 pregnancies (0.8%). The authors concluded that, when applied to high-risk pregnancies, measuring maternal plasma DNA detects nearly all cases of Down syndrome at a very low false-positive rate. This method can substantially reduce the need for invasive diagnostic procedures and attendant procedure-related fetal losses [66].

Using the same cohort of patients, Palomaki et al. reported additional data indicating that maternal plasma cell-free DNA sequencing also has the capability to detect other aneuploidies, such as trisomy 18 and trisomy 13 [12].

Chiu et al. validated the clinical efficacy and practical feasibility of massively parallel maternal plasma DNA sequencing to screen for fetal trisomy 21 among high risk pregnancies clinically indicated for amniocentesis or chorionic villus sampling with the 2-plex protocol, trisomy 21 fetuses were detected at 100% sensitivity and 97.9% specificity, which resulted in a positive predictive value of 96.6% and negative predictive value of 100%. The 8-plex

protocol detected 79.1% of the trisomy 21 fetuses and 98.9% specificity, giving a positive predictive value of 91.9% and negative predictive value of 96.9%. The authors concluded that multiplexed maternal plasma DNA sequencing analysis could be used to rule out fetal trisomy 21 among high risk pregnancies [67].

Studies evaluating the performance of the Verifi Prenatal Test, all of which involved high-risk patient populations, reported a test sensitivity of 100% for trisomy 21, 97.2% to 100% for trisomy 18 and 78.6% for trisomy 13. The overall false-positive rate was 0%, and the test failure rates ranged from 3% to 4.2% [68].

The MELISSA (MatErnal Blood IS Source to Accurately Diagnose Fetal Aneuploidy) trial was conducted as a prospective, multicenter observational study to determine the diagnostic accuracy of massively parallel sequencing (verifi™) to detect fetal aneuploidy from maternal plasma. The authors reported high sensitivity and specificity for the detection of trisomies 21, 18, 13 in women with singleton pregnancies at an increased risk for aneuploidy.

A team of scientists, led by Diana W. Bianchi, MD, Executive Director of the Mother Infant Research Institute at Floating Hospital for Children at Tufts Medical Center, proceed a clinical trial where they analyzed samples from 1,914 pregnant women, and found that noninvasive cfDNA testing had a ten-fold improvement in the positive predictive value for trisomy 21, commonly known as Down syndrome, compared to standard prenatal aneuploidy screening methods [14]. Importantly, the cfDNA test performed consistently well in a general population of pregnant women, regardless of their risk for fetal chromosomal abnormalities. Previous studies have shown that the tests were more accurate for women who had higher risks for fetal chromosomal abnormalities, but this was the first time that the cfDNA tests were compared in a general obstetrical population to the variety of blood and ultrasound tests that comprise the current standard of care in the United States [69].

"We found that the major advantage of noninvasive prenatal DNA testing was the significant reduction of the false positive rate," said Bianchi. "Prenatal testing using cell-free DNA as a primary screen could eliminate the need for many of the invasive diagnostic procedures (such as amniocentesis) that are performed to confirm a positive screen" [14].

Sehnert et al. evaluated an optimized algorithm for use with massively parallel DNA sequencing of cell-free fetal DNA to detect fetal chromosomal abnormalities. Existing algorithms focus on the detection of fetal trisomy 21 (T21); however, these same algorithms have difficulty detecting trisomy 18 (T18). Sequencing of the independent test set led to 100% correct classification of T21 (13 of 13) andT18 (8 of 8) samples. Other chromosomal abnormalities were also identified. The authors concluded that massively parallel sequencing is capable of detecting multiple fetal chromosomal abnormalities from maternal plasma when an optimized algorithm is used [70].

Studies evaluating the performance of the Harmony Prenatal Test, involving a combination of both high-risk and average-risk populations, reported sensitivity of 100% for the detection of both trisomies 21 and T18, with false-positive rates of 0% to 0.7%. The test failure rate ranged from 0% to 4.9%. A single study examined the ability of the test to detect trisomy 13 and reported a test sensitivity of 80%, a false-positive rate of 0.01% and a test failure rate of 2.6% [68].

Ashoor et al. assessed the performance of the Harmony Prenatal Test for the detection of trisomy 13. The trisomy 13 risk scores were > 99% in eight (80.0%) cases of trisomy 13 [27].

Nicolaides et al. conducted a cohort study of 2049 pregnant women undergoing routine screening for aneuploidies at 11-13 weeks' gestation. Plasma cell-free DNA analysis using chromosome-selective sequencing was used. The authors concluded that noninvasive prenatal testing (NIPT) using chromosome-selective sequencing in a routinely screened population identified trisomies 21 and 18 with a false-positive rate of 0.1%. However, the authors cautioned that because the sensitivity and specificity of NIPT is not 100%, the test should not be considered a diagnostic replacement for invasive testing in high-risk pregnancies [66].

Norton et al. conducted a multicenter cohort study evaluating the performance of a noninvasive prenatal test for fetal trisomy 21 (T21) and trisomy 18 (T18) using cell-free DNA from maternal plasma. The authors concluded that chromosome-selective sequencing of cell-free DNA and application of an individualized risk algorithm is effective in the detection of fetal T21 and T18 [71].

Noninvasive prenatal testing (NIPT) using cell free DNA provides accurate screening for the common trisomies, including trisomy 13 (Patau syndrome), 18 (Edwards syndrome), and 21 (Down syndrome) [72].

In a study titled Rare Chromosome Abnormalities Detected by Current Prenatal Screening Compared to Expected Performance using Non-Invasive Prenatal Testing (NIPT), 68,990 of 1,324,607 women tested positive for trisomy 18 or 21 when they underwent prenatal screening as part of the California Prenatal Screening Program between March 2009 and December 2012. Invasive diagnostic testing with CVS or amniocentesis was performed on 26,059 women who tested positive, and 2993 were found to have abnormal results. Of those chromosomal abnormalities, 2489 (83.2 %) were abnormalities that would be detectable with NIPT, while 16.8 % were less common aneuploidies that would not be detected [72].

One of the study's authors Mary Norton, said that more of the abnormal results were detectable in the women over 40, who are at higher risk for trisomy 13, 18 or 21. Conversely, fewer of the abnormalities in younger women would be detected by NIPT, as the risk for

common trisomies is lower in this group, while the rare aneuploidies are not typically associated with maternal age [71].

Sparks and colleagues described the initial development of the Harmony Prenatal Test. Blood samples were obtained from 298 women with singleton pregnancies at 10 weeks or later. Invasive prenatal diagnosis identified 39 fetuses with trisomy 21 and 7 fetuses with trisomy 18. Samples were analyzed using a novel, highly multiplexed assay, referred to as digital analysis of selected regions (DANSR™). All (100%) aneuploid samples were correctly identified [73].

Sparks et al. evaluated a novel biochemical assay and algorithm for the prenatal evaluation of risk for fetal trisomy 21 (T21) and trisomy 18 (T18) using cell-free DNA obtained from maternal blood. In a blinded analysis of 167 pregnant women, digital analysis of selected regions (DANSR), in combination with a novel algorithm, fetal-fraction optimized risk of trisomy evaluation (FORTE), correctly identified all 36 cases of T21 and 8 cases of T18. The authors concluded that while DANSR and FORTE enable accurate noninvasive fetal aneuploidy detection in a high-risk population, larger studies that include low-and average-risk pregnancies are needed [71].

In a nested case-control study, Ashoor et al. reported that the sensitivity for detecting trisomy 21 was 100% (50/50 cases); the sensitivity for trisomy 18 was 98% (49/50 cases) and the specificity was 100% (297/297 cases) by chromosome-selective sequencing of maternal plasma cell-free DNA [74].

Studies evaluating the performance of the Panorama Prenatal Test, involving a total of 408 pregnancies (most considered high-risk), reported a test sensitivity of 100% for the detection of trisomies 21, 18 and 13, false-positive rates of 0% and test failure rates ranging from 5.4% to 2.7% [68].

Nicolaides et al. validated the Panorama Prenatal Test (Natera Inc.) in a population of 242 women with singleton pregnancies undergoing chorionic villus sampling (CVS) at 11 to 13 weeks of gestation. Results were provided for 229 (94.6%) of the 242 cases. Thirty-two cases were correctly identified as aneuploid, including trisomy 21, trisomy 18, trisomy 13, Turner syndrome, and triploidy, with no false positive or false negative results [75].

Zimmermann et al. evaluated a noninvasive prenatal test using targeted sequencing of SNPs (Panorama Prenatal Test). All aneuploidies were correctly identified for a sensitivity and specificity of 100% [53].

Data regarding the clinical validity of noninvasive prenatal tests (NIPT) is limited by the lack of follow-up information for the majority or pregnancies [68]. In the first study of 284

obstetrical patients who were evaluated by both the Harmony Prenatal Test and traditional first-trimester screening, all patients received NIPT results indicating a low likelihood (< 0.01%) of fetal trisomies 21, 18 or 13. The clinical outcomes for the remaining pregnancies were not known at the time of publication, although the NIPT results were consistent with the risk estimations provided by first-trimester screening [76].

In a second study involving the Harmony Prenatal Test, 1005 pregnant women were tested with both NIPT and traditional first-trimester screening. The detection rates for trisomies 21 and 18 were 100% and 80%, respectively [77].

Analytical validity studies, which compare the results of noninvasive prenatal testing to the standard of care for the diagnosis of fetal aneuploidies, also contribute to the clinical validity of the analysis [68]. Two studies of analytical validity used their data to demonstrate high positive and negative predictive values (PPV and NPV, respectively) for noninvasive prenatal testing [66,71]. These studies found that the PPV for the detection of trisomy 21 was 96.6% for MateniT12 PLUS and 98.8% for Harmony, while the NPV was 100% for both tests. The study of the Harmony Prenatal Test also reported that the PPV and NPV for the detection of trisomy 18 were 94.9% and 99.96%, respectively [71].

Published evidence regarding the clinical utility of noninvasive prenatal testing is limited. Prospective data is needed in which test results are acted upon clinically, showing that results lead to a change in patient management and/or outcomes [78].

Comparison of different test performance is summarized in **Table 4**.

Table 4: Comparison of currently available tests for free DNA screening

	Verifi® Verinata	**Harmony Ariosa**	**MateniT21 Sequenom**	**NIPT Natera**
Result Types	-Aneuploidy Detected -Aneuploidy Suspected -No Aneuploidy Detected	Risk scoreincorporating maternal, gestational age	-Positive -Negative	Risk scoreincorporating maternal, gestational age
Assay Failure Rate	**<0.7%**	4.6 – 4.9%	1%	5.9 – 12.6%
Sample	1 tubematernal blood	2 tubes maternal blood	2 tubes maternal blood	2-4 tubes maternal blood (best with paternal sample)
Egg Donors	Yes (with data)	No	Yes	No
Test Menu	T21, T18, T13 Optional sex chromosome aneuploidies (Published data)	T21, T18, T13 Y chromosome (optional)(not published)	T21, T18, T13 Mandatory sex chromosome aneuploidies (not published)	T21, T18, T13 Sex chromosome aneuploidies (only MX published)
Published Clinical Validation	Large-scale, blinded clinical validation	Large-scale, blinded clinical validation	Large-scale, blinded clinical validation	Small, blinded clinical validation

7. Summary

NIPT technology is likely to become a primary screening method for common chromosomal abnormalities such as Down syndrome in pregnancy, and that it will enhance the information available to pregnant women while reducing unnecessary invasive procedures.

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