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Review article

Strategies for prenatal diagnosis today

Daniilidis A[1], Klearhou N[1], Mauromichali M[2]

[1] *3rd Department of Obstetrics& Gynaecology Hippokration University Hospital*
[2] *Department of Paediatrics Gennimatas General Hospital*

Abstract. *Prenatal diagnosis has become a standard part of modern obstetrics. There is always the risk for a diagnostic error, which could result in a loss of a normal pregnancy or in an avoidable birth of a handicapped child. The various available screening tests which are being used nowadays aim to detect with the highest possible sensitivity the high risk pregnancies. The first trimester screening test combines maternal age, nuchal translucency and the measurements of serum b-hCG, AFP, uE3 and inhibin A. Accurate prenatal diagnosis of chromosomal abnormalities is available by obtaining fetal cells through amniocentesis or chorionic villous sampling with an unavoidable though risk of miscarriage. Undoubtedly family history and also the improvement of ultrasound machines and skills play an important role in the detection of foetal abnormalities. The need of developing non invasive approaches for accurate prenatal diagnosis will become more and more desirable in the future. Hippokratia 2006; 10(1)1:22-27*

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Corresponding author: Daniilidis A, 81 A Nimfeou 56224, Thessaloniki, Greece - E-mail:angedan@hotmail.com - Tel:6932211395

Prenatal diagnosis for fetal abnormalities has become a standard part of modern obstetrics. Nowadays, the delay in childbearing and the trend for smaller family size are factors that impact on the need for accurate prenatal diagnosis. For many couples the availability of screening and diagnosis expands their options regarding pregnancy, since prenatal diagnosis may allow them to undertake a pregnancy that they might not have otherwise undertaken[1] or to terminate a pregnancy if the fetus is affected. Obstetricians offering prenatal diagnosis or screening bear a heavy responsibility. It is a fact that there is always the risk for diagnostic error, which could result, in loss of a normal pregnancy or on the other hand, in an avoidable birth of a handicapped child. Also, there is always the unavoidable dilemma of causing anxiety by offering testing and restricting the woman's choice by failure to offer the appropriate test.

Before the 1960's, the prenatal detection and diagnosis of genetic disorders and birth defects did not exist. The only available method was practically the family history. Practically, couples based on family history would decide either to take the risk of having a possibly affected child or not to become pregnant at all[2]. The association between maternal age and Down syndrome was first described by Penrose in 1933. It was not until 1960s though that that prenatal diagnosis of genetic disorders became a viable option. With the advent of safe technologies, new gene discoveries, improvements in cytogenetic and DNA-based testing for hundreds of genetic conditions, couples have many more reproductive options available than ever before. These technologies coupled with the liberalization of abortion laws in the 1970s have made prenatal screening and diagnosis an integral part of prenatal care[3].

The current state of affairs

Screening for chromosomal abnormalities in an obstetric setting has traditionally meant screening for trisomy 21. It is the single most common cause of mental restriction in school-age children. The distinction between screening and diagnostic tests is often spurious since most tests will be used in both ways in different patients. At present accurate prenatal diagnosis of chromosomal anomalies is only available by obtaining fetal cells through an invasive procedure, such as an amniocentesis or chorionic villous sampling (Table 1). Due to the fact that both procedures are associated with a risk of miscarriage[4] these procedures are currently applied only to small group of women which are in a higher risk of having an offspring with a chromosomal defect in comparison to the general population. In order to determine this "high risk" group some screening approaches have been developed during the years.

Table 1. *Current techniques of foetal cell sampling.*

Technique:	Drawbacks
1 Amniocentesis	Second trimester procedure Miscarriage rate 1% Diagnostic errors due to maternal cells and pseudomosaicism
2 CVS (chorionic villous sampling)	Miscarriage rate 1-2% Mosaicism 0,26%
3 FBS (foetal blood sampling)	Miscarriage rate 5% Preterm delivery 15%

The success of a screening test is not only measured by the number of affected pregnancies detected. Patients should enter a screening programme only on the basis of adequate information about the implications and limitations of the test, so they are not faced with results and decisions for which they are not prepared. The aim of the currently available screening tests is actually to identify, with the highest possible sensitivity and specificity, those women who should be offered the invasive procedure. The risk for many of the chromosomal defects increases with maternal age. Additionally, because fetuses with chromosomal defects are more likely to die in utero than normal fetuses, the risk decreases with gestational age. Maternal age of 35 years at delivery has been the medicoligal standard in USA and Europe for more than 20 years (Table 2). This high risk group constituted 5% of the pregnant population. It was estimated that approximately 30% of trisomy 21 occurred to mothers >35 years old.

Nowadays, such a screening is provided by using the family history, the maternal serum screening and the ultrasonography. Every time a test is carried out the background risk is multiplied by the test factor to calculate a new risk, which then becomes the background risk for the next test. This process is called sequential screening. Although screening tests are not diagnostic they can indeed alter the odds. It is a fact that although the risk of any individual 36 years old is higher than a 26 years old woman, there are so many more pregnancies in the 26 years old group that from a population perspective, most abnormalities (approximately 70%) occur in the 'low risk' population.

The majority of chromosome abnormalities identified in prenatal samples are trisomy for chromosomes 13, 18, 21 and sex chromosome aneuploides. These are associated with the newborn phenotypes, Patau syndrome, Edwards syndrome and Down syndrome (trisomy 13, 18 and 21 respectively), and the less severe Turner (monosomy X) and Klinefelter's(XXY) syndromes. Down's syndrome, with an incidence rate of 1 in 800 pregnancies, is the predominant reason for women seeking prenatal diagnosis. Karyotype analysis of cells by culture is usually available in more than in one week. In order to reduce anxiety and improve pregnancy management, more rapid aneuploidy testing are used. The most widely established method is interphase-fluorescence in situ hybridization (FISH) [5,6] .Here a set of chromosome- specific fluorescence-labelled probes are hybridized to interphase nuclei of uncultured prenatal cells. The number of fluorescent signals in each nucleus represents chromosome copy number. Between 50 and 100 cells are usually analyzed to allow for low-level background and signal overlay that can occur during FISH procedures [7]. A quantitative fluorescence-PCR (QF-PCR) is a more recent addition to aneuploidy diagnosis[8,9] .The technique involves the relative quantification of micro satellite alleles to determine sequence copy number; amplification using fluorescence-labelled primers is followed by size separation and allele peak measurement on a semi-automated genetic analyzer.

Since 1990, a new technique has been developed for pre implantation genetic diagnosis, enabling identification of genetic disorders before the establishment of pregnancy. It is a technique used nowadays following in vitro fertilization procedures. It combines recent advances in molecular genetics and assisted reproductive technologies. One to two blastomeres are removed from early cleavage stage embryos (6-8 cell stage) on the 3rd day post fertilization. The genetic material is then analyzed in order to distinguish any aneuploidies, single-gene and X-linked disorders.

Table 2. *Risk of Down's syndrome and maternal age at EDD expected date of delivery and at the time of amniocentesis*[2].

Age(completed years)	Risk at mniocentesis	Risk at EDD
35	1:285	1:348
36	1:174	1:276
37	1:146	1:216
38	1:122	1:168
39	1:91	1:130
40	1:80	1:99
41	1:67	1:75
42	1:45	1:57
43	1:30	1:43
44	1:23	1:32
45	1:21	1:24
46	1:11	1:18

Family history and ethnical background in prenatal diagnosis

The evaluation of family history and potential heritable conditions has a quite important role in prenatal diagnosis. If a mother has had a previous affected child the risk in subsequent pregnancy is increased and the precise risk depends on whether the affected pregnancy ended in live birth or miscarriage, whether the child had non-disjunction or translocation, and whether either parent is a balanced translocation carrier. In the case that a Down's pregnancy has reached the term and both parents have a normal karyotype, the risk of recurrence is 0.34% above the age-specific risk and 0.75% higher than the maternal and gestational age related risk for trisomy 21 at the time of the test. For the 14/21 balanced translocation the risk is about 10% if the mother is the carrier and approximately 2% if the father is a carrier[2]. In addition, another important area in preconception counselling is the assessment of ethnical background [10]. Genetic diseases more common in specific ethnic groups include the hemoglobinopathies, thalassemias, and cystic fibrosis (Table 3). In screening for thalassemia, which occurs in broad band from Mediterranean to the Middle East and Sough East Asia [11] a screening complete blood count specifically looking at the mean cell volume (MCV) is a determining factor in deciding for electrophoresis.

Table 3. *Genetic disorders and Ethnic Origin.*

Ethnicity	Disorder	Carrier frequency	Incidence of disease
4 Greek	A Thalassemia	1/25	1/2500
	B Thalassemia	1/30	1/3600
5 Italian	B Thalassemia	1/30	1/3600
6 SE Asia	A Thalassemia	1/20	1/2500
7 S.China	A Thalassemia	1/20	1/2500

Cystic fibrosis is also an autosomal recessive condition. One in 22 in UK is asymptomatic carrier and chances of two such carriers mating assuming no consanguinity is 1 in 484 [12]. According to the recommendations of the American College of Obstetricians and Gynaecologists screening for cystic fibrosis should be offered to:

1 Individuals with family history of CF

2 Reproductive partners of individuals who have CF

3 Couples in whom one or both partners are Caucasian and are planning pregnancy

4 Couples in specific ethnic groups (Table 4)

Table 4. *Chance to have a child with CF based on ethnicity [3].*

Ethnic group	Carrier Frequency	Incidence
1 Ashkenazi Jewish	1/29	1/3300
2 Caucasian	1/29	1/3300
3 African American	1/65	1/17000

Screening involves the 25 disease causing mutations for CF that are known to have an allele frequency of greater than 0.1% among North American patients with CF [13].

First trimester screening (11-14 weeks)

Nowadays prenatal screening and diagnosis between 11 and 14 weeks of gestation is becoming more and more available and efficient worldwide. Recent studies concluded that the sensitivity of first trimester screening is nearly as sufficient as the second trimester test. Several maternal serum markers have been used and PAPP-A (pregnancy associated plasma protein) and b-HCG (free b-human chorionic gonadotropin) have been the most promising so far. It is found that maternal serum concentration of b-HCG is increased during first (1.83 MoM) and second trimester in cases of Down's syndrome and that PAPP-A on the contrary is decreased (0.38). By using PAPP-A as a screening marker in combination with maternal age approximately 50% of cases of Down's can be detected [13, 14]. By using maternal age combined with b-HCG about 45% of cases of Down syndrome are detected [13, 14]. The use of all three factors combined offers a detection rate of 65% [13, 14]. In the last few years an ultrasonografic finding has been added as a quiet important marker for the screening of Down's syndrome [15] and other fetal anomalies such as trisomy 13, 18, Turner syndrome, triploidy and heart defects [16]. That is the nuchal translucency (NT), which is actually the measurement of the subcutaneous collection of fluid at the back of the fetal neck. The measurement is performed ideally between 11-13+6 weeks and a cut-off based on gestational age is used [17], since NT normally increases with gestation. The minimal CRL (crown rump length) should be 45mm and the maximum 84 mm, a good sagittal section of the fetus should be obtained, the fetus should be in a neutral position, the magnification should be such that the fetus occupies three-quarters of the image and fetal skin and amnion should be distinguished. Fetal nuchal translucency thickness increases with CRL and therefore it is essential to take gestation into account. Studies showed that at 10-14 weeks the observed numbers of fetuses with trisomies 21, 18, 13 with NT measurements of 3 mm, 4 mm, 5 mm, and > 6mm were 3 times, 18 times, 28 times and 36 times higher than numbers observed on the basis of maternal age. A measurement of >6 mm identifies about 40% of cases with trisomy 21 in a high risk population. In the Foetal Medicine Foundation Multicenter Project of screening for trisomy 21 by a combination of maternal age and NT at 10-14 weeks, 325 with chromosomal abnormalities other than Down's were found and 70.5% of these had NT above the 95th centile (Table 5).

Fetal abnormalities other than Down's syndrome associated with increased NT are summarized in table 6. Cardiac defects are found in about 1% of live fetuses. Evidence suggests that about 50% of all cardiac defects are in the subgroup with NT above the 95thcentile. The combination of maternal age and NT leads to a detection rate of 75% for Down syndrome and false positive rate of 5%[15,16,18]. By combining the first trimester biochemical screening with maternal age and the NT the detection rate for Down syndrome is estimated to be about 90% and the false positive rate 5% [19,20,21] (Table 9).

Table 5. *Nuchal translucency thickness above the 95th centile and abnormalities other than trisomy 21.*

Trisomy	18 74.8%
Trisomy	13 71.7%
Turner Syndrome	87.0%
Triploidy	59.4%
Deletions, partial trisomies, unbalanced translocations, sex chromosome aneuploidies	55.4%
Total	70.5%

Table 6. *Conditions associated with increased nuchal translucency.*

- 1 Cardiac defects
- 2 Diaphragmatic hernia
- 3 Exomphalos
- 4 Achondrogenesis type II
- 5 Achondroplasia
- 6 Asphyyxiating thoracic dystrophy

Prenatal screening and diagnosis has definitely some obvious advantages in comparison to the second trimester firstly that provides the option of early termina-

tion of pregnancy, secondly earlier reassurance [22] . It should be mentioned though that it is not possible to perform screening for neural tube defects in this early stage of pregnancy.

Second trimester screening

Ultrasound examination in the second trimester of pregnancy is a really important tool for prenatal diagnosis. In order to achieve a comprehensive and maximum sensitivity ultrasound study the level II (detailed) ultrasound should be performed at 18-22 weeks of gestation. The sensitivity for anencephaly is near 100%, for spina bifida between 60- 96%. A lot of other anomalies either isolated or part of chromosomal defects (i.e. congenital heart defects, omphalocele, and diaphragmatic hernia) can be detected with satisfactory sensitivity at this gestational age. Recently a new class of ultrasound findings, referred as markers for Down's syndrome, has been under investigation. These markers as isolated findings are not diagnostic for trisomy but it is observed that they are quite frequent findings in fetuses with Down's syndrome[23-25]. These markers are: shortened femur, intracardiac echogenic foci, echogenic bowel, and renal pyelectasis and increased nuchal fold. Several studies estimate that a detailed ultrasound examination detects only 50% of major malformations[26] and major abnormalities in less than 25% of fetuses with Down syndrome. The last increases to 50% if the ultrasound examination includes systematic review of the markers[23]. Of course there are limitations in ultrasound examination and certainly the every day clinical practice proves that ultrasound cannot be a definite and 100% diagnostic tool for prenatal diagnosis.

Various maternal serum markers have been used for screening for fetal chromosomal abnormalities and in particular Down syndrome but also for Neural tube defects (NTD). It was in 1972 when it was firstly observed the association of high maternal serum a-fetoprotein and increased risk neural tube defects. Once other factors are excluded, such as gestational dating, multiple pregnancy, fetal death any woman with elevated serum- AFP should be offered an ultrasound study for spina bifida. Low maternal serum AFP is associated with increased risk for Down syndrome. AFP is a fetal -specific protein that is produced by the fetal yolk sac and liver. By using AFP as a screening marker the detection rate for trisomy 21 is approximately 40%. Other serum markers the serum b-HCG, the unconjugated estriol (uE3) and lately inhibin A (INH-A) are also used for screening purposes. The triple test involves the combination of maternal age with s-hCG, uE3 and MSAFP and provides a detection rate of 65% with a FPR of 5%. The quadruple screen includes also inhibin A, and gives detection rate of 75% [27] (Table 8). Lately are being used combinations of measurements of PAPP-A and NT in first trimester and of MSAFP, hCG,uE3 and INH-A, with a detection rate of 94% and a FPR of 5% [13] . The serum markers are measured between 15 and 19 weeks and the results are expressed as multiples of the median (MoM) for unaffected pregnancies at the gestation. MoM rather than centiles or ratios to the mean were chosen early in the screening programme in the 1970s because laboratories with small experience can produce a stable median relatively easily. Pregnancies affected with open NTD on average have a MSAFP of 4 MoM, and detailed USS or amniocentesis should be offered with levels over a certain cut-off, typically 2.0 or 2.5 MoM.(table 7) A special algorithm is designed in order to combine the woman's age related risk with her serum results to generate a patient specific risk figure [28]. Trisomy 18 is associated with lower levels of AFP, decreased hCG and inhibin A [29, 30]. By taking into account also maternal age a risk greater than 1/250 for trisomy 21 is an indication for amniocentesis.

Accurate assessment of gestational age by USS is a requirement in order to accurately calculate the patient specific risk. Several other factors apart from gestation are affecting the levels of serum markers, like ethnic origin, weight, smoking, diabetes, twin pregnancy. The indication for the use of invasive technique for prenatal diagnosis is that the risk for fetal anomaly is at least the same or greater than that for fetal loss from the procedure.

Table 7. *Median multiples of Median for second trimester markers in pregnancies with Down's syndrome.*

Marker	MoM
AFP	0.75
hCG	2.06
Free hCG	2.20
uE3	0.72
Inhibin A	1.92

Table 8. *Various screening markers combination in second trimester*

screening combination	sensitivity (%)
age	30
age+AFP	39
age+AFP+hCG	59
age+AFP+hCG+uE3	69
age+AFP+hCG+inhibin A	68
age+AFP+hCG+uE3+inhibin A	76

Table 9. *Various screening markers combination in first trimester.*

screening combination	sensitivity(%)
age+free bhCG	38
age+PAPP-A	52
age+PAPP-A+free hCG	60
age+NT	77
age+NT+PAPP-A+free bhCG	89

Diagnostic procedures (Amniocentesis and CVS)

The procedure of amniocentesis became an available option for genetic diagnosis for the first time in 1966 by Steele and Breg.When it was first introduced

into clinical practice it was used for the investigation of Rh isommunisation and later for the short-lived technique of estimating the lecithin-sphingomyelin ratio. It is normally performed between 15 and 20 weeks of gestation. The use of amniocentesis prior to 14 weeks lead to higher pregnancy loss rates(7.6%), increase in talipes (1.3% as opposed to 0.1%) and an increase in amniotic fluid leakage(3.5% as opposed to 1.7%). The aim is to obtain fetal cells derived from skin, mucous membranes, amnion and umbilical cord for karyotyping or DNA analysis. It is performed under continuous ultrasound guidance by inserting a 22-gause spinal needle transabdominally and infiltrating a sample of amniotic fluid. The operator should try to avoid the placenta but it is more important to gain access to a deep, clear pool of liquor even if that means a trans-placental approach. Fetal viability should always be checked before and after the procedure. Local anesthesia is not necessary. Usually, 20mls of amniotic fluid are enough. If the patient is Rh negative then 250 IU of anti-D immunoglobulin should be administered. The major risk of this procedure is of miscarriage which is estimated between 0.5%-1 percent [31]. Other potential side effects include maternal infection, injuries and preterm labour. Amniocentesis remains the most common invasive prenatal diagnostic procedure today.

Chorionic villous biopsy is performed between 9 and 14 weeks of gestation. It was first described in China in 1975 as a mean for sex determination. It was developed by Brambati in Italy in 1991 under continuous ultrasound guidance sampling of the villous area is performed either trans-abdominally or trans-vaginally. The first route has become lately more popular. Although it is possible CVS is not performed before 9 weeks due to increased incidence of foetal limp abnormalities [32]. The placenta is easily identifiable at this gestation and the thickness is sufficient for a safe procedure. Trans-abdominal technique is the technique of choice nowadays although transcervical route is needed in low lying placentas. For the trans-abdominal route local anesthetic is used and then with an 18-20 gauze spinal needle under continues ultrasound guidance placenta tissue is aspirated. The aspirating needle is moved up and down for about ten times. Anti-D will be needed for Rh-negative patients. In comparison to amniocentesis the major advantage of CVS is that it is performed quite early in pregnancy reducing anxiety and allowing a first trimester stopping of pregnancy if needed. It has higher risk of miscarriage from amniocentesis which is estimated to be 1%-2%[33] Chromosomal mosaicism in about 2% of cases is other disadvantage [34] . Contraindications for trans-abdominal CVS are bowel adhesions on anterior abdominal wall and multiple fibroids. Vaginal or cervical infection is a contraindication for the trans-cervical route.

Need for non invasive prenatal diagnosis

At present prenatal diagnosis of foetal chromosomal anomalies is obtained by invasive procedures which entail a risk of miscarriage. That is the reason that the indication for CVS or amniocentesis is restricted to defined high risk groups according the up to day available screening tests. The available screening tests don't have a100% sensitivity and also there is always a small false positive rate.

These facts make the need for developing non invasive approaches for prenatal diagnosis in the low risk population more than desirable. An alternative approach would be to separate physically the rare fetal cells circulating within maternal blood for antenatal genetic analysis. If chromosome and single gene analysis could be accomplished without invading the uterus, prenatal diagnosis could be offered to all pregnant women without consideration of their presumptive risk of having an affected fetus.

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