NON-INVASIVE SCREENING FOR PRENATAL GENETIC DIAGNOSIS

Report of a WHO Temporary Adviser

by

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1. **INTRODUCTION**

Screening has been defined as the systematic application of a test or inquiry to identify individuals at sufficient risk of the specific disorder to benefit from further investigation for direct preventive action among patients who have not sought medical attention on account of that disorder.\(^1\) Screening should be based on explicit personal choice, both at the time of invitation to screening and at each stage of the screening and diagnostic processes.

Screening pregnant women of advanced maternal age has been available for over 25 years. First amniocentesis and now chorionic villus sampling have been offered to such women for detecting fetal chromosomal abnormalities. Approximately half of these women accept the procedure-related risks inherent with any invasive procedure. Because relatively few women under age 35 are candidates, the birth prevalence of Down syndrome in the general population will decrease relatively little (20%) given that only 10-20% of births occur to women 35 years or older.

Several different screening approaches to detect, for example, Down syndrome, are possible. Second trimester maternal serum screening is already accepted in many countries. Ultrasonographic detection of anomalies associated with aneuploidy is an attractive option, given the widespread use of ultrasound for other obstetrical indications. An exciting new application under clinical evaluation is recovery and analysis of fetal cells in maternal blood.

An International Working Group was convened in Tel-Aviv, Israel, on 22 May 1994\(^*\), with co-sponsorship of the World Health Organization, in order to review each of the above approaches, to define their current status and to offer recommendations for current application.

2. **MATERNAL SERUM SCREENING**

Since the late 1980s various maternal serum markers, principally alpha-fetoprotein (AFP) and human chorionic gonadotropin (HCG), have been used in combination with maternal age to screen for Down syndrome. The detection rate (sensitivity) is approximately 60% for all age groups, ranging from >90% for women >35 years to less than 25% for those <25 years.\(^2,3\) This 60% detection rate is achieved at a cost of an amniocentesis rate of about 5%. Overall, serum screening at 15-22 weeks can identify more pregnancies associated with Down syndrome for a given number of women identified as being at high enough risk to justify an invasive diagnostic procedure (e.g., amniocentesis) that can be achieved using only an age threshold (e.g., 35 years) as a cut-off. Alternatively, for the same number of women identified as having a pregnancy with Down syndrome, fewer women need to have an invasive diagnostic procedure with serum screening than with age threshold maternal age screening. That is, maternal screening for Down syndrome detection should be more effective and indeed has been shown to be so.\(^4\)

\(^*\) Please see attached annex for a full list of the participants.
The following conclusions and recommendations were thus made:

- Practice differs from country to country, reflecting clinical preferences and jurisprudence. For example, in the USA the policy of offering an invasive procedure to women over age 35 was recently recodified. In countries that retain age 35 as a threshold for offering amniocentesis or CVS, women should probably be given the option of serum screening before making the decision on an invasive procedure; however, in such circumstances it should be noted that sensitivity of serum screening in that age group is not 100% but 90%. Irrespective, all women under age 35 years should be offered maternal serum screening. Other countries no longer routinely offer an invasive procedure above a specific age, but rather pursue universal maternal serum screening. This policy should maximize detection of Down syndrome. If such a policy is extant in a given country, some older women may insist on an invasive diagnostic procedure outside the maternal screening programme. This request should also be regarded as an individual clinical matter on a case-by-case basis.

- Serum screening for Down syndrome should be based on multiple serum markers in addition to maternal age. There is no unanimity concerning whether the serum markers should consist of the "double test" of human chorionic gonadotropin (HCG) and alpha-fetoprotein (AFP) or the "triple test" of hCG, AFP and unconjugated estriol ($\mu E_3$). Most published experience is based on the "triple test", from which better screening performance can be expected. Additional serum markers or alternative approaches should be used only if justified in terms of increased screening performance, medical efficacy and financial cost effectiveness.

- Screening should only be offered as an integrated service. Resources must be identified for coordination, provision of information, counselling, pregnancy management, professional education and programme monitoring.

- Screening before 15 weeks of gestation is still investigational. However, preliminary date with selected analytes (free ß-hCG, AFP, PAPP-A) suggest that detection rates approximate those in the 15-22 week interval.

3. ULTRASONOGRAPHY

Ultrasound in clinical medicine has been available for about 30 years. During the past 20 years improvement of technology and the widespread availability of instrumentation has made the use of ultrasound in pregnancy an integral part of clinical care. The general value of obstetrical ultrasound includes identification of placenta previa, assessment of gestational age, identification of fetal number and position, documentation of fetal life, assessment of amniotic fluid volume, and biophysical assessment of the fetus. Ultrasound has also been used to evaluate the uterus, cervix, adnexa, and fetal anatomy, identifying malformations as early as 12 weeks of gestation. All these benefits are universally accepted as information that should be obtained as part of the complete obstetrical ultrasound examination.

To date, the benefit of routine ultrasound screening in low risk patients remains controversial. Notably, the USA Routine Antenatal Diagnostic Imaging with Ultrasound Study (RADIUS) failed to demonstrate improvement in morbidity or mortality in low risk
patients. Critics of the RADIUS study point out serious methodological limitations. However, the RADIUS study demonstrated a significant difference between the detection of birth defects in patients undergoing routine screening compared to patients undergoing ultrasound for pre-established indications. The study also demonstrated a significant difference in detecting serious malformations between centres in which fetal ultrasound was performed by specialists compared to centres in which ultrasound examinations were performed by obstetricians or community based radiologists.

Overall, there is universal acceptance that ultrasound in indicated cases is beneficial, especially when there is an increased risk of structural malformations. More uncertain is the role of ultrasound in the detection of aneuploidy, the current topic.

It is clear that some aneuploid fetuses may exhibit either ultrasonically detectable structural changes or abnormalities of growth. Abnormal measurements of the femur and humerus in the first trimester were among the first indices proposed, but these have not gained wide acceptance because investigations have been unable to reproduce results from initial studies. Unfortunately, investigators usually use different cut off values for the proposed measurements, further confusing the issue. Because there is no doubt that various ultrasound measurements are indeed associated with aneuploidy, quantification of these relationships by comprehensive prospective investigation is awaited.

Additional ultrasound markers of aneuploidy in the second trimester include occipital subcutaneous edema, which when greater than 6 mm has been associated with an increased prevalence of trisomy 21. Other investigators confirmed this finding, whereas still others were unable to document a relationship between nuchal thickening and Down syndrome. Some investigators have pointed out technical difficulties, such as the ability to create spuriously "the sign" by angling the transducer caudally instead of approaching the head axially at the level of the cerebral hemispheres. Other proposed ultrasound markers include hypoplasia of the middle phalanx of the fifth digit and clinodactyly. However, the size of the middle phalanx is quite small during the second trimester; thus, detection on the basis of decreased size of these bones may not be reliable.

Studies regarding first trimester ultrasound findings in aneuploidy have also been published. Aneuploid embryos tend to have smaller crown rump lengths, but variability exists in measurements for fetuses with aneuploidy; thus, this technique is not commonly used. Nicolaides et al first reported that nuchal translucency of 3 mm or more had 75% sensitivity for the identification of Down syndrome in the first trimester; however, Brambati et al found only an 18.6% prevalence of chromosomal abnormalities when nuchal translucency was 3 mm. To add confusion, different criteria have been used to define abnormal nuchal translucency. Another proposal is to use doppler flow studies, but again standard criteria do not exist.

The other general ultrasonographic approach is to search for structural malformations. One example is a cardiovascular defect. Up to 50% of Down syndrome fetuses will have a cardiac defect in the second trimester, whereas 30% manifest such a defect at term. Similarly, up to 90% of trisomy 18 and trisomy 13 fetuses are said to have cardiac defects. Usefulness of the four chamber view of the heart in screening for Down syndrome will once again depend upon the accuracy of detecting cardiac anomalies. The RADIUS trial showed a very poor detection rate for cardiac malformations when the examination was performed by non-tertiary centres in a general
obstetrical population. Other investigators, however, have demonstrated that approximately 33-63% of cardiac defects can be detected in a routine scan.18,19

Nicolaides et al were the first to report an association between choroid plexus cysts and trisomy 18,20 a finding confirmed by others.21,22 Other findings associated with Down syndrome include pyelectasis, echogenic bowel, enlarged cisternal magna, ventriculomegaly, and wide distance between the first and the second toe. However, many reports show differences in detection rates based on gestational age of the fetus, as well as fetal sex. Still other investigators have suggested that in the absence of any other anatomical defects amniocentesis would not be indicated. A problem with this recommendation is that inability to identify heart defects may result in failing to perform an amniocentesis to detect trisomy 18.

Investigators have usually focused upon only a single ultrasound marker (e.g., pyelectasis, hyperechoic bowel, abnormal nuchal fold) to detect Down syndrome, rather than looking for multiple markers. Ultrasonographic detection rates for aneuploidy might be strengthened if a constellation of signs were detected. Several such signs occurring simultaneously would be a stronger indication of Down syndrome than any single sign alone. The cardiovascular system in particular has been inadequately evaluated in women at increased risk for trisomy 21 on the basis of maternal serum screening (>1:270). DeVore and Alfi17 reported an ultrasound sensitivity of 87% for trisomy 21, with a false positive rate of 11%.

As a result of the factors cited above, ultrasound markers alone will not at present replace maternal serum screening in the majority of centres. However, as groups better define descriptive methods and publish the sensitivity, specificity, and predictive values, ultrasound is likely to play an increasing role in detecting malformations in both low as well as high risk patients. Some areas of the world have already implemented routine early ultrasound screening.

Finally, ultrasound screening by one or more modalities may be used adjunctively with biochemical testing. Circumstances include (1) pregnancies of anxious patients identified as "at risk" by non-ultrasound tests, (2) pregnancies in which structural malformations associated with aneuploidy have been identified, (3) pregnancies in which further information is needed before eschewing invasive testing in women over 35 who show lower than expected aneuploidy risks by maternal serum screening, (4) pregnancies in which further information is needed concerning the need for invasive testing in women who despite being less than 35 show higher than expected aneuploidy risks by maternal serum screening.

As a result of the above, the following conclusions were made.

- Ultrasound is an integral part of serum screening for Down syndrome, particularly in defining gestational age for serum screening markers.
- Ultrasound is essential in identifying structural malformations. A decision about the specific anomaly or anomalies to seek remains uncertain because detection sensitivities are not adequately defined.
- Routine ultrasound screening cannot be recommended at the current time because of unresolved issues concerning timing during gestation, equipment, operator experience and image quality.
The inclusion of abnormal ultrasound findings into serum analyte risk assessment programmes is an important research project that should be given highest priority.

4. FETAL CELLS IN MATERNAL BLOOD

Obstetricians and pathologists have long known that fetal cells occasionally enter the maternal circulation, as indicated by the phenomenon of Rhesus isoimmunization and amniotic fluid embolization. Twenty-five years ago Walkowska et al. reported recovering fetal cells in maternal blood, based upon apparent 46,XY metaphases. In 1979 Herzenberg et al. used flow-sorting technologies to recover Y-chromatin positive cells from women whose fetus presumably inherited a paternal human leukocyte antigen (HLA) differing from that of its mother. However, no consensus existed that fetal cells were truly present in maternal blood until Lo et al. used nested primer PCR to detect Y-DNA sequences in the maternal blood of women pregnant with male fetuses. Subsequently, the detection of single gene (Mendelian) mutations has been achieved through analysis of both sorted and unsorted maternal blood. PCR-based technology reveals fetal cells to be present as early as 35 days gestation, and consistently present by the end of the first trimester. The fetal cell type being detected in these studies is still unclear.

Cell enrichment techniques, followed by in situ hybridization (FISH) with chromosome-specific DNA probes, have demonstrated that fetal cells are present in the maternal circulation during the first and second trimesters. Prenatal detection of fetal chromosome abnormalities has been reported. For fetal cytogenetic diagnosis, selecting a specific fetal cell type almost certainly is necessary because the relative excess of maternal cells in the maternal sample necessitates cell enrichment. Potential fetal cell types include nucleated red blood cells, trophoblasts, lymphocytes, and granulocytes. The greatest successes to date have been achieved with nucleated red blood cells. It is with this cell type that fetal trisomies were first detected in maternal blood, using different density gradient separations, different technologies or magnetic activated cell sorting) and different positive and negative selection criteria. FISH analysis with chromosome-specific DNA probes was used to make a precise diagnosis.

Less experience exists with other fetal cell types. Presence of fetal lymphocytes in maternal blood has apparently been made by PCR detection of a paternally transmitted HLA allele; however, trisomies have not been detected. Perhaps a more attractive cell type is the trophoblast. Accepted as an ineluctable consequence of placentation, trophoblasts are removed rapidly by the maternal lungs. Moreover, trophoblast-specific monoclonal antibodies have proved difficult to generate. Nonetheless, several groups have enriched maternal blood with trophoblast specific antibodies and detected Mendelian disorders. One group reported obtaining karyotypes.

Although recovery and analysis of fetal cells in maternal blood appears promising, key biological questions remain. These include determining the optimal fetal cell type for isolation, frequency of fetal cells in maternal blood, optimal timing during gestation for fetal cell isolation, and likelihood of persistence of cells after delivery. As noted fetal cells of unknown type can be detected early in gestation, and by 10 weeks are consistently present. The frequency increases with gestation, but fetal cells in maternal blood remain rare events. A concern is that fetal cells could persist months if not years after delivery. The origin of such cells is through clones established in the maternal hematopoietic system. This could potentially result in a future erroneous
diagnosis. It is because lymphocytes are considered the cell type most likely to persist that erythroblasts or trophoblasts are the preferred cell type for analysis.

Recently, the USA National Institute of Child Health and Human Development funded four Centres to recruit approximately 3,400 women for clinical evaluation of methods to isolate and analyze fetal cells from maternal blood. Simultaneously, information will be gathered concerning attitudes, preferences, and behaviour of pregnant women towards such non-invasive techniques for prenatal diagnosis. Possible adverse effects (e.g., coercion) are being established. The diagnostic accuracy of cytogenetic studies performed will be compared to those of invasive techniques (e.g., amniocentesis, chorionic villus sampling). If the accuracy is equivalent, fetal cell isolation from maternal blood could serve as a definitive diagnostic test for fetal aneuploidy, in lieu of invasive procedures. Even if such a stringent requirement for accuracy is not fully met, fetal cell isolation could still play an important role in prenatal screening for fetal genetic disorders, either as an independent test, or in combination with other tests such as maternal serum markers.

The following conclusions and recommendations were made:

- Fetal cells definitely exist in the maternal blood during the first and second trimester of pregnancy, offering the potential for diagnosis of fetal genetic disorders including aneuploidy.

- The optimal cell type for isolation and analysis is not known. Both trophoblasts and erythroblasts are attractive candidates, and their recovery and analysis are not mutually exclusive. For example, detecting Mendelian disorders or Rh-status early in pregnancy on unsorted cells could be based on presence of trophoblasts, whereas aneuploidy might better be detected by enrichment for erythroblasts.

- Sensitivity, specificity, positive predictive value and negative predictive value of screening based on this approach is unknown. Until resolved, isolating and analyzing fetal cells from maternal blood for detecting aneuploidy must be regarded as investigational. Clinical decisions should not be based exclusively on results of such investigations.

5. REFERENCES


ANNEX

List of Participants in the International Working Group on Non-Invasive Screening for Prenatal Genetic Diagnosis
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