On first trimester Down syndrome screening

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Chapter 1

General introduction and thesis outline

Introduction

In 1922, Painter studied human chromosomes derived from testis biopsies, and wrongly established the number of chromosomes in humans to be 48. The discovery of the correct number of chromosomes in humans to be 46 (Tjio and Levan, 1956), was the starting point for subsequent developments in human chromosome studies. The first autosomal trisomy was described by Lejeune et al., (1959), who found that Down syndrome was caused by the presence of three copies, as opposed to the normal two, of one of the smallest human chromosomes.

Nowell (1960) was able to culture lymphocytes using a substance of beans, phytohemagglutinine (PHA). That same year, the “Chromosome conference” decided to order the human chromosomes according to their size. With the discovery of a specific banding pattern on each chromosome (Caspersson, 1968), each chromosome could be uniquely identified.

Chromosomal anomalies

Down syndrome

Trisomy 21, one of the most common chromosomal abnormalities detected in pregnancy or at birth, causes Down syndrome (mongolism). It occurs in 1.2 per 1000 life births (Hook, 1992), with a life expectation of about 60 years.

John Langdon Haydon Down was the first to describe Down syndrome. In 1866, Down wrote in his paper “Observations on an ethnic classification of idiots”:

"I have for some time had my attention directed towards the possibility of making a classification of the feeble-minded, by arranging them around various ethnic standards. I have been able to find among the large number of idiots and imbeciles, which came under my observation, that a considerable portion can be fairly referred to one of the great divisions of the human race other than the class from which they have sprung. The great Mongolian family has numerous representatives, and it is to this division, I wish, in this paper, to call special attention. A very large number of congenital idiots are typical Mongols. So marked is this, that when placed side by
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side, it is difficult to believe that the specimens compared are not children of the same parents. The number of idiots who arrange themselves around the Mongolian type is so great, and they represent such a close resemblance to one another in mental power, that I shall describe an idiot member of this racial division”.

At the end of this paper he wrote: “The life expectancy, however, is far below the average, and the tendency is to the tuberculosis, which I believe to be the hereditary origin of the degeneration.”

It took almost hundred years to prove the latter believe of Down to be incorrect.

Clinical features
It is known that pregnancies with Down syndrome are selectively miscarried throughout pregnancy. From about 10 weeks of gestation to term, approximately 40 per cent of pregnancies with Down syndrome will end in a miscarriage or stillbirth (Morris et al., 1999). From 16 weeks of gestation to term, an estimated 20 per cent of pregnancies will end in a miscarriage or stillbirth, and 10 per cent of Down syndrome pregnancies will result in a stillbirth or a neonatal death.

Individuals with Down syndrome often have specific major congenital malformations. The clinical picture includes mental retardation, cardiac defects and malformations of the gastrointestinal tract, immunological deficiencies and premature aging with similarities to Alzheimer disease. There is an increased incidence of leukemia in Down syndrome. In particular acute megakaryocyte leukemia occurs 200 to 400 times more frequently in Down syndrome than in chromosomally normal people.

The features of the face are very characteristic and point towards the diagnosis. The infants have epicanthic folds, oblique palpebral fissures and a flat nasal bridge. The neck is short, often with an excess of skin. The tongue often protrudes and may be thick. The ears are small and often abnormally shaped (McKusick, 1994).

Other chromosomal anomalies
Chromosomal anomalies can be divided into two main categories: numerical anomalies and structural anomalies of individual chromosomes.

Numerical anomalies occasionally found before or after birth are trisomy 18 (Edwards syndrome), trisomy 13 (Patau syndrome), and sex-chromosomal anomalies, such as Turner syndrome (45,X), Klinefelter syndrome (47,XXY), Triple-X (47,XXX) or Double-Y (47,XYY). Most other numerical autosomal anomalies are lethal in embryos. Trisomy 18 has an incidence of approximately 1 in 7,000 live births (Hook, 1992). This condition can manifest with isolated growth retardation and/or multiple structural
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anomalies. The infants are always severely psycho-motorically retarded and mortality amounts to almost 100% before the first year of live. Trisomy 13 has an incidence of approximately 1 in 12,500 (Hook, 1992). This condition is also associated with multiple congenital anomalies, such as holoprosencephaly, facial midline defects and renal and cardiac anomalies, and death usually occurs a few weeks after birth.

Sex chromosome aneuploidies are the most frequent chromosome disorders in live-born children, with an incidence of 1 in 500 live births (Hook, 1992). These disorders, such as Turner syndrome (45,X), Klinefelter syndrome (47,XXY), 47,XXX and 47,XYY are compatible with life but may cause problems with fertility.

Structural deviations of chromosomes arise from one or more breakpoints in one or more chromosomes during DNA replication, leading to translocations, inversions, deletions, insertions or ring chromosomes. These structural deviations can be balanced, i.e. the total amount of DNA is not altered and the phenotype is normal, or unbalanced, which leads to an abnormal phenotype. The overall incidence of these structural deviations of chromosomes, including supernumerary marker chromosomes, is 1 in 365 (Hook, 1992). Clinical features of these structural chromosomal abnormalities vary between none or minor disabilities to severe (mental) handicaps.

Prenatal Diagnosis

General aspects

The possibility of obtaining fetal cells during pregnancy has made prenatal diagnosis of chromosomal abnormalities possible. The first technique that was introduced to acquire fetal cells was amniocentesis (Steele and Breg, 1966). In the early eighties a technique to sample cells from the placenta (chorionic villus sampling) became available (Simoni et al., 1983). Because the miscarriage risk that is involved in these invasive procedures, and the costs involved with karyotyping, these procedures are not offered to all pregnant women. Therefore screening methods are required to detect those women at higher risk for fetal chromosomal abnormalities.

The incidence of Down syndrome is related to maternal age (Penrose, 1933; Hook, 1981). In the Netherlands, invasive prenatal testing for chromosome analysis is offered to pregnant women aged 36 years or older at 18 weeks of gestation (Kloosterman, 1987). This age limit was chosen, because at this age the risk of carrying a child with a chromosomal abnormality equals or becomes larger than the average population risk of a chromosomal abnormality (0.5 per cent). By chance, this risk also equals or becomes larger than the amniocentesis related risk for a ‘spontaneous’ abortion.
Women eligible for invasive prenatal diagnosis are those women with an increased risk of chromosomal abnormalities, which is in the majority of cases because of advanced maternal age. Other indications are parental carrier status of a balanced chromosomal anomaly, a previous child with a chromosomal abnormality or structural defects seen at ultrasound examination suspicious for a chromosomal abnormality.

**Amniocentesis**

Amniocentesis was initially described in the nineteenth century (Prochownik, 1877; Lamb, 1881; Schatz, 1882; Henkel, 1919) in the management of polyhydramnios, but it was not until the middle of this century that the technique was applied to prenatal diagnosis and management of Rhesus isoimmunization (Liley, 1961). Bevis et al., (1953) demonstrated that spectrophotometric analysis of amniotic fluid could be used to predict the severity of Rh-isoimmunization.

A further advance was made when it became possible to culture and karyotype fetal amniotic fluid cells taken from high-risk pregnancies (Steele and Breg, 1966; Jacobson and Barter, 1967) and to diagnose Down syndrome (Valenti et al., 1968).

Apart from the cytogenetic information available from amniotic fluid cells, amniotic fluid may also be sampled for other fetal products which can be used for prenatal diagnosis. Before 20 weeks of gestation the concentration of alpha-fetoprotein in amniotic fluid can be measured to detect open spina bifida and anencephaly. At a later gestational age the concentration of (pulmonary) phospholipids can be determined to assess fetal lung maturity.

**Sampling procedure**

Nowadays amniocentesis is performed under direct ultrasound guidance so that the position of the needle and the fetus and the placenta is known (figure 1). Although amniocentesis is technically possible from 8 weeks onwards, it is usually performed around 15 weeks of gestation. Culturing amniotic fluid cells takes on average 10-14 days, with the result from karyotyping being available in most clinics after 2-4 weeks, at a gestational age of 17 to 19 weeks. In case an affected fetus is found, the pregnancy can be terminated by induction.

**Complications**

The risk of a subsequent abortion following amniocentesis is considered to be between 0.3% and 0.5%, after correction for the background miscarriage rate (Leschot and Kleiverda, 1999; Kuliev et al., 1996). This procedure-related risk is derived by
subtracting the natural background abortion risk from the total number of abortions occurring after amniocentesis. However, this natural background abortion risk is difficult to establish as no randomized controlled trials are available. These procedure-related risks are slightly affected by the operator’s experience (Leschot et al., 1985) and the number of needle insertions (Marthin et al., 1997). Other complications, as neonatal lung function abnormalities and sepsis, are rare.

The hazards of the procedure to the mother are difficult to assess. Psychological stress is a common feature in all forms of prenatal diagnosis. A retrospective study of Rothman (1986) reported that amniocentesis changes the experience of pregnancy, of women ‘denying’ their pregnancy until after the receipt of a favourable result. Women were reporting similar anxieties before the procedure (about whether it would hurt them, harm the baby or cause a miscarriage) but overwhelmingly they recalled the stress of waiting for the results. This dissatisfaction with the need to wait until the mid-trimester for prenatal diagnosis, lead to the consideration of first trimester amniocentesis.

The procedure of early amniocentesis is similar to late amniocentesis, except that less amniotic fluid is removed (10 ml instead of 20 ml). A limitation to perform earlier sampling was the finding that aspiration of large amounts of amniotic fluid earlier in gestation would be more likely to cause fetal loss, neonatal limb defects and respiratory complications (Calhoun et al., 1994; Nicolaides et al., 1994; Johnson et al., 1996; Wilson et al., 1997). The Canadian Early and Mid-trimester Amniocentesis Trial Group (CEMAT, 1998) has performed a study to assess the safety and cytogenetic accuracy of early-amniocentesis as compared to mid-trimester amniocentesis. They reported an
increased risk of talipes equinovarus (an incidence of 1.3%, as compared to 0.1% in the mid-trimester amniocentesis group) to be associated with early amniocentesis, as well as an increased risk of a spontaneous miscarriage (4.4%). Amniotic fluid leakage was more common, and the success rate for culturing the amniocytes and providing a diagnosis was lower with early amniocentesis than with mid-trimester amniocentesis. There was no difference between early and mid-trimester amniocentesis regarding respiratory problems.

**Chorionic Villus Sampling (CVS)**
In 1968, Mohr introduced the concept of chorionic biopsy for fetal diagnosis. He used hysteroscopy and was remarkably successful in obtaining villi from patients before termination of pregnancy. In 60% of the cases chorionic villi could be obtained, but in 50% there was a failure to culture these villi. Suspected damage to the membranes, possibly caused by poor visibility due to maternal bleeding, resulted in the loss of 10 out of 27 pregnancies within one week after biopsy. The reported complications and the rapid acceptance of amniocentesis delayed any major research in this area for approximately 10 years.

The increasing demand for fetal diagnosis for high risk conditions along with progress in DNA technology and the addition of ultrasound guidance led to the renewed interest in first trimester CVS. In 1975, a group of Chinese investigators (Teitung Hospital), in an attempt to establish a safe and easy method for first trimester sex determination, reported their experience with "blind" aspiration of chorionic villus material. Kazy *et al.* (1982) were the first to introduce the real time ultrasonic guidance for placental biopsy. The authors found that visualization of the placenta improved the success rate of CVS and reduced the incidence of procedure-related complications. Further interest in CVS was aroused when Simoni *et al.* (1983) demonstrated that villus material could be used for cytogenetic and biochemical diagnosis without the need to culture the cells.

**Sampling procedure**
The procedure may be carried out from 6 weeks onwards. Transcervical, transabdominal and transvaginal approaches have been described (Ward *et al.*, 1983; Smidt-Jensen *et al.*, 1984); the first two are most commonly used. The transabdominal method can be used throughout pregnancy. Before sampling, ultrasound evaluation is performed to establish the localisation of the placenta. CVS can be performed by either insertion of a biopsy instrument (forceps or
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cannule) through the cervix in the placenta, or insertion of a needle through the abdominal wall (figure 2).
Specimens yielded by CVS can be examined directly to give a karyotype within 24 to 48 hours. Alternatively, they can be cultured; this takes 8 to 10 days. In case an affected fetus is found, the pregnancy can be terminated using suction before 14 weeks of gestation.

Figure 2. Transcervical (A) and transabdominal (B) CVS (from: birth defects, AMC, 1997).

Complications
The main complication of CVS is fetal loss following the procedure. This CVS-associated fetal loss rate (0.5%) is comparable to that after amniocentesis (Kuliev et al., 1996; Leschot and Kleiverda, 1999). Again it is difficult to assess the true abortion rate caused by CVS, given the relatively high background spontaneous miscarriage rate in the first trimester of pregnancy. Risk factors for fetal loss after CVS are a history of either a first-trimester miscarriage or delivery between 16 and 27 weeks and multiple insertions required to obtain sufficient villus material (Lunshof et al., 1995). Alfirevic et al. (1996) found in a review which compared CVS with mid-trimester amniocentesis, a statistically significant increase in miscarriages in the CVS group that was procedure related.

CVS performed before 10 weeks of gestation gives an increased risk of limb reduction defects (Firth, 1997). Therefore it is currently performed between 10 and 12 weeks of
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gestation. At this gestational age there is no increased risk of limb reduction defects (Kuliev et al., 1996).

Other technical problems associated with CVS are maternal cell contamination, chromosomal mosaicism, chromosome anomalies confined to the placenta, and false-negative and false-positive results. Mosaicism may be found either as an artifact of culture (pseudo-mosaicism), or as a genuine difference between placenta and fetus (confined placentomal mosaicism), or as true mosaicism in the fetus. In a large study (62,865 CVS samples), Hahnemann and Vejerslev (1997) found either a normal karyotype (true negative result) or a non-mosaic chromosomal aberration (true-positive result) in 98.5% of cases. A true fetal mosaicism was diagnosed in 0.15% of the samples, while confined placentomal mosaicism occurred in 1.0%. False positive (0.15%) and false-negative CVS results (0.03%) were also observed, as well as failure to produce a diagnosis (0.15%). False-positive results, mainly trisomy 18 and monosomy X, and false-negative results were only found in this study after direct preparation. This occurs as a result of postzygotic nondisjunction in the aggressively dividing cytotrophoblast cells (Bianchi et al., 1993).

Ultrasound examination

Obstetric ultrasound started in the late 1950s with the development of the static scanner. It became possible to quantify in vivo fetal growth by measuring distances and circumferences in particular of the head, the abdomen and the long bones. With the introduction of real-time imaging, many images could be obtained per second, the image resolution was improved and the information on fetal development became more detailed. With the improvement of ultrasound technique, ultrasonographers focused on the visualization of fetal structures and organs. Detailed information on development and anatomical structures have been recognized by ultrasound. The introduction of vaginal probes made earlier examination possible.

The efficiency of (routine) ultrasound examination for fetal abnormalities has been extensively studied. The RADIUS study (Routine Antenatal Diagnostic Imaging with Ultrasound), including more than 15000 women without a specific indication for ultrasonography, showed that routine ultrasound examination has no clinically significant benefit (Ewigman et al., 1993; LeFevre et al., 1993 Crane et al., 1994).

However, in a high-risk population the accuracy of ultrasound in diagnosing congenital anomalies is more than 90% (Garmel and D’Alton, 1994). Moreover, ultrasound appears to be the preferred strategy for most women to get reassurance about their child’s wellbeing (Romano and Waitzman, 1998).
Other prenatal diagnostic procedures

**Cordocentesis**
Cordocentesis is an invasive technique to obtain fetal blood. The first fetal blood samplings were performed during labor (Saling, 1966). Blood obtained through a small incision in the skin of the fetal scalp exposed by an amnioscope after rupture of the membranes could be used for evaluation of acid-base status. This became, and still is, a standard technique of assessing fetal well being during labor. Valenti, in 1973, was the first to sample fetal blood during pregnancy. Fetal blood was obtained under direct vision (fetoscope) by puncture of the chorionic plate vessels (Hobbins and Mahony, 1974) or the umbilical cord vessels (Rodeck and Campbell, 1978). This approach first allowed for direct intravascular transfusion to correct fetal anemia due to Rh isoimmunization (Rodeck et al., 1981). Improvements in ultrasound technology enabled the introduction of ultrasound guided puncture of the umbilical cord (cordocentesis), which is the current method of fetal blood sampling.

Fetal blood sampling is used to assess the fetal karyotype in the presence of structural defects diagnosed by ultrasound which are known to be associated with an increased risk of chromosomal abnormalities (exomphalos, non-immune hydrops, cystic hygroma, obstructive uropathy, cardiac malformations, etc.). Such rapid karyotyping is not only indicated in the mid-trimester, when termination of pregnancy in case of an aneuploid fetus is still an option, but also in the third trimester, when knowledge of a serious chromosomal defect may guide antenatal and antepartum management, including the mode of delivery.

Other reasons to sample fetal blood sampling are cases in which a haemoglobinopathy or congenital infection (as toxoplasmosis, rubella, CMV or herpes) is suspected. The risk of fetal loss following cordocentesis is comparable with the loss rate after CVS or amniocentesis (Antsaklis et al., 1998).

**Uncultured amniocytes**
The relative late gestational age at which amniocentesis is performed, and the relative long time before the diagnosis is known, led to the interest of prenatal diagnosis on uncultured amniocytes. With fluorescent in situ hybridization (FISH), a technique that can highlight specific chromosomes in non-dividing (interphase) cells, a limited diagnosis using amniocentesis can be available within three days.

Van Opstal et al. (1998) used FISH for the rapid detection of chromosome aberrations in uncultured amniocytes in cases of ultrasound abnormalities. In 196 cases, the amniocytes were screened with probes specific for chromosomes X, Y, 13, 18 and 21.
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These chromosomes account for the great majority of all chromosome aberrations found in chromosomally abnormal fetuses presenting with ultrasonographic detected anomalies (Wladimiroff et al., 1995). In 46 of 196 cases a chromosomal anomaly existed, that could theoretically have been detected with FISH. Three of these detectable chromosome aberrations were missed; there was one false negative result (a failure to detect trisomy 13), and 2 uninformative results due to technical failures. Van Opstal et al. concluded that FISH on uncultured amniocytes provides a rapid and accurate method for prenatal diagnosis of chromosomal aneuploidy in pregnancies complicated by fetal anomalies. However, the reliability, and therefore the clinical utility, will depend on the specificity and hybridisation efficiency of the probes. Also Eiben et al. (1998) detected with FISH on uncultured amniocytes all cases with a chromosomal aberration, with the exception of one case (of trisomy 21) which was due to a technical problem. Neither false-positive nor other false-negative results were obtained and they also concluded that FISH is a valuable and reliable method for rapid prenatal diagnosis.

Prenatal screening

Screening is not diagnosis

"Screening means the identification among apparently healthy individuals, of those who are sufficiently at risk of a specific disorder to justify a subsequent diagnostic test or procedure, or, in certain circumstances, direct preventive action. The screening procedure may take the form of a simple inquiry (e.g. determining maternal age in case of Down syndrome screening) or a special test (e.g. maternal serum alpha-fetoprotein estimation in case of neural tube defect screening). It differs from a diagnostic procedure in that there is no intention to offer therapeutic intervention solely on the basis of a positive screening result." (Cuckle and Wald, 1984).

Screening may lead to unnecessary interventions that pose a health risk to the patient. The Committee Genetic Screening of the Health Council in the Netherlands has published a report with criteria that should be considered before the introduction of a screening program. Most of the criteria described in that report are based on principles first suggested by Wilson and Jungner (1968):

"The condition screened for should be an important health problem. There should be an accepted treatment for patients with recognized disease and there should be an agreed policy on whom to treat as patients. Facilities for diagnosis, a suitable and generally acceptable test, and facilities for treatment should be available. The natural history of the condition, including a recognized latent or early..."
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symptomatic stage and development from latent to declared disease, should be adequately understood. The cost of case-finding (including diagnosis and treatment) should be economically balanced in relation to possible expenditure on medical care as a whole. Case finding should be a continuing process and not a “once and for all” project.”

Down syndrome screening may be considered in perspective of the above described criteria (Wildhagen et al., 1996).

“Down syndrome is an important health problem. In Down syndrome screening the mother carrying the Down syndrome fetus should be seen as “patient”. With a screening test being the assessment of maternal age or a blood sample, a patient can reliably be identified as being at high risk. These tests are quite acceptable for the patient. The diagnosis Down syndrome can be made reliably by karyotyping using amniocentesis, CVS or cordocentesis. Actually, there is no prenatal “treatment” besides termination of the pregnancy, but prenatal diagnosis may lead to more options in choice.”

Prenatal screening for Down syndrome is directed towards identifying women at increased risk of having a Down syndrome fetus and to offer them the option of invasive prenatal diagnosis for fetal karyotyping in order to enable them to make a decision about the continuation of the pregnancy.

Screening test characteristics

The clinical use of a screening test is dependent, besides on the incidence of the condition screened for, on the test-characteristics. The most important characteristic is to what extent the test discriminates between affected and unaffected individuals, i.e. the accuracy. This can be expressed by the sensitivity, i.e. the proportion of affected individuals with a positive test result, and the specificity, i.e. the proportion of unaffected individuals with a negative test result, or by the false-positive rate (the number of unaffected individuals with a (incorrect) positive test result) and the false-negative rate (the number of affected individuals with a (incorrect) negative test result. The proportion of individuals with a positive test result that were actually affected, is expressed as the positive predictive value. And vice versa, the proportion of individuals with a negative test result that were unaffected can be expressed as negative predictive value. The positive and negative predictive values directly assess the usefulness of the test in practice, but are dependent on the prevalence of the condition tested for in the study population.
The detection rate (sensitivity) and the false-positive rate are influenced by the chosen cut off level, the level at which a result is considered as positive. If a higher false-positive rate is accepted, the detection rate will increase.

**Maternal age**

With the introduction of invasive prenatal diagnostic tests it was clear that karyotyping all fetuses was practically and financially impossible. Therefore a method to identify women at highest risk was sought. The only known risk factor for having a child with Down syndrome at population level was maternal age (Penrose, 1933; Hook, 1992). Maternal age is used as selection criterion or screening method (figure 3).

![Figure 3: Estimated rate per 1000 of a Down syndrome live birth. Data from Cuckle et al. (1987), Hook and Chambers (1977), and Huether et al. (1981). The space between the double line represents the range.](image)

At this moment approximately 14% (CBS, 1997) of the live births in the Netherlands is from women 36 years or older, and this percentage is still rising. The incidence of Down syndrome increases with this increasing mean maternal age in a population. However, the incidence of Down syndrome live births has been stable over the last 25 years (EUROCAT). This can be attributed to the performances of prenatal diagnosis, for a growing proportion of women are eligible for invasive prenatal diagnostic testing. The uptake rate of invasive prenatal diagnostic tests has decreased slightly over the past few years. In 1991, 47% of the women aged 36 years or older underwent an invasive prenatal diagnostic procedure, as compared to 45% in 1996 (WPD, 1999).
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Biochemical screening

Second trimester maternal serum screening

In a screening program for Neural Tube Defects (NTD), by determination of maternal serum alpha-feto-protein (AFP) concentration, a relation was detected by chance between a low AFP level and Down syndrome (Merkatz et al., 1984). By combining the information derived from the AFP level with the patient's age related risk an individual risk estimate for each pregnancy could be established (Cuckle et al., 1984).

In 1987, the finding of higher maternal serum levels of human chorionic gonadotrophin (hCG) in women carrying a fetus with Down syndrome was reported (Bogart et al.). This glycoprotein hormone consists of an α- and a β-subunit. The α-subunit is virtually the same as in luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyrotrphin stimulating hormone (TSH). The β-subunit is pregnancy specific. Total hCG is produced by the syncytiotrophoblast cells and it appears in the maternal circulation shortly after implantation. The free β-subunit is more specific in detecting Down syndrome than total hCG (Spencer et al., 1992). The underlying pathology and biochemistry for this increase in (free β-) hCG is unknown.

The fetal liver also participates in the synthesis of the fetoplacental hormone estriol. Canick et al., (1988) studied unconjugated estriol (uE3) in maternal serum and found lower levels in fetal Down syndrome. As each marker had a low sensitivity and specificity in screening for Down syndrome, measurement of these three markers (AFP, hCG and uE3) was combined with maternal age to produce a “triple-test”, capable of detecting 60% of Down syndrome pregnancies in the general pregnant population, for a 5% amniocentesis rate (Wald et al., 1988).

At present, AFP, hCG and uE3 form the basis for second trimester prenatal serum screening for Down syndrome. The Gaussian height for the normal and the Down syndrome group can be calculated for example for the AFP concentration (figure 4). The ratio between the two calculated heights is the likelihood ratio (LR). Down syndrome risk is estimated by multiplying the age-related risk for carrying a Down syndrome fetus (pre-test odds) with the likelihood ratio. The following formula is used:

$$\text{Pre-test odds} \times \text{likelihood ratio} = \text{post-test odds}$$

$$(\text{maternal age-related risk} \times LR = \text{Down syndrome risk})$$

The concentrations of the analytes used to calculate the LR is dependent of gestational age. Therefore, the results of the measurements of the analytes are expressed as multiples of the gestation specific median (MOM).
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Recently (dimeric) inhibin-A has been added to the existing maternal serum screening programs. Cuckle et al. (1996) has estimated that adding the measurement of inhibin-A to AFP, hCG and uE3 can increase the Down syndrome detection rate with 7%.

![Gaussian frequency distributions of a hypothetical test in affected and unaffected populations. The likelihood ratio (LR) is calculated from the height of the affected curve divided by the height of the unaffected curve; LR = b/a.](image)

Factors influencing second trimester biochemical screening

With the aim of optimizing the screening, factors that could influence the results of the biochemical tests have been investigated.

Maternal weight is known to influence the levels of biochemical markers. An increase in maternal weight gives a decrease in the concentration of the biochemical markers for the same amount of substance is diluted in a greater maternal blood volume. In an overweight woman, the test results in a lower AFP level, putting her at increased risk of a fetus with Down syndrome, but her hCG concentration is also lower, thereby lowering her risk of Down syndrome. There is a small improvement in screening efficiency after adjusting for maternal weight (Wald et al., 1992), so in most maternal serum screening programs marker levels are adjusted for maternal age.

Maternal smoking also affects maternal serum screening results. Due to smoking during pregnancy, hCG and uE3 are reduced (Bernstein et al., 1989) and AFP levels tend to increase (Cuckle et al., 1990).

Other variants known to influence maternal serum concentrations are race (Watt et al., 1996), insulin-dependent diabetes mellitus (Wald et al., 1992b), gravidity and parity (Barkai et al., 1996), the result from maternal serum screening in a previous pregnancy (Holding and Cuckle, 1994; Dar et al., 1995), and twin pregnancies (Neveux et al., 1996).
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1996). Adjusting maternal serum levels for these factors has been shown to have a marginal effect on screening performance in the second trimester.

First trimester maternal serum screening
With the advances in second trimester maternal serum screening for fetal Down’s syndrome and the introduction of CVS, the increasing demand for early prenatal diagnosis stimulated the search for biochemical markers in the first trimester of pregnancy. Screening in the first trimester appears to have many advantages, in particular because of earlier and technically easier termination in case of an affected pregnancy and earlier reassurance (Kornman et al., 1997).

In 1986, the association between low maternal serum AFP in the first trimester and fetal aneuploidy was reported (Brambati et al., 1986). The other biochemical markers used in the second trimester do not work as well in the first trimester as in the second trimester. Other serum markers have been studied, as pregnancy-associated plasma protein A (PAPP-A), Schwangerschafts protein 1 (SP1), cancer antigen 125 (CA 125), free-beta hCG, free-alpha hCG, inhibin and inhibin-A (Cuckle and Van Lith, 1999). Of all these markers PAPP-A and free beta-hCG turned out to be useful in discriminating Down’s syndrome pregnancies from unaffected pregnancies at 8-14 weeks of gestation. PAPP-A levels are lower and free-beta hCG levels higher in Down syndrome pregnancies as opposed to pregnancies with normal fetuses. The combination of PAPP-A, free-beta hCG and maternal age can achieve a detection rate in the first trimester of pregnancy comparable to that in the second trimester of pregnancy (Casals et al., 1999).

Urine screening
Most Down syndrome screening protocols have focussed on the analysis of maternal serum markers. Screening based on a urine sample has several logistical advantages. Beta-core is the major degradation product of free β-hCG. Several groups have measured this degradation product in maternal urine, and median MoM values in Down syndrome cases varied between 4.38 and 6.28 (Iles, 1996). These results suggest that urinary β-core is superior to any single serum marker. However, these early reports are in contrast to later reports, which show variable and inconsistent results (Cole et al., 1999). If allowed for the individual urinary volume output, or if samples are used that are frequently frozen and thawed, results are less impressive.

Urinary analysis in the first trimester of pregnancy has been shown to be of no value in discriminating Down syndrome from unaffected pregnancies (Kornman et al., 1997b).
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Ultrasound screening

Second trimester ultrasound screening for Down syndrome

Many ultrasound markers for Down syndrome have been suggested, as for example nuchal fold, hydronephrosis, echogenic bowel, shortening of the humerus and femur length, duodenal atresia, cardiac defects, hypoplasia of the middle phalanx of the fifth digit, sandal gap, clinodactyly, wider fetal iliac angle, frontal lobe shortening and mild ventriculomegaly. Although these findings occur more often in Down syndrome than in chromosomally normal fetuses, it is difficult to estimate their positive and negative predictive values. To denote when karyotyping should be offered, at what ultrasound abnormalities, Benacerraf (1996) suggested a scoring system where a score of two or more is reason to offer an invasive procedure. Recently, this sonographic scoring index was adjusted (Bromley et al., 1997), and was capable of detecting 75% of Down syndrome fetuses to a false-positive rate of 5.7%. Second trimester ultrasound screening for Down syndrome is shown to be cost-beneficial only when its overall sensitivity is over 74% (Vintzileos et al., 1998).

First trimester ultrasound screening for Down syndrome

Improvement in ultrasound resolution made fetal anomalies visible in the first trimester. In 1990 the association between a subcutaneous nuchal fluid accumulation in the first trimester and trisomy 21 was reported (Szabo and Gellen, 1990). In the first prospective study by Nicolaides et al. (1992), measurement of this nuchal fluid accumulation or nuchal translucency, as this ultrasound feature was called according to its appearance, between 10 and 14 weeks of gestation was shown to be a useful marker for fetal aneuploidy, especially Down syndrome. This finding was confirmed by many other studies (Nicolaides et al., 1994b; Brambati et al., 1995; Taipale et al., 1997; Pajkrt et al., 1998; Snijders et al., 1998). Despite all these efforts, the exact performance of nuchal translucency measurement in detecting Down syndrome is still unclear. The two largest studies (Taipale et al., 1997; Snijders et al., 1998) report detection rates between 54% and 72% in an unselected pregnant population.

The exact morphological background and the aetiology of the nuchal translucency remain unclear. Some authors suggest that nuchal fluid accumulation may be the consequence of hemodynamic readjustments during cardiovascular development. Hyett et al. (1998), indeed found that 56% of fetuses with major defects of the heart and great arteries showed an enlarged NT at 10 to 14 weeks of gestation. However, most of the types of congenital heart defects described in that study would not be expected to produce heart failure during prenatal life (Simpson, 1999).
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Rare genetic syndromes can present prenatally with an enlarged NT at 10 to 14 weeks of gestation. Amongst these syndromes detected prenatally or at birth are Smith-Lemli-Opitz syndrome, Frijns syndrome, EEC syndrome, Stickler syndrome, arthrogryposis, Noonan syndrome, Jarco-Levin syndrome and Joubert syndrome (Bilardo et al., 1998; Soukka et al., 1998).

Mostly, fetuses showing an enlarged nuchal translucency at first trimester ultrasound examination are affected by Down syndrome. Chromosome 21 contains the gene that codes for type VI collagen. It is conceivable that one sub-unit of this collagen can be over-expressed in Down syndrome, resulting in connective tissue that is more elastic (Berger, 1999), and thus can contain more fluid.

Most pregnancies affected by Down syndrome presenting with an enlarged nuchal translucency are being terminated. Only one study did report on the intrauterine lethality in Down syndrome fetuses in relation to nuchal translucency (Hyett et al., 1996). Fetuses with an enlarged NT showed an almost doubled risk of fetal loss, suggesting that the detection rate of Down syndrome fetuses that result in a live birth of children with Down syndrome, would be not as high as reported.

Fetal cells in maternal blood

Despite many years of research, using a variety of ingenious approaches, the transfer of nucleated cells across the placenta from fetus to mother remains a topic of great interest and active research, since many problems are as yet unsolved. Attempts of isolating fetal nucleated cells from maternal blood samples have been carried out in the hope of using these cells to perform prenatal diagnosis by a non-invasive procedure.

The presence of fetal cells in the maternal circulation was first described in 1893 by Schmörl, a pathologist who found trophoblast cells in the peripheral circulation of women who had died from pre-eclampsia. The first cytogeneticists who began to search for fetal cells in maternal blood were Walknowska et al. (1969), who found lymphocytes in the maternal circulation containing five acrocentric chromosomes, that were thus derived from a male fetus. In the subsequent years, similar findings were reported (Grosset et al., 1974; de Grouchy and Trebuchet, 1974). However, the presumptive male cells were also observed in blood samples from mothers who later delivered a female fetus. The most plausible explanation is that these authors were looking at fetal cells from a previous pregnancy.

In 1979, Herzenberg et al. described a cell sorting technique, fluorescent activated cell sorting (FACS) that made it possible to sort for rare cells. Fetal cells are very rare in maternal blood, but identification with antibodies against the HLA type of the father
Chapter 1

was possible. The idea was to sort cells by flow-cytometry containing a paternal allele not present in the mother. Only a few sorted cells were confirmed as being fetal.

With the introduction of the polymerase chain reaction (PCR) many groups renewed interest in fetal cells in the maternal circulation. Lo et al. (1989) were the first to use PCR to indicate Y-sequences in whole blood of pregnant women carrying male fetuses. The original work of this group was performed on unsorted cells. Their findings were confirmed by others (Bianchi et al., 1990; Wachtel et al., 1991). Then FISH (fluorescent in situ hybridization) was introduced, which made it possible to make a diagnosis of aneuploidy on non-dividing, uncultured cells. Many groups were encouraged by this new application. Different cell types, enrichment procedures, detection methods and monoclonal antibodies were tested, but without conclusive results to which of these was the best strategy.

Table 1. Published trials on nucleated red blood cells isolated from maternal blood.

<table>
<thead>
<tr>
<th>Year (Ref)</th>
<th>Enrichment procedure</th>
<th>Antibodies</th>
<th>Detection</th>
<th>N</th>
<th>Males found</th>
<th>No. of fetal cells</th>
<th>GA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1991¹</td>
<td>FACS*</td>
<td>CD-71-GPA</td>
<td>PCR (Y)</td>
<td>18</td>
<td>12 of 12</td>
<td>9 of 14</td>
<td>10-18</td>
</tr>
<tr>
<td>1993²</td>
<td>MACS*</td>
<td>CD-71</td>
<td>FISH (XY)</td>
<td>10</td>
<td>6 of 6</td>
<td>5-23</td>
<td>16-36</td>
</tr>
<tr>
<td>1993³</td>
<td>FACS</td>
<td>CD71-CD36 GPA</td>
<td>PCR (Y)</td>
<td>49</td>
<td>13 of 24</td>
<td></td>
<td>8-19</td>
</tr>
<tr>
<td>1993⁴</td>
<td>MACS</td>
<td>CD45-CD32 HbF</td>
<td>FISH (XY)</td>
<td>6</td>
<td>4 of 5</td>
<td>2-8</td>
<td>13-17</td>
</tr>
<tr>
<td>1994⁵</td>
<td>MACS</td>
<td>CD45-CD14/CD71</td>
<td>PCR (HLA)</td>
<td>11</td>
<td>7 of 11</td>
<td></td>
<td>12-25</td>
</tr>
<tr>
<td>1994⁶</td>
<td>Unsorted</td>
<td></td>
<td>FISH (XY)</td>
<td>59</td>
<td>12 of 19</td>
<td>1-12</td>
<td>17-39</td>
</tr>
<tr>
<td>1995⁷</td>
<td>Unsorted</td>
<td></td>
<td>FISH (XY)</td>
<td>30</td>
<td>12 of 16</td>
<td>1-57</td>
<td>16-20</td>
</tr>
<tr>
<td>1995⁸</td>
<td>FACS</td>
<td>CD45-CD71 HbF</td>
<td>FISH (XY)</td>
<td>19</td>
<td>12 of 19</td>
<td>1-8</td>
<td></td>
</tr>
<tr>
<td>1997⁹</td>
<td>MiniMACS</td>
<td>CD71-GPA/CD36</td>
<td>FISH (XY)</td>
<td>8</td>
<td>7 of 17</td>
<td>1-16</td>
<td>15-19</td>
</tr>
<tr>
<td>1997¹⁰</td>
<td>MACS</td>
<td>CD45-CD14</td>
<td>FISH (XY)</td>
<td>34</td>
<td>4 of 17</td>
<td>1-4</td>
<td>11-14</td>
</tr>
<tr>
<td>1998¹¹</td>
<td>CFS°</td>
<td></td>
<td>FISH (XY)</td>
<td>17</td>
<td>9 of 10</td>
<td>2417</td>
<td>10-18</td>
</tr>
<tr>
<td>1998¹²</td>
<td>Unsorted</td>
<td>DAB-HbF</td>
<td>FISH (XY)</td>
<td>20</td>
<td></td>
<td></td>
<td>10-14</td>
</tr>
</tbody>
</table>

*Fluorescent activated cell sorter (FACS), † Magnetic Activated Cell Sorter (MACS), ‡ Charged Flow Separation (CFS). 1; Price et al., 2; Gänshirt-Ahlert et al., 3; Bianchi et al., 4; Zeng, 5; Büsch et al., 6; Björkqvist et al., 7; Hamada et al., 8; Simpson and Elias, 9; Campagnoli et al., 10; Jansen et al., 11; Wachtel et al., 12; Oosterwijk et al.
Among the cell types that have been addressed as target cells are lymphocytes, granulocytes, trophoblast cells and nucleated red blood cells. A disadvantage of lymphocytes is their long survival in the maternal circulation. Bianchi et al. (1996) found fetal lymphocytes in the maternal circulation as long as 36 years after delivery, which makes them useless for prenatal diagnosis. Trophoblast cells also have disadvantages. A highly specific antibody is not available to sort for these cells. As is known from CVS, trophoblast cells can give discrepant results (as confined placental mosaicism) since they originate from the placenta. Nucleated erythrocytes (NRBCs) stood out to be the best candidate. In table 1 the results of the published trials on fetal nucleated red blood cells (NRBCs), the cell type that turned out to be the best candidate, are listed.

Although NRBCs are rare in the maternal circulation of pregnant women, they are even rarer in non-pregnant women. Furthermore, there are several antibodies available for NRBCs, such as the transferrin receptor, glycophorin-A or fetal hemoglobin. Moreover, NRBCs have a very short life span (up to 90 days), so it is impossible that NRBCs persist from a previous pregnancy.

The exact number of fetal cells circulating in maternal blood is very difficult to estimate. Estimations from recent studies vary between 0 and 5.7 fetal cells per milliliter maternal blood (Hamada et al., 1995; Cheung et al., 1995; Little et al., 1997; Sohda et al., 1997; Bianchi et al., 1997). A much higher frequency was reported by Wachtel et al. (1996), who used a charged-flow-separation technique, and estimated that several thousands of fetal cells were present in maternal blood samples. Various factors are thought to influence this number. A previous invasive procedure can cause a fetomaternal transfusion (Jansen et al., 1997), with a significant increase in fetal cells. Recently Bianchi et al. (1997) showed an increase in fetal cell equivalents in aneuploid pregnancies using quantitative PCR.

Psycho-social aspects of prenatal screening

Besides medical and technical aspects of screening for aneuploidy, there are psychosocial aspects to be considered. These aspects, perhaps even more than technical ones, determine the overall acceptability of screening in prenatal diagnosis and play a major role in deciding what screening policy will be preferred.

Each woman has to make her own decision about whether or not to take tests, after being fully informed about her possibilities (informed consent). A woman’s motive may be quite different from those of the providers of screening tests. Tests may tell a woman that her baby has the particular abnormality screened for, or they may reassure her that
it does not. A woman who is ambivalent about screening for fetal abnormalities may still choose to have a screening test because her need for reassurance takes priority. A number of factors contribute to a woman’s decision about screening tests. One important factor is what Tijmstra (1990) called the “decision regret”; a choice is made partly in order to avoid future regret at not having made that choice. Negative feelings might arise if it should appear that the “wrong” decision was made. Women may fear, and try to anticipate on, the regret they might feel if they avoid prenatal screening or diagnosis and then give birth to a child with Down syndrome. When offering screening to the whole population, it has to be avoided that screening gets an imperative nature (Tijmstra, 1991). Society may feel that it is forced into preventive action. Any handicap might be seen as failed prevention or even worse, the result of a patient's refusal to comply with preventive tests.

Each program, which aims at finding fetal anomalies, is likely to produce stress and anxiety. One advantage of maternal age screening is the fact that pregnant women know in advance whether or not they are at a higher risk of having a child with Down syndrome. With serum or ultrasound screening, any woman may turn out to be at an increased risk for this disorder. Many studies have been performed about the anxiety level induced by screening. It is known that the anxiety of screened women rises after a screen-positive result, but returns to normal or an even lower level within the second trimester of their pregnancy (Marteau, 1992).

It is important to realize that screening can do harm. Tijmstra (1991) called this the prevention paradox: “Screening programs bring much benefit to the population but offer little to each participating individual; it is not possible for one person to profit without another losing out”. All women participating in screening programs must understand the distinction between screening and diagnosis; the purpose, limitations and risks of the procedures that they undergo and the options for further testing. Inadequate understanding may result in uninformed uptake of screening tests as well as uninformed decline. Giving adequate information on prenatal screening might not be easy in the Dutch prenatal care system (Leschot, 1991), and it must be stressed that the procedures offered are screening tests and not diagnostic tests.

Aim of this thesis

Second trimester maternal serum screening for Down syndrome clearly shows the feasibility of this screening method and its superiority above maternal age screening in terms of detection efficiency. A disadvantage of second trimester screening is the relative late gestational age at which maternal blood is sampled. Therefore, we
investigated whether it is possible to move prenatal screening for Down syndrome into the first trimester of pregnancy.

The aim of the thesis is to answer the following questions:

1. Can measurement of the nuchal translucency in the first trimester of pregnancy be combined with first trimester maternal serum screening for Down syndrome?
2. Can measurement of the nuchal translucency thickness be introduced in routine ultrasound practice?
3. Can fetal cells be isolated from the maternal peripheral circulation and be used in prenatal diagnosis for Down syndrome?
4. What prenatal diagnostic or screening test do pregnant women prefer, and at what gestational age?

Thesis outline

In chapter 2 the efficiency is studied of combined screening with the first trimester serum markers PAPP-A, free β-hCG and AFP, and the ultrasound marker nuchal translucency. This is done by a retrospective case-control study.

Chapter 3 focuses on the co-variables that are known to have an influence on the markers used in second trimester maternal serum screening. The influence of maternal weight, smoking habits, gravidity and parity on the first trimester markers PAPP-A, free β-hCG and AFP is determined.

In chapters 4 the average extra time needed to measure the nuchal translucency thickness on top of a routine ultrasound examination is studied. The ultrasound examinations of women coming for nuchal translucency measurement are recorded, and the time interval between the different measurements calculated.

In chapter 5 the best gestational age at which to measure the nuchal translucency thickness is determined. The nuchal translucency thickness is measured weekly in pregnant women at a gestational age between 10 and 15 weeks.
Chapter 1

In chapter 6 the effect is studied of fetal position on the measurement of the nuchal translucency thickness. Is the thickness of the nuchal translucency influenced by a prone or supine fetal position.

In chapter 7 is reported of a pregnancy in which an enlarged nuchal translucency was seen at 12 weeks, but karyotyping revealed a normal chromosome pattern. The child turned out to be affected by Zellweger syndrome.

Chapter 8 deals with a new approach in prenatal “diagnosis”; the isolation of fetal cells in maternal blood. A novel enrichment procedure and automated detection system were tested and evaluated.

In chapter 9 a pregnancy with fetal triploidy is described in which fetal cells isolated from the maternal circulation are found with the described method.

In chapter 10 the opinions, wishes and needs of pregnant women regarding prenatal testing for Down syndrome are evaluated. The knowledge about existing prenatal diagnostic and screening tests, and the preferences of pregnant women about screening tests that will be available in the near future is determined.

References


Introduction


Chapter 1


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