Chapter 1
Introduction
1.1 Prenatal cytogenetic diagnosis

In many parts of the world prenatal diagnosis has been part of antenatal care for already thirty to forty years. The purpose of prenatal diagnosis is to determine whether a foetus, believed to be at risk for a certain disorder, actually has that disorder and to inform the parents about the possible course, seriousness and treatment of the disorder. On the basis of that information they can decide to continue the pregnancy or not. In the Netherlands prenatal diagnosis is performed in 5% - 10% of all pregnancies (Niermeijer et al., 1997; Nagel et al., 2004). Women with an increased risk of bearing a foetus with a chromosomal abnormality can opt for a prenatal cytogenetic diagnosis. The purpose is to detect or exclude a chromosomal abnormality of the foetus. For that, sufficient metaphase chromosomes of an appropriate quality are needed. Current routine procedures to obtain cells for prenatal cytogenetic diagnosis are amniocentesis (AC) and chorionic villus biopsy (CVB). In the Netherlands, 8370 ACs and 3230 CVBs were performed in 2002 (Dutch Prenatal Working Party, 2002). Thus, second trimester amniocentesis, normally performed between 16 – 18 weeks of pregnancy, when 15 – 20 ml amniotic fluid is drawn under direct ultrasound guidance, is the preferred technique in most centres for prenatal diagnosis. There is an estimated procedure-related risk of abortion of 0.5% - 1% (NICHD, 1976). Because of the introduction of first trimester serum screening combined with an early cytogenetic analysis in Groningen, CVB is there, in contrast to other centres, the most used invasive prenatal procedure for cytogenetic diagnosis. CVB is usually performed transcervically or transabdominally between 10 – 12 weeks of pregnancy, when under ultrasound guidance 20 – 100 mg chorion villi are obtained for karyotyping. There is an estimated risk of procedure related abortion of 0.5% - 1.0% (Brambati et al., 1991), i.e. similar as the risk associated with AC.
1.1.1 Procedure of cytogenetic diagnosis on amniotic fluid cells

Amniocentesis was first used in Germany in the early 1880s to treat hydramnions (Lambl, 1881). In the 1950s amniotic fluid was used for monitoring bilirubin levels in foetuses with Rhesus isoimmunization (Bevis, 1952). This development was followed by the determination of foetal sex (Fuchs and Riis, 1956) in amniotic fluid (AF) cells. The first chromosomal analysis was performed in 1966 by Steele and Breg, and the first chromosomal abnormality, a balanced translocation, was detected by Jacobson and Barter in 1967. In 1968, both Valenti et al. and Nadler et al. reported in utero detection of Down syndrome, with confirmation after elective abortion. The relative safety and accuracy of the procedure was demonstrated in a study on 142 patients during 155 pregnancies (Nadler and Gerbie, 1970). In the same period, AF was used for prenatal detection of biochemical abnormalities (Galjaard, 1972). Brock and Sutcliff (1972) found an association of a raised concentration of alpha-fetoprotein in AF and open neural tube defect.

AF is suggested to be a mixture of transudate of the maternal plasma, urine and other body secretions from the foetus, accumulating into the AF space (Behrman et al., 1967). It contains cells that can be precipitated by low-speed centrifugation. The cell pellet consists of at least three cell types that can be morphologically distinguished in culture; fibroblast-like (F) cells, epithelioid (E) cells and amniotic fluid (AF) type cells (Hoehn et al., 1975). The precise foetal origins of these cells have still not been resolved. AF cells are thought to originate from trophoblast tissue as it has been demonstrated that these cells produce chorionic gonadotropin (Laundon et al., 1981), a product of trophoblast tissue. E-type cultured cells probably come from foetal skin and AF-type cells are from mesenchymal origin. Also kidney tissue might contribute to cells in amniotic fluid, since it has been implicated as a source of trisomy 20 cells (Hsu et al., 1991). For cell culture either the in-situ method or the flask method are applied as basic techniques. For in-situ culture, cells are grown on coverslips and harvested in-situ, on average after 6-14 days. The in-situ harvest method has first been described by Cox et al. (1974), who used clones of AF cells grown and harvested in Petri dishes.
The sides of the dish were removed and the bottom on which the cells were growing was taped on a slide for microscopy. Peakman et al. (1977) modified this method and grew cells on coverslips inside Petri dishes. The coverslips were ‘harvested in-situ’ in the dishes, removed and attached to slides for microscopy. Cells cultured according to the flask method are harvested from a cell suspension, which is obtained by trypsinisation of the cells growing attached to the bottom of the flask. Since a proportion of cells is lost in this procedure, more cells are needed, which implies a longer culture time.

Whatever the culture method, before harvesting cultures are treated overnight with the metaphase arrester colcemid to obtain adequate numbers of cells in metaphase. Hypotonic treatment is performed the following day and cells are fixed several times with a mixture of methanol and glacial acetic acid. At last, the fixative is removed and the cells are allowed to dry under specific climate conditions to achieve well-spread metaphase chromosomes. After "ageing" either overnight or for a short period at 90°C, the slides are ready for histochemical treatment in order to produce discriminating banding patterns on the chromosomes.

From the stained slides a certain number of metaphases will be analysed. For preparations from in-situ cultures a single metaphase per clone is used for analysis.

1.1.2 Procedure of cytogenetic diagnosis on chorionic villi

In 1968, Mohr reported a new method as an alternative to second trimester amniocentesis. In this method chorionic villi were aspirated transcervically. A test series was made prior to elective abortion. Only half of the samples, however, were found to contain chorionic tissue. Complications as puncture of the amniotic sac and membranes aspirated instead of villi were encountered. Reports on larger series (Hahnemann, 1974; Kullander and Sandahl, 1973) had to contend with maternal bleeding and infections, absence of real time ultrasound and low success
rate of chorionic villus cell culture. Still, interest in first trimester diagnosis and improvement of ultrasound machines resulted in 1982 in the first report of placental biopsies using ultrasound guidance (Kazy et al., 1982), followed by a publication of Old et al. (1982) on genetic diagnosis of hemoglobinopathies. Simoni et al. (1983) developed a method for a rapid, direct preparation of chromosomes that took advantage of spontaneous mitoses in the rapidly dividing villi. This direct method yielded a cytogenetic diagnosis within a couple of hours after sampling. Moreover, maternal cell contamination, often encountered in chorionic tissue culture, was not a problem anymore.

Chorionic villi have a central mesenchymal core with foetal blood vessels. This core is covered by a trophoblastic epithelium which consists of two layers; an inner layer of single cells, which is called the cytotrophoblast and a "multinucleated" outer layer, which is called the syncytiotrophoblast. The trophoblast outer layer already exists four to six days after fertilisation as a layer that surrounds the blastocyst. After embedding of the blastocyst in the endometrium, trophoblast cells will fuse, forming the syncytiotrophoblast layer. Non-fusing trophoblast cells will grow into this syncytiotrophoblast layer. Stromal cells originating from the extraembryonal mesoderm will invade the cytotrophoblast after about 13 days. From this stage on, new placental tissue is formed by proliferation and branching (Figure 1).

After aspiration of the chorionic villi, the biopsy is checked under a microscope for quality and quantity of the cell material and carefully cleaned from maternal decidua. Chorionic villi can either be cultured or used for direct preparation.
Chapter 1

Figure 1: Early embryonic development, according to Crane and Cheung, 1988

Direct preparation of chorionic villi cells

For direct preparation, cells from the cytotrophoblast are used. The great advantage is that culturing of trophoblast cells is not necessary for getting metaphase cells. Thus, a cytogenetic result can be rapidly obtained. Colcemid is added to the villi to obtain adequate numbers of cells in metaphase. After approximately one hour, hypotonic treatment is performed and chorionic villi are fixed by methanol and glacial acetic acid. The cells are released from the villi by a brief exposure to 60% acetic acid. The suspension is spread over a slide using a bent tip of a Pasteur pipette (Simoni et al., 1983). Since no culturing is involved, an effect of maternal cell contamination is limited to a minimum. At least 10 mg chorionic villi are needed. However, the banding quality of the chromosomes is poor and sometimes not enough metaphases are available. Therefore, as an
alternative to this method, a semi-direct preparation or short-term culture of chorionic villi is used. The difference between direct and semi-direct methods is a 24h incubation of chorionic villi in fluorodeoxyuridine (Gibas et al., 1987). This causes a block in DNA synthesis, which is subsequently released by adding thymidine at high concentrations as well as colcemid, so that synchronisation is reached. Although theoretically this should result in a higher yield of metaphases and an improvement of the morphology of the chromosomes, the overall quality improvement, however, appears to be minimal.

Culturing of chorionic villi cells

For culturing of chorionic villi, the mesenchymal core is used. After the first attempts to culture trophoblast cells (Kullander et al., 1973), culture methods were improved considerably by the introduction of hormone-supplemented medium (Chang et al., 1982; Czepulkowski et al., 1986). To avoid maternal cell contamination, the outer layer of chorionic villi is stripped with trypsin. Mesenchymal cells are further desegregated by collagenase (Jackson, 1989) and the resulting suspension is cultured. A cytogenetic diagnosis can be made after 7-10 days. Very few mg of chorionic villi are sufficient for successful culturing. However, for quality reasons at least four cultures in two different incubators should be initiated. The percentage of maternal contamination turned out to be relatively low, between 0.1% to 2.16% (Roberts et al., 1988; Ledbetter et al., 1990 and 1992). By the application of a long-term culture technique problems involved in the direct method, namely poor banding quality and a low number of metaphases, can be overcome.

For harvesting chorionic villus cultures, either the in-situ or the suspension method can be used. The harvest procedure, as well as the procedures for slide making, staining and analysis, are in principle the same as described for AF cell cultures.
1.2 Quality control and assessment in prenatal cytogenetic diagnosis

Quality assessment in cytogenetics is still somewhat in its formative stage. The best developed quality control and assurance programmes can probably be found in the United Kingdom and the United States, where every cytogenetic laboratory must participate in external proficiency testing (Watson, 1997). This includes inspection of the facilities and making cytogenetic diagnoses of different test samples every other year. As a consequence, only qualified and certified personnel are permitted to sign chromosome analysis reports. Moreover, in the United Kingdom, the Association of Clinical Cytogeneticists (2001) has formulated guidelines for cytogenetic diagnosis. In the Netherlands a report “Advice concerning Genetic Counselling” published in 1977, described for the first time the indications for post- and prenatal cytogenetic diagnosis and the requirements for personnel and equipment. A report from the Working Party of the Dutch Association of Clinical Cytogeneticists (Mellink et al., 2000) was published in 1995 and revised in 2003, formulating minimal standards for cytogenetic diagnosis. A minimum number of metaphases for analysing and karyotyping, a minimum number of chromosome bands, a maximum turn around time for different indications and a minimum qualification of the personnel are prescribed. These standards are, however, insufficient to cover all quality aspects of the cytogenetic process. In particular, aspects of culturing and harvesting are missing in these guidelines. Factors involved in handling material for cytogenetic diagnosis have also not been spelled out, nor has the effect of such factors on cytogenetic quality been considered. Nevertheless, such factors are essential in determining the quality needed for adequate analysis. Therefore, the following gives an overview of which factors are involved and what is their effect on quality during the process of cell culturing, slide preparation and chromosomal analysis.
1.2.1 Quality control of culturing AF cells and chorionic villi

Culture quality can be measured as the culture time required for slide preparation, as the number of metaphases on a slide, as the number of culture failures and more specifically for AF fluid cells; as the number of clones. Culture quality is mainly dependent of the operator who performs AC or CVB, on the condition of the submitted material and on the culture conditions. Although these aspects count for AF cell cultures as well as for chorionic villus cultures, we will focus on the culture of AF cells, because this has been more extensively investigated.

With respect to the operator, different studies (Silver et al., 1998; Horger et al., 2001; Blackwell et al., 2002) have demonstrated that the caseload influences the sampling efficiency and that successful aspiration of clear AF increases with experience in amniocentesis. Therefore, only an experienced obstetrician should perform amniocentesis (Elias et al., 1998). Failure to obtain AF is then less than 1 percent (Tabor et al., 1986). In order to be allowed to perform amniocentesis an obstetrician in the Netherlands has to perform at least 30 amniocenteses per year (Dutch Society of Obstetrics and Gynaecology, 1997). However, neither the influence of the caseload of the operator on culture quality, nor the minimal amount of AF necessary for a reliable cytogenetic diagnosis have been investigated. Well-defined guidelines for the transport of AF (at which temperature and for how long) are also missing.

For the submitted material, the gestational age at which the sample is taken, the amount and appearance of AF as well as the reason for sampling might influence the quality of culture. Analysis of the effect of these variations might retrospectively explain culture failures or delay in culture time. Some publications have appeared in which these aspects were investigated in relation to culture quality. Although the total number of cells in AF increases with gestational age, the number of viable cells decreases (Elejalde et al., 1990). Moreover, only a fraction of these cells will form clones, on average no more than 10 cells/ ml AF (Hoehn et al., 1974). At 7 to 9 weeks of gestation 7.7 to 12.2 clones were counted per ml AF (Kennerknecht et al., 1992); at 14 to 16 weeks 3.1 +/- 1.2 clones and at 24 to 32 weeks less than 1.5 clones were counted (Rischkind and Risch, 1990).
A red or brown coloured appearance of AF due to the presence of haemoglobin negatively influences cell culture quality according to Golbus et al. (1979), who observed a higher percentage of foetal demise and culture failures among these samples. In a series of Hankins et al. (1984), however, no advanced risk on pregnancy nor foetal problems were detected. Chang and Jones (1991) as well as Seguin and Palmer (1983) reported inhibition of cell growth in red coloured AF. Persute and Lenke (1995) concluded that the risk of culture failure is higher in cases of foetal aneuploidy. However, Reid et al. (1996) substantiated further that it was not merely a higher percentage of foetal aneuploidies, but more in general the reason of referral “structural abnormalities at ultrasound”, resulting in a higher percentage of culture failures. However, for none of the above-mentioned variations of the submitted AF the effect of these variations on cell culture quality has been systematically investigated.

In most laboratories the in-situ method is used because of its reduced turn-around time compared to the flask method. Furthermore, after harvesting one is still dealing with clones, which is preferable for analysis. Various protocols describe how many cultures need to be initiated, which type of culture medium has to be used and what should be further environmental conditions during culturing (Priest and Rao, 1997; Keagle et al., 1999; Saunders and Czepulkowski, 2001). Cell culture quality has to be monitored at least by culture time and number of culture failures. On average, 0.5% culture failure is considered as acceptable (Lam et al., 1998).

Crucial for success and minimal duration of AF cell cultures is the (type of) culture medium and its supplements, in particular the foetal calf serum. Mostly used are commercial media, such as Chang medium, Amniochrome or Amniomax that include growth factors and foetal calf serum. The growth potential of different lots or types of medium can differ. To minimise culture failures, use of two different (lots or types) of medium per sample is recommended, as well as culturing in separate incubators. All the used reagents, particularly each lot of medium and serum, need to be tested on sterility and ability to support growth (Priest and Rao, 1997). However, an absolute and easy to use procedure for such testing is not available.
In order to control all aspects that influence culture quality, protocols for collecting AF and chorionic villi, guidelines for handling these and protocols for culturing are necessary. To define such guidelines, knowledge and registration of the potential influence of different factors on culture quality is essential. Some of these factors have been well investigated and some guidelines are already existing. This is e.g. the case for the influence of the operator on the material which is submitted and for some factors during culturing. A systematic investigation of the effect of quality variations in the submitted material on culture quality is missing, however, as are guidelines for transport of the material and for testing the growth potential of the culture medium. Therefore, there is a strong urge for investigating these factors in order to formulate guidelines.

1.2.2 Quality control during slide preparation
Slide preparation quality can be measured as the degree of spreading of the metaphase chromosomes and the presence or absence of cytoplasm over the metaphases. It will likely depend mainly on conditions during harvesting (hypotonic treatment and composition of fixative) and on climate conditions during the process of slide preparation. Slide preparation is a rather complex process. Individual laboratories have their own “tricks” to obtain appropriate metaphase quality. Several authors have investigated factors involved in this process. Claussen et al. (2002) found hypotonic treatment to be essential for obtaining well-spread metaphase cells. Without such a treatment, chromosomes become preferentially positioned close to each other in the cell centre. The slide preparation process starts with a water-induced swelling of mitotic cells during evaporation of the fixative from the slide (Claussen et al., 2002). Methanol from the fixative evaporates early during drying. This is followed by a water-induced swelling through the hygroscopic effect of the gradually evaporating acetic acid and leads to a stretching of the chromosomes via their flattening (Hliscs et al., 1997). For this process a relative humidity of at least 21% is needed, otherwise no swelling will occur. Thus, from these studies it may be concluded that hypotonic treatment,
evaporation of acetic acid and a certain percentage of relative humidity are essential for well-spread metaphases.

Spurbeck et al. (1996) determined the optimal climate conditions for slide preparation from cells from suspension and secondary AF cell cultures and found that the largest metaphase area was reached at 55% relative humidity and a temperature of 20°C. Deng et al. (2003) demonstrated a strong influence of relative humidity on spreading of cells from suspensions. However, in none of these studies the effects of the investigated factors have been determined quantitatively. Furthermore, in-situ cultures, for which the slide preparation process is much more complicated than for cells from suspension due to variable retention of nuclear matrix proteins over the metaphase plate, have not been investigated in these studies. For primary in-situ cultures effects of different variables during harvesting and slide making need, therefore, to be investigated in order to define optimal harvest conditions and to establish rational guidelines.

1.2.3 Quality control of chromosomal analysis
The quality of analysis can be measured as sensitivity and specificity of diagnosis. This in turn mainly depends on the banding resolution of the chromosomes and on the number of analysed metaphases. The chromosomal analysis will reveal or exclude structural and numerical abnormalities (in case of mosaicism with a given likelihood for exclusion).

Some minimal banding resolution is one quality criterion for an accurate analysis. The International System for Human Cytogenetic Nomenclature (ISCN) defined a total number of bands in human metaphases in 1995. In the Netherlands, the Working Party of the Dutch Association of Clinical Cytogeneticists formulated a minimal banding quality for cytogenetic diagnosis. For prenatal diagnosis, the minimum resolution has been determined at 400 bands (Mellink et al., 2003). This is in general the resolution of metaphases from chorionic villus cultures. Metaphases from direct preparations of chorionic villi usually have less than 400 bands. In metaphases from AF cell cultures up to 550 bands are visible.
A chromosomal abnormality which is detected in only a proportion of the investigated cells, is called chromosomal mosaicism. A mosaic pattern results from an abnormal cell division, which leads to the presence of two or more cells with an identical abnormality among the other (normal) cells. This can either reflect a true foetal mosaicism or be confined to the extra embryonic tissue or represent a culture artefact (pseudomosaicism). Pseudomosaicism is believed to usually originate in vitro, without clinical consequences. Incidence and interpretation of mosaicism is difficult and different for AF cells and chorionic villi.

Chromosomal analysis in AF cell cultures

Mosaicism can be excluded to a certain degree by analysing a given number of metaphases. Hook (1977) published the number of cells that should be analysed from peripheral blood cultures for exclusion of 10%, 20% and 30% mosaicism at 90%, 95% and 99% confidence levels, as derived from Newton's binomial formula. Based on this model also the number of metaphases to be analysed from in-situ cultures and flask cultures in order to exclude mosaicism in AF cell cultures were similarly determined (Claussen et al., 1984; Richkind and Risch, 1990; Featherstone et al., 1994). In current practice, for the flask method 20 cells from at least two flasks and for the in-situ method 10 –15 cells from different clones should be analysed. These guidelines allow exclusion of more than 20% mosaicism with a confidence level of 95% to 99% (Rischkind and Risch; 1990).

In terms of a laboratory result, mosaicism can be classified at three different levels (Hsu et al., 1992; Hsu and Benn, 1999): (1) a single cell is abnormal; (2) two or more cells in the flask method, a single clone or two or more clones within the same culture dish in in-situ cultures are abnormal; (3) two or more cells/ clones from independent cultures are abnormal. Only the last situation is considered to reflect true mosaicism. In AF cell cultures true mosaicism is rare with an occurrence in 0.1% to 0.3 % of samples (Bui et al., 1984; Hsu; 1984; Worton and Stern, 1984). The mosaic pattern is confirmed in the foetus in about 70% of the cases. Pseudomosaicism of level (1) occurs in 2.47% to 7.10% and of level (2)
in 0.64% to 1.10% of cases, including more structural than numerical abnormalities. In order to discriminate pseudomosaicism from a potentially true mosaicism one can increase the number of analysed metaphases by analysing metaphases in a culture dish different from the one in which the abnormal cells/clones were present. Hsu et al. (1992; 1999) developed guidelines how to deal with different chromosomal abnormalities. Depending on which abnormality was detected, no analytical work up, moderate (12 extra clones) or extensive work up (24 extra clones) should be done on metaphases in other dishes than the initial dish in which the abnormal cells/clones had been found (Table 1). The use of these guidelines results in a reliable diagnosis of pseudomosaicism or true mosaicism. In case of a work up sometimes, however, an insufficient number of metaphases is available on the regularly harvested in-situ dishes and a back-up culture needs to be trypsinised and analysed. In such a situation guidelines on how many metaphases should be analysed from trypsinised back-up cultures are missing.

Chromosomal analysis in chorionic villi

True mosaicism in chorionic villi can be confined to the placenta, to the foetus or be present in both foetus and placenta, i.e. be a generalised mosaicism. Kalousek and Dill (1983) demonstrated mosaicism confined to extra-embryonic tissue in two out of 46 spontaneous abortions. Although foetus and placenta originate from the same zygote, their chromosomal constitution can be different. Soon after the introduction of CVB in routine practice, it turned out that the cytogenetic diagnosis from direct preparations did not always reflect the chromosomal constitution of the foetus. Reports appeared on false positive results (Bartels et al., 1986; Breed et al., 1986; Leschot et al., 1987), followed by reports on false negative results (Eichenbaum et al., 1986; Linton et al., 1986; Martin et al., 1986). Collaborative studies (Ledbetter et al., 1992; ACC, 1994; Hahnemann and Vejerslev, 1997) showed mosaicism confined to the placenta in 1% to 2% of all cases.
Table 1: Guidelines for work-up to exclude or detect pseudomosaicism or mosaicism, from Hsu and Benn (1999)

<table>
<thead>
<tr>
<th>Flask method</th>
<th>In-situ method</th>
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<tbody>
<tr>
<td><strong>A. Indications for extensive work-up</strong></td>
<td><strong>A. Indications for extensive work-up</strong></td>
</tr>
<tr>
<td>1. Autosomal trisomy involving a chromosome 21, 18, 13 or 2; 5, 8, 9, 12, 14, 15 16, 20, 22 (SC, MC)</td>
<td>1. Autosomal trisomy involving a chromosome 21, 18, 13 or 2; 5, 8, 9, 12, 14, 15 16, 20, 22 (SCI, MCI)</td>
</tr>
<tr>
<td>2. Unbalanced structural rearrangements (MC)</td>
<td>2. Unbalanced structural rearrangements (MCI)</td>
</tr>
<tr>
<td>3. Marker chromosome (MC)</td>
<td>3. Marker chromosome (MCI)</td>
</tr>
<tr>
<td><strong>B. Indications for moderate work-up</strong></td>
<td></td>
</tr>
<tr>
<td>4. Extra sex chromosome (SC, MC)</td>
<td>4. Extra sex chromosome (SCI, MCI)</td>
</tr>
<tr>
<td>5. Autosomal trisomy involving a chromosome 1, 3, 4, 6, 7, 10, 11, 17, 19 (SC, MC)</td>
<td>5. Autosomal trisomy involving a chromosome 1, 3, 4, 6, 7, 10, 11, 17, 19 (SCI, MCI)</td>
</tr>
<tr>
<td>6. 45,X (MC)</td>
<td>6. 45,X (SCI, MCI)</td>
</tr>
<tr>
<td>7. Monosomy (other than 45,X) (MC)</td>
<td>7. Monosomy (other than 45,X) (SCI, MCI)</td>
</tr>
<tr>
<td>8. Marker chromosome (MC)</td>
<td>8. Marker chromosome (MCI)</td>
</tr>
<tr>
<td><strong>C. Standard, no additional work-up</strong></td>
<td></td>
</tr>
<tr>
<td>10. 45,X (SC)</td>
<td>10. Unbalanced structural rearrangements (SCI)</td>
</tr>
<tr>
<td>11. Unbalanced structural rearrangements (SC)</td>
<td>12. Break at centromere with loss of one arm (SC)</td>
</tr>
<tr>
<td>13. Break at centromere with loss of one arm (SC)</td>
<td>13. All single-cell abnormalities</td>
</tr>
</tbody>
</table>

MC: multiple cells (single flask), MCI: multiple clones (single dish), SC: single cell, SCI: single clone

A model of Crane and Cheung (1988) can explain the discrepancy between the chromosomal constitutions of foetus and placenta. According to their model (Figure 2), from the first five cell divisions following fertilisation only two of the 32 resulting cells will give rise to the formation of the embryo. All other cells are responsible for the formation of the trophoblast and the extraembryonic tissues.
A possible failure (nondisjunction) in an early cell division may lead to an abnormal cell in a specific cell line. A false positive result can be explained when in a normal foetus a localised nondisjunction in the chorionic villi occurs, which may cause an aberrant cell line in the direct preparation or culture, but not in the foetus. A false negative result can be explained by the occurrence of a meiotic nondisjunction, which may lead to a trisomic fertilised ovum, and a subsequent mitotic nondisjunction in the chorionic villi leading to a normal diploid cell line in the placenta, whereas the foetus remains fully trisomic.

Based on the position of the different cell lines and on the chromosomal constitution of the foetus six major categories of mosaics can be distinguished (Table 2).
Table 2: Types and frequencies of placental-fetal discordance at chorionic villus sampling, modified from Hahnemann and Verjerslev (1997)

<table>
<thead>
<tr>
<th>Type</th>
<th>Nature</th>
<th>Trophoblast</th>
<th>Villus core</th>
<th>Embryo/foetus</th>
<th>Relative frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CPM</td>
<td>Abnormal</td>
<td>Normal</td>
<td>Normal</td>
<td>48</td>
</tr>
<tr>
<td>II</td>
<td>CPM</td>
<td>Normal</td>
<td>Abnormal</td>
<td>Normal</td>
<td>25</td>
</tr>
<tr>
<td>III</td>
<td>CPM</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Normal</td>
<td>16</td>
</tr>
<tr>
<td>IV</td>
<td>TFM</td>
<td>Abnormal</td>
<td>Normal</td>
<td>Abnormal</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>TFM</td>
<td>Normal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>4</td>
</tr>
<tr>
<td>VI</td>
<td>TFM</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>7</td>
</tr>
</tbody>
</table>

Trophoblast, preparations from direct/short term culture; Villus core, preparations from long term culture; CPM, confined placental mosaicism; TFM, true foetal mosaicism (generalised foetal-placental mosaicism). An abnormal karyotype could be mosaic or non-mosaic. A normal embryonic/foetal karyotype could reflect normal biparental disomy or uniparental disomy.

This table also shows the relative frequencies of the different types of mosaicism (Hahnemann and Vejerslev, 1997). The results of direct preparation alone (type I) were less reliable than those of the method using culturing (type II). More cases of mosaicism confined to the placenta were detected in the direct preparation alone, which would in a number of cases lead to unnecessary amniocentesis as follow-up. False negative cases occur at a very low frequency (Saura et al., 1998), but (nearly) all of the cases were found in direct preparations (type IV versus type V). The model of Crane and Cheung (1988) can also explain these differences.

Trophoblast cells (used in direct preparation) are forming the first line of differentiation from totipotent cells of the morula and have therefore a greater chance of failures of cell division. Mesodermal core cells (used in culture) reflect the later segregating line of the extraembryonic mesoderm. These cells are closer related to the cells that form the embryo than trophoblast cells. Therefore, the use of a direct preparation alone should be avoided and reliability of the cytogenetic diagnosis improved by the combined use of a direct preparation and a long-term culture (Hahnemann and Vejerslev, 1997).
Once an abnormality is detected, the question arises of the probability, i.e. a calculated predictive value, of an abnormal foetal karyotype. In general, the predictive value is high in case of a non-mosaic abnormality found in culture (83%-100%), low in case of a mosaic abnormality in culture (24%) and zero in case of a(n) (mosaic) abnormality detected only in a direct preparation (Hahnemann and Vejerslev, 1997). Of special interest is the predictive value of a particular chromosome abnormality for adequate counselling of the parents about follow-up options, such as amniocentesis, ultrasound investigation or termination of pregnancy. However, because of the small number of aberrant cases for most chromosomes, predictive values are imprecise and additional cases are needed to improve that.
1.3 Scope of the thesis

The aim of prenatal cytogenetic diagnosis is to identify a possible chromosomal abnormality of the foetus. The most important quality criteria for diagnosis are sensitivity, i.e. the overall detection rate of abnormalities, and specificity, i.e. true occurrence of the detected abnormality in the foetus. For a reliable result, sensitivity and specificity need to be as close to 100%. Moreover, a result should be given within the shortest time possible on a minimum of material and at the lowest costs. To realise this, knowledge of all factors involved in the process of diagnosis is needed, as is a determination of their effects. Based on this knowledge, guidelines for handling prenatal material can be established. In particular aspects of culturing and harvesting are missing in the previously described cytogenetic procedures and factors involved in handling prenatal material have not been spelled out nor has the effect of these factors on cytogenetic quality been considered. This is particularly important in prenatal cytogenetic diagnosis, where because of the risk for both the pregnant woman and the foetus in obtaining the required material, the material is very precious. Moreover, in case of a detected chromosomal abnormality, a following decision to terminate or continue the pregnancy needs to be based on a reliable diagnosis.

The scope of the thesis is, therefore, to establish guidelines for quality controlled processing of amniotic fluid cells and chorionic villi for purposes of prenatal cytogenetic diagnosis. We will investigate which factors are of critical importance in handling these materials and will quantify their influence. Depending on their influence we will advice which factors need to be monitored carefully and we will develop some new guidelines in case the existing ones are insufficient. Our goal is to determine the limits between which reliable prenatal cytogenetic diagnosis is possible. For this purpose we divide the cytogenetic process in three distinct main parts, namely cell culture, slide preparation and chromosomal analysis.
Chapter 1

Culture quality can be measured as the culture time required for slide preparation for analysis and for AFcell culture more specifically as the number of clones and as the number of culture failures. The culture quality depends mainly on the condition of the submitted material and on the culture conditions. Therefore, we will first investigate the effect of variations in the submitted amniotic fluid (i.e. volume, appearance) and in culture medium on cell culture quality (Chapter 2.1). Second, we want to determine the minimal volume of AF required for a reliable cytogenetic diagnosis, avoiding repeated amniocentesis (Chapter 2.2). Finally, we want to develop an absolute procedure for testing the growth potential of reagents involved in culturing and of other factors influencing cell culturing. Using such a procedure, we want to test the influence of a decreased oxygen tension during culturing and to determine the effect on culture quality (Chapter 2.3).

The quality of slide preparation can be measured as the degree of spreading of the metaphase chromosomes and the presence or absence of cytoplasm over the metaphases. It mainly depends on conditions during harvesting (hypotonic treatment and composition of fixative) and the process of slide making (climate conditions). We will investigate the effect of these variables on primary in situ cultures (Chapter 3). Once we know the quantitative effects of ambient temperature, relative humidity, air flow and composition of fixative, we will be able to give some recommendations on how to achieve a consistent high quality of preparations.

The quality of analysis can be measured as sensitivity and specificity of diagnosis. It mainly depends on the banding resolution of the chromosomes and the number of metaphases analysed. For amniotic fluid culture, probability tables exist to determine the number of metaphases that should be analysed from either the in-situ or the flask method of culturing in order to exclude a certain degree of mosaicism. For trypsinised in-situ cultures, however, such a table is missing. We will, therefore, construct a probability table to determine the number of metaphases that need to be analysed in order to detect mosaicism when using trypsinised back-up cultures complementing in-situ AF cell culture analysis (Chapter 4.3).
For chorionic villi analysis, sensitivity and specificity need careful consideration, starting from the selection of the cell type which should be analysed from the villi; trophoblast cells in direct preparations, mesenchymal core cells in culturing or a combination of direct preparation and culturing. Since routinely we are using culturing only, we will investigate the results of that method in terms of success rate, maternal cell contamination, chromosomal aberrations observed and predictive value (false positive results). These results will be compared with those from the literature on direct preparations alone and on the combination of the direct preparation and the culturing method (Chapter 4.2).

Eventually, we will try to formulate guidelines for maintaining a good quality in prenatal cytogenetic analysis of amniotic fluid culture and chorionic villi and suggest possible further investigations (Chapter 5).
1.4 Literature


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