Quality assessment of prenatal cytogenetic diagnosis
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Chapter 5

Summary, future prospects, and suggested guidelines
Chapter 5

5.1 Summary

The scope of the thesis is to establish guidelines for quality controlled processing of amniotic fluid cells and chorionic villi obtained for purposes of prenatal cytogenetic diagnosis. We, therefore, have investigated which factors are of critical importance and we have quantified their influence. We have introduced controlled experimental variations in the workflow in order to find main and possibly interactive effects. The results will be discussed with respect to the three parts that can be distinguished in the cytogenetic process, namely cell culture, slide preparation and chromosomal analysis.

Factors influencing the quality of cell culture

Amniotic fluid as obtained by amniocentesis following the guidelines of the Dutch Society of Obstetrics and Gynaecology (1997) will be in general a good source for culturing amniocytes for cytogenetic diagnosis (Chapter 2.1). The volume of the admitted material, the reason for amniocentesis, gestational age at amniocentesis as well as the chromosomal diagnosis, appeared as no major critical factors where small variations would lead to unacceptable variation in outcome. We found that only the variation in appearance of the amniotic fluid has a relatively major impact. However, the effect of bloody or brown amniotic fluid was an extended culture time rather than culture failure (Chapter 2.1). The minimal amount of amniotic fluid required for culturing leading to a reliable cytogenetic result appeared to be 3.2 ml (Chapter 2.2). Commercial hormone-supplemented media are generally of sufficient quality for prenatal cytogenetic purposes. However, because of the inclusion of in-vivo produced serum, intrinsic variations in quality may occur that make acceptance testing necessary. To address this issue, we have developed an acceptance test based on the principle of stress testing (Chapter 2.3). We established the validity and sensitivity of this test by investigating the influence of decreased oxygen tension during culturing and by comparison with standard growth curve determination. A reduced oxygen tension during culturing caused a
2.5-fold increase in the number of AF cells within a clone. The number of clones, however, is only slightly increased (Chapter 2.3).

Factors involved in culturing chorionic villi have not yet been systematically investigated. To shorten the culture time and to increase the number of metaphases per preparation, a systematic investigation of factors involved in this process, similar to what we have done for amniocytes, is recommended.

Factors influencing the quality of slide preparation
In preparations from primary in-situ cultures of AF cells and chorionic villi we found that metaphase quality is only to a limited degree affected by the composition of fixative, the relative humidity, the ambient temperature and the air flow at harvesting (Chapter 3). Variations in the conditions commonly used in prenatal cytogenetic diagnosis have a limited influence and cannot be considered as critical for the quality of slide preparation. Thus, expensive measures for fine control of these conditions are not necessary at all. Within this limited influence it is the composition of fixative which mostly affects chromosome spreading. The presence or absence of cytoplasm over the metaphase is for amniotic fluid cells most affected by the relative humidity and for chorionic villi by the air flow (Chapter 3). We have not been able to discover factors that could lead to less variation in quality.

We recommend in-situ harvesting rather than suspension type of harvesting, since the turn-around time is much shorter and less chromosomal analyses have to be made for a reliable diagnosis of in-situ cultures.

Factors influencing the quality of chromosomal analysis
All previously mentioned factors lead to the final quality of the chromosome preparation; an appropriate number of metaphases on the slide, a sufficient length of the chromosomes and within a short turn-around time. The required conditions have already been well-defined in existing guidelines (Hsu et al., 1992; Hsu and
Benn, 1999; ACC Working Party, 2001; Mellink et al., 2003). Following these guidelines will result in an accurate and reliable detection or exclusion of structural and numerical chromosomal abnormalities. However, an additional guideline for the exclusion of chromosomal mosaicism in AF cell cultures would be desirable. We, therefore, developed a probability table which may help laboratories to standardise the confidence levels of prenatal diagnosis regarding mosaicism when, despite routinely using the in-situ method, trypsinised cultures need to be used to complete the analysis (Chapter 4.3). This probability table can of course also be applied when using solely the flask method.

For chorionic villi, chromosomal analysis from both a direct preparation and a culture is recommended. Provided that maternal contamination is limited, the culture method alone is sufficiently reliable for prenatal diagnosis to be used as the sole method (Chapter 4.2). The risk of a false negative result when using a direct preparation has been calculated as 1-3 percent per 1000 and is negligible when analysing preparations from cultures (Chapter 4.1). The risk of a false positive result (positive predictive value) is in general higher in analysis from direct preparations (Hahnemann and Vejerslev, 1997) and depends on the type of abnormality (Chapter 4.2).

5.2 Future prospects

Amniocentesis and chorion villus biopsy will in the foreseeable future remain the methods of choice in prenatal diagnosis. It is to be expected that the cytogenetic diagnosis of AF cells and chorionic villi will increasingly be supplemented by molecular techniques. For a more rapid test and a reduction of maternal anxiety, detection of the most common aneuploidies, such as trisomies 13; 18; 21 and sex chromosomal aneuploidies, interphase fluorescence in situ hybridisation (FISH) has been introduced (Klinger et al., 1992; Bryndorf et al., 1997; D’Alton et al., 1997; Pergament et al., 2000). In this technique, a set of chromosome-specific fluorescence-labelled probes is hybridised to interphase nuclei of uncultured prenatally obtained cells. The number of fluorescent signals in each nucleus
represents the copy number of the chromosome. A result is ultimately available after two days, however, due to the significantly higher costs and labour requirements this testing is offered only for a subset of prenatal referrals, mainly for the indication ‘foetal structural abnormalities found upon ultrasound examination’.

More recently, quantitative molecular tests, such as quantitative fluorescent (QF)-PCR or multiplex ligation-dependent probe amplification (MLPA), have become available for high-throughput, reliable, fast and robust aneuploidy detection (Mann et al., 2004; Slater et al., 2003). MLPA is a PCR-based method for relative quantification of up to 40 DNA sequences by means of a simple single-tube assay (Schouten et al., 2002). During the procedure, several sequence-specific probe sets are hybridised to genomic DNA, covalently ligated into single probes after successful hybridisation, and only than amplified using just one primer set. Afterwards, the resulting PCR-products are analysed semi-quantitatively using a capillary sequencer. In practice, a first study on MLPA for detection of aneuploidy of chromosomes 13; 18; 21; X or Y in almost 600 AF samples, leading to no false negative or false positive results, has been successfully conducted (Slater et al., 2003).

QF-PCR involves the relative quantification of microsatellite alleles to determine sequence copy number. Multiplex amplification using fluorescence labelled primers is followed by size separation and allele peak measurement on a semi-automated genetic analyser. In testing more than 7000 prenatal samples for trisomy 13; 18 and 21 no false positive nor false negative results were detected for non-mosaic trisomies (Mann et al., 2004).

DNA-microarray analysis is another molecular technique which allows a simultaneous quantitative analysis of genomic copy numbers, using many probes consisting of mapped DNA fragments that are spotted on a glass slide (Pinkel et al., 1998; Snijder et al., 2001). Microarray technology was initially conceived to detect the expression of thousands of genes simultaneously, but quantification of genomic copy numbers is also possible. The resolution of an array depends solely on its contents. Presently, subtelomere-specific arrays (Veltman et al., 2002),
Chapter 5

chromosome-specific arrays (Veltman et al., 2004) and whole genome arrays, with resolutions varying from 2Mb to tiling path density, are being used in diagnostic practice. The comparative nature of the technique implies that only net gains and losses can be found. Balanced rearrangements will remain undetected. In prenatal diagnosis, routine microarray analysis of AF cells or chorionic villi is too labour-intensive and too expensive to date. However, this technique can be used for further investigation of small chromosomal unbalances at a higher resolution when an apparently balanced karyotype is found in the group of referrals with ‘abnormalities at ultrasound’, or it can be used to determine whether a de-novo structural abnormality is balanced or not.

Cytogenetic analysis of AF cells and chorionic villi is associated with invasive techniques to obtain the material. These techniques carry a risk for the mother and the foetus. Therefore, they need to be restricted to women whose increased risk of having a foetus with an abnormality outweighs the risk of the procedure. Introduction of a better risk estimator than maternal age will reduce the number of invasive tests, especially in the group of women with advanced maternal age (Beekhuis et al., 1994; Kornman et al., 1995). At the same time the detection rate for Down syndrome for each procedure as well as the absolute number has increased (Cornel et al., 1993). One approach is biochemical screening of maternal serum possibly combined with a measurement of thickening of the nuchal fold at ultrasound (nuchal translucency). This procedure for prenatal detection of Down syndrome is estimated to result in a detection rate of 93% with 5.9% false positive rate (Steenhouse et al., 2004). Only if this screening indicates an increased risk for Down syndrome, subsequent cytogenetic diagnosis by use of AF cells or chorionic villi should be performed. Recently, a model for contingent screening already in the first trimester was recommended which avoids further testing in up to 75% of unaffected pregnancies (Wright et al., 2004). In most European countries screening for Down syndrome is offered to all pregnant women. In the Netherlands the advice of the Health Council (2004) was in this line. However, the last population screening act (Ministry of Health, 2004), passed after this advice, allowed health care providers only to offer screening for Down syndrome to
pregnant woman older than 36 years of age. Pregnant women younger than 36 years of age have to be informed about screening, but the use is at their own expense. Since most Down syndrome children are born to younger mothers, this policy means a highly ineffective way of screening for Down syndrome.

Alternative non-invasive techniques for prenatal diagnosis that can be offered to all pregnant women are actively explored. Isolation of intact foetal cells from the maternal circulation has been extensively studied (Adinolfi, 1995; Bianchi et al., 1999; Simpson and Elias, 1993). The major limitation in applying this technique clinically, however, is the in general low number of foetal cells found in the maternal circulation; approximately one foetal cell per ml of maternal blood (Bianchi et al.; 1997, Hamada et al.; 1993). Furthermore, foetal cells can persist in the circulation, sometimes many years after delivery (Bianchi et al.; 1996). As an alternative, Lo et al. (1997) was the first to describe the presence of cell-free foetal DNA in maternal plasma and serum. Cell-free foetal DNA is cleared from the circulation in less than an hour (Lo et al., 1999). Several clinical applications for cell-free foetal DNA have been described, such as foetal sex determination (Sekizawa et al., 2001) and foetal Rhesus- D genotyping (Faas et al., 1998). Maternal plasma/ serum analysis has the advantage of being rapid, reliable, reproducible, and easily carried out for a large number of samples. The main limitation at present appears to be the availability of uniquely foetal gene sequences that identify and/or measure the presence of foetal DNA in both male and female foetuses (Bianchi, 2004; Wataganara et al., 2004).

For the further future combining the isolation of foetal DNA from maternal serum/plasma with molecular techniques such as MPLA or whole genome microarrays, would certainly be a desirable way to go, in order to perform prenatal diagnosis without any risk to mother and foetus.

As an alternative to prenatal diagnosis, preimplantation genetic diagnosis (PGD) was introduced at the beginning of the 1990s to prevent termination of pregnancy in couples with a high risk for offspring affected by a sex-linked genetic
disease (Sermon et al., 2004). It is a kind of early prenatal diagnosis, in which at least one cell of an 8-cells in-vitro embryo is analysed for well defined genetic defects. Only embryos free of this defect are replaced in utero. Recently, PGD for aneuploidy screening has been applied to improve a low success rate of in-vitro fertilisation (IVF) attributed to chromosomal aneuploidies in the embryos, as is sometimes the case in woman older than age 36 –40 years (Wilton et al., 2002). In the Netherlands, a blind randomised study has been started to answer this question (Mastenbroek et al., 2004). However, PGD will be restricted to women undergoing IVF and to a few couples with a high risk of an affected foetus for well-defined diseases.

Reducing the costs of health care is an important issue since the budget is not increasing at the same rate as the demands. From a medical and financial point of view, a screening program is the most cost-effective policy (Wortelboer et al., 2000). It leads to a better use of cytogenetic diagnosis, with more benefit and less harm to the pregnant women and their babies. Furthermore the question rises whether a complete cytogenetic diagnosis is essential for the indication ‘advanced risk at screening’ (Hulten et al., 2003). A diagnosis for just the common aneuploidies, such as 13; 18; 21; X and Y by a molecular technique, e.g. MLPA, might be sufficient. In a retrospective study among 65,189 cases in the advanced maternal age group 0.2% false negative or ‘clinically relevant’ abnormalities would have been missed if women would have had the MLPA test for chromosomes 13; 18; 21, X and Y instead of karyotyping (Niewint et al., 2004). The false negative rate could be further reduced by the use of an MLPA test with probes for the subtelomeric regions of all chromosomes (Suijkerbuijk et al., 2004).

Although (prenatal) cytogenetics is moving into the direction of molecular approaches, these developments by no means imply that ‘conventional’ cytogenetic diagnosis will disappear from the laboratories in the near future. Only by regular karyotyping, an overview of the complete human genome is obtained and balanced aberrations, such as translocations, inversions or insertions can be visualised, whereas DNA techniques can only detect unbalanced aberrations.

128
In summary, possibilities for prenatal diagnosis will further increase in the future. The choice of the method will remain difficult. On the one hand there is the wish of pregnant women to get maximal certainty of a healthy baby, on the other hand there is a limited health care budget. Moreover, with every introduction of a new technique the question should be discussed whether the test should be available to all pregnant women. Professionals in the field of prenatal diagnosis should inform and advice the government, health care providers and pregnant women on the implications of the different tests. Eventually, it is up to the society to determine whether all questions in prenatal diagnosis should be answered, which tests should be offered to whom and at which costs. The answer to these questions might be different for different countries.

5.3. **Suggested guidelines for handling amniotic fluid and chorionic villi**

In general, our prenatal cytogenetic practice has proven to be robust and reliable. Yet, based on our findings a few additional guidelines are formulated.

Guidelines for culturing AF cells:

It can be concluded that AF can be stored, if necessary, for at least two days either at room temperature or in the refrigerator without any decrease of growth potential (Chapter 2.3). The desired amount of AF is 6 ml. According to our standard procedure for culturing, four in-situ cultures will be initiated with 1.5 ml AF per dish (Chapter 2.2). However, 3.2 ml AF, 0.8 ml AF per in-situ dish, are sufficient for a reliable diagnosis, while culture time is extended with two days. We suggest to register a bloody or brown appearance of AF and AF sample volumes less than 3.2 ml for explaining a possible delay in culture time or culture failure (Chapter 2.1). A new lot or type of culture medium needs to be subjected to stress testing before introducing it in the routine practice (Chapter 2.3). The growth potential of AF cells, as assessed by an increase of the number of cells within a clone, can be enhanced 2.5-fold under a decreased oxygen tension (Chapter 2.3).
Guidelines for harvesting and slide preparation of in-situ cultures:

As demonstrated in Chapter 3, metaphase quality is only to a limited degree affected by the composition of fixative, the relative humidity, the ambient temperature and the air flow at harvesting. Within this limited influence, the following recommendations can be given. (1) The higher the concentration of acetic acid before slide preparation the better the spreading of chromosomes. (2) The presence of cytoplasm in in-situ preparations is slightly influenced by relative humidity and is minimal between 30% and 40%. (3) Extra air flow of 0.6 m/s further decreases the presence of cytoplasm for chorionic villi preparations. Expensive instruments in climate control during harvesting and slide preparation are not necessary.

Guidelines for chromosomal analysis:

Guidelines for analysing preparations from AF cells and chorionic villi have already been well defined. Therefore, only two additional suggestions are given. (1) The table for exclusion of mosaicism, especially developed for preparations from trypsinised in-situ AF cell cultures, should be used to complete analysis from in-situ culture dishes in case an insufficient number of clones is obtained in the regularly harvested dishes. (2) Metaphase preparations from cultured mesenchymal cells from chorionic villi alone can be analysed for cytogenetic diagnosis provided that maternal cell contamination is limited.
5.4 Literature


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Chapter 5


