Chromosomal mosaicism in the placenta. Presence and consequences
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Chapter 1

General introduction
1.1 Conventional cytogenetics

The term "cytogenetics", a combination of cytology and genetics, was first introduced by Sutton in 1903. Only few studies on human cytogenetics were published until the fifties, and accurate studies on human chromosomes became only possible after several technical developments. Taking advantage of improvements in cell culturing and chromosome spreading, Tjio and Levan (1956) were able to demonstrate that the human diploid chromosome number is 46. Purely on morphological criteria the 23 chromosome pairs could be classified in seven groups (A-G), as originally suggested by Patau (1960). A system for nomenclature, using this classification, was proposed at what became known as the "Denver Conference of 1960" and was officially sanctioned by the "London Conference" in 1963 (ISCN, 1995). In 1959 the first aberration was reported: Lejeune et al. (1959) discovered an extra chromosome (belonging to the G-group) to be present in patients with Down syndrome. This was soon followed by the description of a number of syndromes involving the sex chromosomes. In 1960 two more autosomal trisomies were reported. Patau et al. (1960) described a trisomy with an extra D-group chromosome, while Edwards et al. (1960) described a patient with a trisomy for an E-group chromosome.

A major advance was the observation of Caspersson et al. (1968) of a kind of banding pattern, when staining plant chromosomes with fluorescent quinacrine compounds. This resulted in two articles by Caspersson et al. (1970a, 1971) in which it was shown that such a staining produced consistent fluorescent banding patterns (Q-bands) along each human chromosome. This unique banding pattern made possible the identification of all human chromosomes. Other banding techniques soon followed, utilizing either fluorescent dyes or the Giemsa stain resulting in G-bands (Drets and Shaw, 1971). A basic system for designating not only individual chromosomes, but also chromosome regions and bands was proposed in a report of the "Paris Conference" of 1971. With revisions during the years this system has become a widely accepted nomenclature for human chromosomes (ISCN, 1995). By using this banding phenomenon, Patau syndrome proved to be a trisomy 13 and Edwards syndrome was seen to be a trisomy 18. With this technique of differential staining one could distinguish chromosome 21 from the other G-group chromosome (chromosome 22) and recognise different segments on chromosome 21. Caspersson et al. (1970b) formulated the following: "this implies that the genetic material, which in triplicate gives Down's syndrome, is on the distal part on the long arm of chromosome 21". In this way, many more chromosomal abnormalities and syndromes could be identified.

1.2 Fluorescence in situ hybridization

In situ hybridization (ISH) was a true impulse for the further development of cytogenetics. In this technique cytogenetics and molecular biology merged. In conventional cytogenetics one is looking at bands, which may contain numerous genes. Nowadays, it is assumed that the human genome consists of approximately 30,000 genes (International Human Genome Sequencing Consortium, 2001). When one can distinguish a thousand bands in a karyogram, it means that
on the average each band consists of some 30 genes. Routinely 300-550 bands can be visualized, so, actually karyotyping is a very crude method to establish possible genetic anomalies. In molecular cytogenetics using in situ hybridization we can detect single genes. The main principle of the in situ hybridization technique, in short, is denaturation to make target-DNA as well as (labelled chromosome specific) probe-DNA single-stranded, followed by hybridization of probe-DNA to target-DNA. The target-DNA can consist of DNA fragments, nuclei or metaphases.

In situ hybridization was introduced in 1969 by Gall and Pardue (1969), at that time with radioactive detection (radiolabelled in situ hybridization, RISH). Application of in situ hybridization in clinical genetics, however, became simpler and more practical with the introduction of fluorescent dyes, called fluorescence in situ hybridization or FISH (Cremer et al., 1986; Hopman et al., 1986). Compared to RISH methods fluorescent detection of hybridization sites has advantages in resolution, contrast, speed and safety.

In short the FISH technique implies the following:

1. Material (tissues, cells, and chromosomes) is fixed.
2. Treatment (heat / formamide) to denature the DNA in order to make it single-stranded (target DNA)
3. Hybridization with probe DNA (single-stranded) tagged with a hapten (eg. biotin or digoxigenin) or directly labelled with a fluorochrome.
4. In case of a hapten this is bound to an antibody tagged with a fluorescent dye.
5. Visualization with fluorescence microscopy.

Besides DNA as a target, also RNA or gene expression may be depicted. FISH thus soon became a valuable additional tool in cytogenetic laboratories with a number of applications:

In metaphases:
FISH can be used to clarify translocations (fine-detection of breakpoints), to detect deletions and to solve the origin of marker chromosomes. Sometimes even duplications can be visualized.

In interphases:
FISH can be used for detection of numerical aberrations. The possibility of studying interphases is a major advantage in cases where no metaphases are available or when analysis of large numbers of cells is required for a reliable result. The reporting time may be shortened for there is no need to culture.

Three categories of probes can be distinguished (Blancato, 1999):

1. Satellite sequence probes. Satellite sequences are polymorphic repetitive DNA sequences that are present in the genome, but do not code for gene products. Alpha-satellite DNA is found in centromeric or peri-centromeric regions of human chromosomes. Due to variations in these regions most chromosomes can be distinguished individually, which is useful to determine the ploidy of chromosomes in metaphases as well as in interphases. These probes require a short hybridization and usually generate strong signals. Certain chromosomes show homology in their centromeric region and cannot be individually
discriminated (chromosomes 13 and 21, and chromosomes 14 and 22). At the end of both arms of all chromosomes telomeric repeats can be found. Repeat sequence probes for those regions can also be used for ploidy determination.

2. Whole chromosome painting probes. Each painting probe is composed of a mixture of many probes (unique and repetitive sequences) along an entire chromosome or a chromosome arm. These probes are mainly suitable for metaphases and can be well used for the detection of translocations and insertions, and for some deletions and duplications. They can well be used to determine the chromosome composition of a marker chromosome and to analyse complex rearrangements.

3. Unique sequence probes. These probes can identify specific targets, unique sequences that are not repeated in the genome and may code for genes. They are especially suitable to be applied on metaphases for analysis of deletions and breakpoints in translocations. Most currently used probes are aimed at identification of microdeletions. The signal may be too small for detection in interphase nuclei. Also the so called “subtelomeric probes” belong to this group. They are unique sequence probes, hybridizing on the gene rich subtelomeric region. These probes are suitable for establishing deletions in these areas or for detecting cryptic translocations.

1.3 Prenatal diagnosis

1.3.1 Amniocentesis

In the late sixties Steele and Breg (1966) demonstrated that amniocytes could be cultured successfully and if sufficient mitoses were available, these could be used to determine the karyotype of the fetus. So, one would be able to detect chromosomal disorders prenatally (numerical as well as structural), which would make it possible for women/couples to decide on termination of an affected pregnancy. Second trimester amniocentesis was the first prenatal diagnostic test to be introduced in order to establish the fetal karyotype.

Prenatal diagnosis in the Netherlands is routinely offered to women on strict indications only. The largest indication group is the group of women opting for a prenatal test because of advanced maternal age (over 35 years). Around 85% of the total number of samples examined at our laboratory belong to this category. The reason for offering amniocentesis to these women, is mainly because the risk of bearing a child with a numerical chromosomal disorder increases with elevated maternal age. Maternal meiotic errors predominate in this indication group (Nicolaides et al., 1998; Robinson et al., 1999). With elevated maternal age the number of failures during cell divisions, prior to the formation of gametes, rises. The orderly segregation of daughter chromosomes during meiosis I or of sister chromatids during meiosis II may fail. Such errors in meiosis (non-disjunction) result in gametes that contain abnormal numbers of chromosomes and, following fertilization, produce aneuploid conceptuses. Gain of a chromosome leads to a trisomy, whereas loss of a chromosome leads to a monosomy. Although advanced maternal age is a well documented risk factor for maternal-meiotic non-disjunction, there still is a surprising lack of understanding of the mechanisms behind the
maternal age effect (Nicolaides et al., 1998). So far, it has been demonstrated that aneuploidy in embryos generated in vitro, increases notably with maternal age (Munné et al., 1995; Dailey et al., 1996).

The best-known and most frequent chromosomal disorder is Down syndrome (trisomy 21), which occurs in about 1.2 per 1000 live births (Goujard, 1988; Hook, 1992). Recent figures for the Netherlands show an expected prevalence at birth for children with Down syndrome of 1:518, based on the age distribution of the population in 1999 (Health Council of the Netherlands, 2001). Other numerical aberrations, such as trisomy 13 (Patau syndrome) and trisomy 18 (Edwards syndrome) are observed less frequently. These syndromes are less compatible with life. Many of these trisomies end in a spontaneous abortion or late fetal death, and of the liveborns many die within their first hours or days of life. Patau syndrome is calculated to occur in 1:12,000/12,500 (Hook, 1980; 1992) to 1:29,000 (Goldstein and Nielsen, 1988) live births, respectively. Incidental cases of longer survival, up to 19 years, have been reported (Singh, 1990; Zoll et al., 1993). Edwards syndrome is the second most common autosomal trisomy seen in 1:7,000 newborns (Nielsen and Silleisen, 1975; Goldstein and Nielsen 1988; Hook, 1992). Here, too, a few patients with somewhat longer survival have been reported (Carter et al., 1985; Mehta et al., 1986). In trisomy 13 or 18 patients with prolonged survival, especially when some of the typical symptoms of these syndromes are missing, one might suspect mosaicism. Chromosomal mosaicism is characterized by the presence of two or more karyotypically different cell lines within one individual. Mosaicism for trisomy 18 was illustrated by a study of Bass et al. (1982): in a 19-year old male, non-mosaic trisomy 18 was seen in blood and skin fibroblast at different ages; skin fibroblasts from a new biopsy at the age of 18, only revealed normal 46,XY cells. Numerical aberrations of the sex chromosomes are also frequently seen (1:500; Hook, 1992), but usually no mental retardation is observed in these patients. Advanced maternal age is not associated with an increased incidence of Turner syndrome (45,X). In Klinefelter syndrome (47, XXY) approximately 50% of the additional X chromosomes come from the mother and advanced maternal age is associated with 47,XXY, but to a lesser degree than in autosomal aneuploidy. In case of 47,XXY the extra Y chromosome is paternally derived in all cases. In case of triple X (47,XXX), the extra X chromosome is maternally derived in over 90% of the cases and advanced maternal age is a factor in meiosis I, but not in meiosis II (Robinson et al., 1998).

Amniocentesis and the analysis of cultured amniocytes, proved to be a relatively safe, reliable and accurate tool for prenatal diagnosis, for numerical as well as structural aberrations. Cultured amniocytes can also be used for biochemical and molecular tests. Large studies report a 0.5% risk of abortion by this procedure (NICHD study group, 1976; Simpson et al., 1976; Working Party on amniocentesis, 1978; Leschot et al., 1985; Elias et al., 1986). The accuracy of fetal karyotyping is high, possible discrepancies are mainly found among mosaic cases, where two or more different cell lines can be discriminated within one individual. The overall incidence of mosaicism reported among approximately 60,000 cases was 0.25 per cent (Hsu and Perlis, 1984). Single cell abnormalities are rarely an indication of true fetal mosaicism. Mosaicism involving multiple cells in more than one culture should be considered as a strong indication of true fetal mosaicism (Worton et al., 1984).
Amniocentesis nowadays is performed under ultrasound guidance, usually at 15-16 weeks of gestation, and about 15-20 ml of amniotic fluid is aspirated. This is approximately 12.5% of the total volume of amniotic fluid at the time (Finegan, 1984). Cells are cultured and different colonies from different cultures can be analysed (in situ or colony method) so also mosaicism can be detected. Also when the flask method is used and colonies can not be analysed individually, discriminating between true mosaicism and false-positives is possible, if adequate numbers of cells are analysed (Worton et al., 1984).

A disadvantage of amniocentesis is the fact that it is not performed before approximately 14 weeks of pregnancy. The relatively long waiting time before the outcome of the test is known, is another disadvantage. This is mainly due to the fact that cells have to be cultured, which usually takes 7-10 days; so the final result is known 2-3 weeks after the sample has been taken. When a couple is confronted with an adverse outcome, they may decide on termination of pregnancy (TOP). At midtrimester, TOP will be performed by means of induced abortion.

1.3.2 Chorionic villus sampling

An additional prenatal test became available in the seventies. A method described by Mohr (1968) to use chorionic villi for chromosomal analysis of the fetus was refined and improved by other groups. Results from a Chinese group (Tietung Hospital, 1975) showed that chorionic villi could be aspirated in the first trimester of pregnancy and that this tissue could be used for fetal sex prediction. The sample was taken transcervically. A metal canula was used, with an inner fine suction tube connected to a syringe. A major breakthrough was made by Simon et al. (1983) who introduced the so-called "direct method" for cytogenetic analysis, in combination with transcervical chorionic villus sampling. Cytotrophoblast cells yielded relatively high proportions of mitoses, which could be used directly for chromosome analysis. The method implies a one-hour incubation in colcemid, followed by a short hypotonic treatment after which the cells are fixed in methanol/acetic acid (3:1). Trophoblast cells are released from the villus core by using 60% acetic acid. The released cells (in interphase or metaphase stage) can be spread on slides. The advantages of chorionic villus sampling (CVS) are obvious: an early (first trimester) prenatal test, of which the results can be known within a few days time. In the Netherlands the center for prenatal diagnosis in Rotterdam was the first to offer CVS in 1984 (Galjaard, 1985; Sachs et al., 1985), two other centers soon followed and CVS became a frequently used procedure. A chorionic villus sample is taken transcervically or transabdominally under ultrasound guidance at 11-13 weeks of gestation. The amount of villi taken at first trimester sampling is about 10 mg-50 mg out of a total amount of 20 grams of villi at this stage of pregnancy.

Short-term culture

The direct method for the chromosomal analysis of chorionic villi was modified by Gibas et al. (1987), and this improved method became known as the semi-direct method or short-term culture (STC). The main modifications were a 24h incubation in fluorodeoxyuridine, the block in DNA synthesis was subsequently released using thymidine, and the mitotic cells were
arrested in metaphases using a high concentration of colcemid. Synchronization is thus reached, resulting in a higher yield of metaphases and an improvement of the morphology of the chromosome preparations. Combined with a different G-banding method this also led to a better quality of banding.

Patients undergoing CVS are informed about the test results within 7-10 days after sampling, which will usually be around the 12-13th weeks of gestation. In case of an abnormal laboratory result they may choose for termination of pregnancy (TOP). In this stage of pregnancy this can usually be done safely by dilatation and vacuum curettage.

The main concern at first, with respect to prenatal diagnosis of chorionic villi, was the safety of the sampling procedure. All initial reports about CVS studied the procedure-related risk of an abortion (Rhoads et al., 1989; Crane et al., 1988; MRC Working Party on CVS, 1991). Different sampling procedures (transcervical versus transabdominal) were compared with relation to the risk of an abortion (Brambati et al., 1991). Both techniques proved to be equally safe and effective.

**Long-term culture**

Kullander and Sandahl (1973) were the first to culture mesenchymal cells of villi. They came across various problems in these so-called long-term cultures (LTC): growth failure, a low mitotic index and contamination of the cultures by cells of maternal origin (which is extremely rare in short-term cultures). In a study by Williams et al. (1987) maternal cell contamination (MCC) was seen in 13.1% of the long-term cultures in a group of 45 patients. MCC was especially observed after prolonged cultivation time (24.2 +/- 6.8 days) compared with non-contaminated cultures (14.1 +/- 4.4 days) and proved to be a major problem in chorionic villus cultures. It may lead to incorrect sex determination (Hogge et al., 1985; Canadian collaborative CVS-Amniocentesis Clinical Trial Group, 1989) and potentially to false-negative diagnoses.

**1.4 Development of the placenta during early pregnancy**

**1.4.1 Normal development**

In the various prenatal tests used for chromosomal analysis of the fetus (amniocentesis, CVS-STC and CVS-LTC), different cells are analyzed. Knowledge of the origin of these cells and the way they relate to true fetal cells is important for interpretation of the prenatal findings, especially in case of abnormal findings. Description of normal early human development can be found in many textbooks (Kaufmann, 1981; Moore, 1988; Drews, 1995), but nowadays numerous websites with pictures, drawings, histological slides and video's also elucidate this process (e.g. http://anatomy.med.unsw.edu/cbl/embryo; University of New South Wales, Sydney, Australia).

In Figure 1 a schematic model is shown of the different cell lineages in early human development: different cell lineages, which are analyzed with the various prenatal tests, are indicated. With reference to these books and website, early human development, with
emphasis on the development of the placenta, can be described in the following steps (also depicted in Fig. 1):

1. Three days after fertilization the morula (16 cells) has developed. Additional cell divisions produce the blastocyst (four to six days). By the time implantation starts, the blastocyst consists of 50-100 cells. It consists of an Inner Cell Mass (ICM), and a trophoblast outer layer, which surrounds the blastocyst cavity.

2. After seven to eight days of development the embryonic disc is apparent, which is composed of two layers: the epiblast and the hypoblast. The hypoblast will give rise to the formation of the yolk sac, whereas cells from the epiblast will develop into the embryo and into amniotic ectoderm.

3. Between 7-12 days after ovulation the blastocyst will become completely embedded in the endometrium. Trophoblast cells will fuse and form the syncytiotrophoblast or syncytiu. Non-fusing trophoblast cells will grow into this syncytiotrophoblast layer. Labyrinthine cavities (lacunae) appear in the syncytiu. These first three steps are also shown in Fig. 2.

4. About 13 days after fertilization the cytotrophoblast is invaded by stromal cells originating from the extraembryonal mesoderm. From this stage on, new placental tissue is formed by proliferation and branching of the villous tree.

5. At first, chorionic villi surround the gestational sac completely. From the sixth gestational week onwards (i.e. four weeks after fertilization) the evenly distribution of villi disappears and villi opposite the site of implantation start to degenerate. The villi at the implantation site rapidly proliferate invading the maternal stroma.

6. In the 9th week of gestation the villi form a layer of 1-1.5cm in thickness, the so-called chorionic frondosum. From this layer chorionic villus samples are taken of pregnancies of over 10 weeks of gestation. At 12 weeks villi are solely found at the implantation site.

7. Between the sixth and 16th week of gestation the trophoblastic layer consists of a monolayer of syncytiotrophoblast and a monolayer of cytotrophoblast. A basement membrane separates the stromal tissue from the cytotrophoblast (Gerbie et al., 1968).

8. After the 16th week the cytotrophoblast layer largely disappears, syncytiotrophoblast flattens and villous vessels line the basement membrane, to facilitate transport between the intervillous maternal component and the intravillous fetal component.

9. At three months septa are formed to divide the placenta in units. Around the 18th week of pregnancy the definite architecture of the placenta has been attained.

10. After approximately 40 weeks of pregnancy, the child and the placenta are born. After a short inspection (merely for completeness) the placenta usually gets thrown away.

Normal placental development is crucial for continued fetal growth and well being leading to the birth of a healthy infant at term. Failure of placentation or disordered placentation may cause (serious) complications, ranging from miscarriage to pre-eclampsia and intrauterine growth retardation (Kingdom, 1998).
Figure 1. Model of cell lineages in the human embryo, based on Crane and Cheung (1988); and Bianchi et al. (1993). Cell lineages used in various prenatal tests are indicated.

Figure 2. Schematic representation of early human development, based on Moore (1988).
1.4.2 Abnormal morphology of the placenta in relation to an abnormal karyotype

Carr (1967) was the first to describe a relationship between abnormal chromosomal constitution and abnormal development of the fetoplacental unit. Infants born with autosomal trisomies, generally, have lower birth weights than those with a normal chromosome number (Linn and Evans, 1984). Various abnormal morphological features have been described for placentas with an abnormal karyotype. This may range from changes in chorionic villus size to decrease in number of bloodvessels. Also alterations in the outline of villi and many other histological variations have been reported (Honoré et al., 1976; Röckelein et al., 1989; Göcke et al., 1985; Minguillon et al., 1989; Rochelson et al., 1990; Salafia et al., 1993). Many of these features have been claimed to be associated with abnormal karyotype, but many placent al and decidual histological findings can be seen in both chromosomally normal and abnormal pregnancy losses. The predictive value of chorionic villus histology seems to be inadequate (Minguillon et al., 1989, van Lijnschoten et al., 1993). A number of these features only turned out to be predictive of a triploid karyotype. Placental abnormalities might conceivably contribute to poor growth in trisomic fetuses, but there has been little experimental study regarding this question, especially in humans.

It has been shown in recent studies of mouse mutants, that embryonic lethality associated with many targeted mutations, involved placental defects (Rossant and Cross, 2001). Mouse mutants have been described for which the mutated genes affected trophoblast and placental development. At present we have candidate regulatory genes for many of the main phases of placental development. Since the placenta is the first organ to form during mammalian embryogenesis, problems in formation and function may well cause early pregnancy loss and later complications in human pregnancy. Data that are provided by studies of mouse trophoblast development may be helpful in the understanding of human placental function.

1.5 Chorionic villus sampling and placental mosaicism

1.5.1 Confined placental mosaicism

As shown in section 1.4 above, cytotrophoblast cells and mesenchymal cells have different embryogenic progenitor cells (Crane and Cheung, 1988, Bianchi et al., 1993). The fact that different cell lineages are investigated using short-term culture and long-term culture has been affirmed by expression studies. In a report by Haigh et al. (1999) cytoskeletal markers were used for the identification of cell strains in first trimester placenta. Cultured mesenchymal cells expressed vimentin and no cytokeratin 7, whereas in trophoblast cells the reverse situation was found.

Already in 1983 Kalousek and Dill (1983) made an important observation. They demonstrated that an aberrant cell line could be confined to extra-embryonic tissues (confined placental mosaicism, CPM). They investigated term placentas of children with unexplained intrauterine growth retardation (IUGR) and found two cases of trisomy (trisomy 22 and trisomy X).
normal diploid karyotype was seen in cultured tissue of amnion and cord blood in both cases and in the trisomy 22 case, cultures of skin tissue also showed a normal karyotype.

Crane and Cheung (1988a) showed a schematic representation of the first five cell divisions after fertilization leading to a total of 32 cells, of which only two to three cells will give rise to the formation of the embryo (Figure 3). All other cells are responsible for the formation of the trophoblast and extraembryonic tissues. This clearly illustrates that possible failure (non-disjunction) in early cell division may lead to an abnormal cell line in a specific cell lineage. For example: a division error in the trophoblast may lead to an abnormal cell line in the placenta, while the embryonic precursor cells, from which the embryo will develop, may well be unaffected. Yet another example: meiotic non-disjunction may lead to a trisomic fertilized egg, subsequent mitotic non-disjunction in the trophoblast may lead to a normal diploid cell lineage in the placenta, while the embryo still is trisomic. These examples explain that both false-positive and false-negative findings may occur in prenatal tests using placental tissue. By false-positive is meant that an individual who is free from the given condition is erroneously classified as abnormal, whereas in case of a false-negative an affected individual is erroneously classified as normal.

Figure 3. Representation of the first five divisions in a human embryo after fertilization, according to Crane and Cheung (1988a). In the 32-cell stage the various cells destined to form different cell lineages are indicated, note that just two cells are destined to develop into the embryo.
In the eighties and nineties, many groups (Simoni et al., 1985; Bartels et al., 1986; Schulze et al., 1986; Verjal et al., 1987; Leschot et al., 1987, 1989; Breed et al., 1990; Sachs et al., 1990; Simoni et al., 1987; Breed, 1992; Pittalis et al., 1994; Wang et al., 1994; Leschot et al., 1996; Los et al., 1998) reported on false-positive findings in first-trimester CVS, due to CPM. Many of these results, combined with new cases, were also mentioned in collaborative studies (Mikkelsen, 1985; Vejerslev and Mikkelsen, 1989; MRC Working party, 1991; Ledbetter et al., 1992; Teshima et al., 1992; ACC Working party, 1994; Wolstenholme et al., 1994; Hahnenmann and Vejerslev, 1997). False-negative findings were also reported (Eichenbaum et al., 1986; Linton and Lilford, 1986; Martin et al., 1986), although these findings proved to be less frequent than false-positives.

CPM can be categorized in three different types (Table 1), according to the tissue in which the aberrant cell line is detected. (Kalousek, 1990; Kalousek et al., 1991; Kalousek et al., 1992; Kalousek, 2000).

### Table 1. Types of confined placental mosaicism in gestations with a diploid embryo/fetus

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotrophoblast</td>
<td>N + A or A</td>
<td>N</td>
<td>N + A or A</td>
</tr>
<tr>
<td>Mesenchymal core</td>
<td>N</td>
<td>N + A or A</td>
<td>N + A or A</td>
</tr>
<tr>
<td>Embryonic/fetal</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

N, Normal (diploidy); A, Abnormal (aneuploidy)

The same types of confined placental mosaicism apply for an aneuploid embryo/fetus but in such cases aneuploidy/diploidy are reversed. Kalousek et al. (1989) reported that a diploid cell line was present in the cytotrophoblast in placentas of a total of 14 viable cases of trisomy 13 and 18. This normal cell line, in their view, “rescued” an otherwise lethal condition. As a consequence, this also suggests that analysis of trophoblast cells in these trisomies is unreliable for prenatal diagnosis. It is repeatedly suggested in various studies, that a normal diploid component of trophoblast might facilitate prolonged intrauterine survival in nonmosaic trisomy 13 or 18 fetuses (Kennerknecht et al., 1990, 1993; Artan et al., 1995; Smith et al., 1999; Wallerstein et al., 2000; Farra et al., 2000). The concept of rescue seemed a reasonable explanation for survival of a specific group of trisomy 13 or 18 pregnancies. Still, it has only been (partly) confirmed in just one other study (Harrison et al., 1993), in which 12 placentas of trisomy 18 cases were systematically analysed for the presence of a diploid cell line.

Especially in the early years of chorionic villus sampling, a number of pregnancies were terminated erroneously based on a false-positive finding with CVS. Later on, aberrant findings at CVS were much reason for concern, leading to difficult counseling and usually follow-up testing (amniocentesis) was advised. In a recent report by Los et al. (1998) the authors even go as far as to recommend CVS (analysing both STC and LTC) only to women with a relatively high cytogenetic risk. In this group of women, with a cytogenetic risk of at least 3 per cent, which is the risk equal to or exceeding that of a 40-year-old pregnant woman, CVS is preferred over amniocentesis. According to their data, only for these patients, the certainty rate and predictive value of abnormal cytogenetic results are acceptable. Others (Sikkema-Raddatz et al., 2000) report that in the recent years the rate of false-positive results with CVS has decreased.
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2000) state to have found no differences in predictive values for different indications and conclude that the positive predictive value does not depend on the indication, but on the cytogenetic result.

Nowadays, in practice, 1-2% of the CVS test results require further examination before a definite prenatal diagnosis can be made. At present, a normal outcome seen in the semi-direct method is considered to be highly reliable. The most common aneuploidy (trisomy 21) is also considered to be a reliable finding. In case of chromosomal mosaicism, or in case of the finding of certain trisomies, follow-up investigations are indicated. In some cases of aneuploidy (e.g. trisomy 13 or 18) ultrasound investigation might be the best choice to confirm the chromosomal findings, by looking for structural abnormalities in the fetus. Still, this may only be done reliably at 20 weeks of pregnancy. For many of these inconclusive cases, though, analysis of amniocytes or karyotyping of fetal cells might be necessary. Finding of an inconclusive result for which follow-up is necessary to conclude whether it is a discrepant or a true finding, causes great concern to the patients involved. In case amniocentesis is performed patients are confronted with an additional invasive test in the second trimester of pregnancy, and a considerable long time-span between the initial prenatal test and the definite prenatal diagnosis.

1.5.2 Combining short-term culture and long-term culture

Reports of false-positive findings in cytrophoblast cells using the direct method or the short-term culture, revived the attention for cell cultures. Because of improved culture media (Chang et al, 1982), resulting in reduction of culture time and a reduction of MCC, as well as an increase in number of mitoses, LTC became an attractive candidate for prenatal diagnosis. Combining the outcome of cultured cells with the results of the STC can be a solution to prevent misdiagnoses using either method.

Applying both methods for each sample, however, is laborious and time-consuming; moreover it considerably delays the reporting time. In quite a number of patients two methods can not be performed, simply because of lack of material. A large U.K. collaborative study (Smith et al., 1999) reported the results on a total of 11,303 analysed chorionic villi samples obtained from 1990-1993. In 70.2% of the samples both methods had been used, in 18.4% the outcome was based on the short-term culture alone and in 11.4% only cultured cells had been analysed. In a U.S. collaborative study on CVS, with cytogenetic data on 11,473 samples these figures were 32% (both methods), 26% (STC only), and 42% (LTC only) (Ledbetter et al., 1992). So clearly, all three different approaches for analysis are being used, with a tendency nowadays towards using the combination of methods. Different approaches can also be observed in different centers in The Netherlands. Since 1997 the center for Prenatal Diagnosis (PD) in Rotterdam combines STC with LTC when at least 20 mg has been sampled and uses STC alone when less tissue is available (van Opstal, 1998; van den Berg et al., 2000). In 84.9% of the reported 1838 samples both STC and LTC-villi slides could be made. The frequency of MCC was low (0.4%).

Another center for PD in the Netherlands (Groningen), who formerly used the short-term culture changed their policy based on the results of collaborative studies and a false-negative
finding in their own laboratory (Sikkema-Raddatz et al., 1997). In 1994 they started using LTC only; using both methods was considered impossible for economic reasons (Sikkema-Raddatz et al., 2000). In their series of 1958 chorionic villus samples no increase in reporting time is seen and a frequency of MCC of 3.6% is found. The percentage of maternal cells ranged from 10%-70%, in no case did this lead to incorrect sex prediction or misdiagnosis. They consider the culture method reliable for prenatal diagnosis and state it can be used as a sole investigative method. Our method of choice (Amsterdam, AMC) during the years has been the short-term culture. When >20mg of villi is obtained 15 mg is used for analysis (STC), the remaining tissue is preserved (Hansson et al., 1995). In case of an ambiguous finding in STC, a culture is initiated and the LTC is also analysed.

Maternal cell contamination using the culture method, as well as possible false-positive or false-negative results in the semi-direct method, are definitely disadvantages of first trimester CVS. Also, the quality of chromosomes compared to those obtained after amniocentesis is somewhat poorer. Still, first-trimester CVS, is a prenatal test that will provide a lot of patients with a test result early in pregnancy. Many pregnant women profit from the fact that they are informed of a normal outcome of the chromosome analysis of the fetus early in pregnancy.

1.6 Uniparental disomy and genomic imprinting

It has long been known that both paternal and maternal genes are required for normal development of the placenta and the fetus. It was first shown in pronuclear transplantation experiments in mice, that embryos containing two maternal pronuclei (gynogenetic) show poor placental development and relatively normal embryos, whereas embryos with two paternal sets of chromosomes (androgenetic) had relatively normal placentas and extra-embryonic membranes, but a poorly developed embryo (reviewed in Solter, 1988). This is in accordance with findings in triploid conceptuses in humans. The parental origin of the additional chromosome set has a major impact on the phenotype of the fetus. When the extra haploid set of chromosomes is of maternal origin (diploidy type), a small placenta is found and severe fetal intrauterine growth retardation is recorded. With an extra paternal haploid set (diandric type) an abnormally large and cystic placenta is seen and a relatively well-grown fetus. Triploidy of maternal origin are seen more frequent than those of paternal origin (Sergi et al., 2000; Baumer et al., 2000). Triploid conceptuses very rarely survive to term and when live-born, the infants generally die within the first hours after birth. As shown, there is a clear phenotypic difference for patients with a triploidy of maternal origin, compared to triploidy of paternal origin. So, obviously differences exist between maternally and paternally derived chromosomes.

Normal development of embryo and placenta require the presence of both a paternal and a maternal set of chromosomes. Phenotypical differences may also occur when both copies of a particular individual chromosome are derived from one parent and none from the other. When two copies of a chromosome are derived from the same parent this is called uniparental disomy (UPD). Engel (1980) hypothesized on the existence of UPD in humans in 1980. After the publication of the concept of UPD (Engel, 1980), five years later Carranach and Kirk (1985) published a study on mice in which they could confirm this viewpoint. Mice that were
isodisomic for specific regions in the genome, which means that a mirror duplication of a chromosome arm or segment is present, often showed an aberrant phenotype.

There are several mechanisms described which may lead to UPD, in all cases two errors (meiotic / mitotic) must take place.

Four different ways of formation of UPD are given by Kotzot (1999):

1. Gamete complementation: a disomic gamete is fertilized by a nullisomic gamete (two meiotic errors), resulting in heterodisomy (two different chromosomes from the same parent).

2. Mitotic duplication: a monosomic gamete is fertilized by a nullisomic gamete and afterwards the monosomic chromosome is duplicated (one meiotic and one mitotic error), resulting in isodisomy (two identical chromosomes derived from one parent).

3. Post fertilization error: normal fertilization with loss of a chromosome followed by duplication of the other homologue (two mitotic errors): isodisomy is seen as a result.

4. Trisomy rescue: a disomic gamete is fertilized by a monosomic gamete, followed by the loss of one of the trisomic chromosomes resulting in UPD in one-third of the cases (one meiotic error and one mitotic): heterodisomy.

These four are probably the most frequent mechanisms resulting in UPD. More complex mechanisms, involving multiple mitotic or meiotic errors, may also exist.

UPD in humans was first ascertained due to the presence of an autosomal recessive disease (cystic fibrosis) in a child whose mother carried the mutant gene, but whose father did not. The locus for cystic fibrosis is situated on the long arm of chromosome 7. The data strongly suggested that the child had received identical copies for at least the segment from the centromere to q22 of chromosome 7 from the carrier mother and none from the father (Spence et al., 1988). Besides cystic fibrosis, the patient also had a short stature, which might be due to a second recessive genetic disorder on chromosome 7. Soon other reports followed, showing association of UPD with specific syndromes. Nicholls et al. (1989) showed that Prader-Willi syndrome was associated with maternal UPD 15, while Malcolm et al. (1990, 1991) reported a case of Angelman syndrome associated with paternal UPD 15. So maternal and paternal UPD involving the same chromosome may lead to distinct and highly different disorders. Henry et al. (1991, 1993) showed that paternal disomy for chromosome 11 is associated with the Beckwith-Wiedemann syndrome and yet another example is the finding of maternal disomy for chromosome 7 in the Silver-Russell syndrome (Kotzot et al., 1993; Preece et al., 1997). Since 1990 many more cases with maternal or paternal UPD of almost all chromosomes have been described (Kotzot, 1999).

UPD may go undetected, for it may have no clinical consequences. It may also have negative effects. Firstly, it is more likely that homozygosity of a harmful recessive gene might appear, since only one parent need be a carrier of a particular harmful gene. This might result in intrauterine death if it is a lethal recessive gene or lead to expression of autosomal recessive disorders, with unpredictable phenotypic outcome (Spence et al., 1988; Voss et al., 1989). Secondly, it may also result in specific disorders as described above.

For the autosomal chromosome pairs, as a rule, one of the chromosomes is of paternal origin and the other one of maternal origin. This, of course, is different for the sex chromosomes.
Males have only one copy of X-linked genes, while in females one of the X-chromosomes is inactivated (silenced). It was shown in mouse studies that the paternal X-chromosome is preferentially inactivated in the extraembryonic cell lineages (Takagi and Sasaki, 1975). The X-inactivation in the embryo itself is random and starts much later. Either the maternal or the paternal X chromosome will become inactivated and daughter cells will show the same inactivation pattern. Ropers et al. (1978) and Harrison et al. (1989) in their studies give evidence that the X inactivation in humans is similar to that in mice. Inactivation of a gene depending on the parent-of-origin is called genomic (or parental) imprinting. DNA-methylation is involved in silencing of genes, but is it not yet clear in what way the primary imprint of a gene is established. During mitosis the imprint remains intact, but during gametogenesis an imprint must be "erased", so it can be remethylated according to the parental gender (Constancia et al., 1998). Genomic imprinting, therefore, is a reversible process, during meiosis.

An increasing number of genes are being identified that are expressed differently depending on whether they are inherited from the mother or the father. Identification of imprinted genes or regions in the mouse has been helpful for the detection of such genes/regions in human. Many of the genes that showed imprinting in mice also tend to be imprinted in human, so imprinting is generally conserved between mouse and human (Searle et al., 1994). It is likely that study of mouse mutants will also lead to information about genes involved in placental anomalies (miscarriage, intrauterine growth retardation, preeclampsia) in human.

When a trisomic zygote is formed, so-called "trisomic rescue" may cause the pregnancy to survive to term. Loss of one of the trisomic chromosomes through mitotic nondisjunction or anaphase lag may result in a diploid cell line in the fetus. Such pregnancies are at risk for UPD. Also in case of a Robertsonian translocation and other structural abnormalities there is an increased risk for UPD (Robinson et al., 1995). However, a search for UPD in carriers of chromosome translocations and an abnormal phenotype (James et al., 1994) and in carriers of supernumerary marker chromosomes (James et al., 1995) showed that UPD was found in only 1/65 and 1/22 cases respectively.

The finding of a trisomy at CVS, followed by a normal outcome in follow-up (which can be achieved with various tissues) is often reason for concern and causes counseling dilemmas depending on the chromosomes involved. An unfavourable outcome of such a pregnancy (e.g. growth retardation, intrauterine death, physical or mental handicaps) may be the result of several events. It may be the consequence of (undetected) low level mosaicism. The mosaic cell line in such cases is not confined to the placenta, but is also present in the fetus, which is called generalized mosaicism. Moreover, UPD / genomic imprinting or the expression of a monogenic disorder may be the cause of the adverse findings, or a combination of these factors. Cases with a particular UPD showing no phenotypic effect must be regarded as a strong indication that for that particular chromosome there are no (paternal and /or maternal) imprinted genes. (Ledbetter and Engel, 1995). When UPD for a certain chromosome is consistently associated with a specific abnormal phenotype one might conclude that this is really the effect of UPD and that the possibilities of a trisomy effect and/or a recessive mutation can be excluded (Ledbetter and Engel, 1995)
1.7  Confined placental mosaicism, uniparental disomy and fetal growth

Intrauterine growth is a complex process, which is in part genetically determined. Pregnancies with intrauterine growth retardation (IUGR), as well as infants that are small for gestational age, are at risk for perinatal death or may show problems on longer term. Identification of underlying causes of growth retardation might result in better understanding, recognition and possibly prediction of abnormal fetal growth.

By using maps of conserved genes, it has been possible to delineate the human chromosomes involved in growth that are homologous to mouse chromosomal fragments carrying imprinted genes (Morison et al., 1998). Overgrowth syndromes, such as Beckwith-Wiedemann syndrome (Elliott et al., 1994) associated with UPD (paternal UPD 11), as well as pregnancies with IUGR in combination with UPD for a specific chromosome (e.g. UPD 16) have been reported. (Kalousek et al., 1993; Robinson et al., 1997).

Pregnancies with IUGR have been studied for the presence of CPM, in order to establish a possible relationship between poor pregnancy outcome and the presence of an abnormal cell line confined to the placenta. So far, the prevalence of CPM detected at term in such pregnancies range from 0% (Kennerknecht et al., 1993) to as high as 60% (Artan et al., 1995).

Type I CPM (confined to the trophoblast) is reported to result in spontaneous abortion, IUGR, intrauterine death or perinatal morbidity in 22% of affected pregnancies. Type II CPM (confined to the mesenchymal core) is mostly found in pregnancies with a normal outcome and rarely with fetal IUGR or intrauterine fetal death. In type III CPM (in both trophoblast and mesenchymal tissue), intrauterine death or IUGR are common (Johnson et al., 1990).

If CPM proves to be a frequent cause of IUGR, it might be worthwhile to establish the influence of abnormal cells on placental functioning and, if present, the possible effect of UPD. In cases with uniparental disomy it is often not entirely clear whether the UPD itself or (hidden) trisomic mosaicism in the fetus is the true reason for IUGR.

1.8  Non-invasive fetal cell diagnosis

Walknowska et al. (1969) described the presence of lymphocytes containing five small acrocentric chromosomes in the maternal circulation. These cells in blood of pregnant women carrying a male fetus thus had to be interpreted as cells derived from the fetus. If such cells could be recognized and isolated, detection of (age-related) trisomies such as Down syndrome would be possible without an invasive procedure during pregnancy. Various fetal cell types proved to be present in the maternal circulation: lymphocytes (Walknowska et al., 1969), trophoblast cells (Covone et al., 1984), granulocytes (Wessman et al., 1992) and nucleated red blood cells (Adinolfi et al., 1989), which could be used for aneuploidy detection. The main problem for all these cell types is the scarcity of the fetal cells in maternal blood. Most groups have focused on trophoblast cells (Mueller et al., 1990; Yagel et al., 1994; van Wijk et al., 1996, 2001; and many more) or nucleated red blood cells (e.g. Bianchi et al., 1992; 1999; Bianchi, 1999; Zheng et al., 1993; Rodriguez de Alba et al., 1999, 2000, 2001; de Graaf et al., 1999).
Trophoblast cells, though, may not be the optimal cells for aneuploidy detection, since these cells derive from the placenta and may give discrepant results, as discussed previously. The true fetal origin of the nucleated red blood cells and their relatively short life span (so no cells of previous pregnancies may influence the test results) makes these cells good candidates for aneuploidy detection. For recognition of fetal cells in maternal blood various antibodies have been tested. Antibodies against embryonic haemoglobin (HbE) proved to be more specific than antibodies against fetal haemoglobin (HbF) in detecting fetal nucleated red blood cells in early pregnancy (Mesker et al., 1998). Anti-HbE proved to be very specific in identifying fetal nucleated red blood cells in the mixture of fetal and maternal cells, that are present in transfer medium of chorionic villus samples (Jakobs et al., 2000). Despite the many different procedures for sorting and cell recognition that have been used, analysis of fetal cells from maternal blood for the detection of numerical chromosomal abnormalities has not led to a clinical application, so far.

Still, research in this particular field has led to the discovery of fetal DNA in maternal plasma, which opens up a new method for non-invasive prenatal diagnosis. (Lo et al., 1997). Fetal DNA in maternal blood has been found to be present in much higher concentrations than fetal cells in maternal blood (Lo et al., 1998a). Various prenatal diagnostic applications of this fetal DNA have already been reported. It has been used in case of sex-linked disorders (Lo et al., 1998a), and for determination of the fetal rhesus status (Lo et al, 1998b). Quantitative abnormalities of fetal DNA in maternal serum have also been reported in case of pre-eclampsia (Lo et al., 1999a), in pregnant women carrying a fetus with trisomy 21(Lo et al., 1999b), and in case of preterm labour (Leung et al., 1998).

Recently it has been shown that part of the fetal DNA that is circulating in maternal plasma exists in the form of apoptotic cells (van Wijk et al., 2000), these cells may also be used for the detection of fetal trisomies. Further diagnostic applications may be developed over the next few years.
1.9 Aim of the thesis

The aim of this thesis is to answer the following questions:
1. Is it possible to locate a cell line with an abnormal number of chromosomes in a term placenta, when such an abnormality was present at CVS?

2. Is it feasible to analyse cells from other cell lineages for follow-up when a chromosomal aberration is seen at CVS?

3. Are viable cases of trisomy 13 or 18 supported by a diploid cell line in the placenta?

4. Is there a correlation between intrauterine growth retardation, chromosomal mosaicism in the placenta and uniparental disomy?

1.10 Outline of the thesis

Chapter 2 shows molecular cytogenetic investigations on placental tissue, performed in cases where a numerical aberrant cell line was detected at chorionic villus sampling.

In chapter 3, two cases are reported with respectively a trisomy 22 and a trisomy 8 detected at chorionic villus sampling. Follow-up analysis of fetal and placental tissues is shown and pregnancy outcome is given.

Chapter 4 records the outcome of pregnancies in which confined placental mosaicism or generalized mosaicism was found at chorionic villus sampling. Furthermore, the analysis and use of mesenchymal cells as well as of fetal nucleated red blood cells is described in case an abnormal cell line is detected in trophoblast cells from chorionic villi.

In chapter 5, the outcome of trisomy 13 and 18 cases detected at first-trimester chorionic villus sampling is given. The presence of diploid cells in placental biopsies of viable cases of trisomy 13 or 18 is determined.

In chapter 6, the relationship between idiopathic intrauterine growth retardation and the presence of a trisomic cell line in the placenta is studied.


Chapter 1


Chapter 1


Chapter 1


